Activation of Transposable Elements in Immune Cells of Fibromyalgia Patients

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Abstract: The development of nucleic acid sequencing technology and the unprecedented availability of metadata has evidenced that 45% of human genome constituted by transposable elements (TEs) is not only transcriptionally active but also physiologically needed. Aberrant regulation of TEs, and of human retroviral endogenous sequences (HERVs) in particular, associates with several neurologic and autoimmune diseases, including the Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) frequently comorbid with fibromyalgia (FM). However, no study has yet addressed whether abnormal expression of these sequences correlates with FM. The work presented here shows, for the first time, that in fact HERVs of the H, K and W types are overexpressed in the cells of the immune system of FM patients with or without comorbid ME/CFS. The patients with increased HERV expression (N=14) presented increased levels of interferon (INF-β and INF-γ) but unchanged levels of TNF-α. In support of our proposal that TE activation is a contributor to FM, we find that the tRNA pools are decreased in comparison to matched healthy participants (N=14). The findings reported here could explain the flu-like symptoms FM patients present with in the absence of concomitant infections. Future work towards identifying specific genomic loci differentially affected in FM and ME/CFS is granted.

Keywords: Fibromyalgia; Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS); HERV (Human Endogenous Retrovirus); Transposable elements; Epigenetics; DNA methylation; tsRNAs (transfer RNA small fragments); Interferon; Non-Hodgkin’s Lymphoma.

1. Introduction

Fibromyalgia (FM) (ICD-10 diagnosis code M79.7) [1] is defined as chronic widespread pain disorder of unknown etiology persisting for more than three months in the absence of any obvious organic lesion. Low pain threshold, joint stiffness, sleep disturbance, cognitive dysfunction, fatigue and depression are symptoms commonly found in FM [2-4]. Changes in nociceptive circuitry and increase in pain sensitivity are mechanisms associating with FM [5].
Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ICD-10 code R53.82 or G93.3 if post-viral) appears as disease frequently comorbid to FM. Although separate clinical diagnostic criteria are described for FM and ME/CFS [2,3,6-10], common features such as alterations of the immune system and overlapping symptoms such as flu-like symptoms are described [11,12]. As such, many authors have investigated a possible viral etiology of the disease [13,14]. However, no clear correlation between FM or ME/CFS and viral infection has yet been established.

A higher prevalence in females has been reported for both FM and ME/CFS with global estimates of 1-5% of population individuals affected of FM and 0.23-5% of ME/CFS depending on geographic areas and the diagnostic criteria applied [15,16].

Most chronic and degenerative disorders seem not to be determined by genetic mutations or polymorphisms deriving from complex gene-environment interactions and leading to aberrant epigenetic changes. The recent report by Polli et al., summarizes the available evidence connecting epigenetic mechanisms to pain [17], particularly on the study of DNA methylation patterns and miRNA interference. DNA methylation profiles and the histone posttranslational modification (PTM) landscape shape mammalian genomes, dynamically controlling gene expression by altering chromatin organization [18], while miRNA profiles (miRNomes) post-transcriptionally regulate target RNA levels [19].

So far, two are the studies that tried to evaluate genome-wide DNA methylation profiles in patients with fibromyalgia [20,21]. The authors found differential methylated regions associating to 47 and 960 genes respectively, with some overlaps. The main affected genes relate to DNA repair, immune system, nervous system & skeletal/organ system development, and chromatin compaction pathways [17,22]. As per miRNA profiling five studies have reported FM miRNomes [23-27], work from our group included.

Like DNA methylation studies, miRNA screenings typically have included low number of participants and yet overlapping deregulated miRNAs are found by more than one group of researchers. For a detailed review of up-to-date results readers are directed to a recent review published by our group [28].

Interestingly, as described for FM, ME/CFS patients have shown predominant hypomethylated DNA patterns within differentially methylated regions [29,30]. Genome hypomethylation can lead to transcriptional activation of regions of the genome otherwise silenced. Although coding genes only constitute about 2% of the genome, they have traditionally taken most of the attention in many differential expression studies. However, after noticing that many of the hypomethylated regions in ME/CFS lie within non-coding regions [30], we set to determine whether these epigenetic marks in ME/CFS could potentially affect the expression of repetitive regions, representing 45% of the genome [32], as shown for other neurological and autoimmune diseases, such as Multiple Sclerosis (MS), Amyotrophic Lateral Sclerosis (ALS), Rett Syndrome or Systemic Lupus Erythematous (SLE) [33].

As published, we uncovered particular patterns of transposable elements (TEs) associating with ME/CFS epigenetic marks leading to the interesting possibility that it is the activation of dormant TEs that may trigger ME/CFS patient flu-like symptoms in the absence of concomitant active infections [34]. Since then, the group led by Dr. Romano at the Universidade de Sao Paulo in Brazil, have informed that the levels of the endogenous retrovirus HERV-K appear in fact upregulated in ME/CFS, as shown by the analysis of PBMCs isolated from ME/CFS patients at the monographic UK ME biobank [35], supporting the hypothesis raised by our group [34]. However, and although FM frequently presents comorbid ME/CFS syndrome, no study has yet determined whether patients with a diagnosis of FM presents similar activation of this group of TEs. Neither the molecular consequences of TE activation in FM or ME/CFS have been investigated.
The aim of the present study was to explore whether HERV elements, constituting an 8% of the human genome, are also activated in FM, with and without ME/CFS comorbidity, and in the case of affirmative findings determine whether the increased levels of HERV transcripts could impact patients’ immune system physiology.

2. Results

2.1. Demographics & other characteristics of participating individuals

Average age of participating patients was 54±7.4 years (range 42-65) and 50.4±10.1 (range 38-65) for the population matched healthy control (HC) group. All subjects were female (N= 28; 14 FM and 14 HCs), and all individuals in the patient group fulfilled the revised American College of Rheumatology (ACR) criteria for the diagnosis of fibromyalgia, as described in Methods [2,3]. In addition, 50 % (7/14) fulfilled the Canadian criteria for ME/CFS and all but one of the 7 presenting comorbid ME/CFS also fulfilled the International criteria for the disease [7,8]. By comparing and contrasting molecular patterns of FM patients presenting or not comorbid ME/CFS we may be able to observe disease-specific changes.

Total FIQ average score of FM participants was 74.88±12.59 (range 56.30-92.93) and MFI’s for general fatigue was 17.31±3.23 (range 10-20). Itemized Fibromyalgia Impact Questionnaire (FIQ), Multi Fatigue Inventory (MFI) and quality of life SF-36 questionnaire scores for participating patients (N=14) are shown on Table 1.

Table 1. Patient assessment with FIQ, MFI and SF-36 [36-39] questionnaires (N=28).

<table>
<thead>
<tr>
<th>Questionnaire</th>
<th>Mean</th>
<th>SD±SE</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIQ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total FIQ</td>
<td>74.88</td>
<td>12.59±3.49</td>
<td>56.30-92.93</td>
</tr>
<tr>
<td>Function</td>
<td>5.76</td>
<td>1.84±0.51</td>
<td>2.64-8.91</td>
</tr>
<tr>
<td>Overall</td>
<td>7.81</td>
<td>3.58±0.99</td>
<td>1.43-10.01</td>
</tr>
<tr>
<td>Symptoms</td>
<td>6.38</td>
<td>2.95±0.82</td>
<td>4.29-10.01</td>
</tr>
<tr>
<td>MFI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>General Fatigue</td>
<td>17.31</td>
<td>3.23±0.89</td>
<td>10-20</td>
</tr>
<tr>
<td>Physical Fatigue</td>
<td>17.00</td>
<td>2.97±0.82</td>
<td>12-20</td>
</tr>
<tr>
<td>Reduced Activity</td>
<td>17.00</td>
<td>2.97±0.82</td>
<td>12-20</td>
</tr>
<tr>
<td>Reduced Motivation</td>
<td>15.62</td>
<td>3.07±0.85</td>
<td>11-20</td>
</tr>
<tr>
<td>Mental Fatigue</td>
<td>16.15</td>
<td>2.97±0.82</td>
<td>12-20</td>
</tr>
<tr>
<td>SF-36</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Physical Functioning (PF)</td>
<td>33.75</td>
<td>17.73±5.12</td>
<td>5-65</td>
</tr>
<tr>
<td>Role Physical (RP)</td>
<td>0.00</td>
<td>0.00±0.00</td>
<td>0</td>
</tr>
<tr>
<td>Bodily Pain (BP)</td>
<td>22.08</td>
<td>17.48±5.05</td>
<td>0-57.5</td>
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<tr>
<td>General Health (GH)</td>
<td>18.33</td>
<td>17.49±5.05</td>
<td>0-45</td>
</tr>
<tr>
<td>Vitality (VT)</td>
<td>12.92</td>
<td>11.37±3.28</td>
<td>0-35</td>
</tr>
<tr>
<td>Social Functioning (SF)</td>
<td>28.54</td>
<td>20.27±5.85</td>
<td>0-77.5</td>
</tr>
<tr>
<td>Role Emotional (RE)</td>
<td>25.00</td>
<td>45.23±13.06</td>
<td>0-100</td>
</tr>
<tr>
<td>Mental Health (MH)</td>
<td>47.67</td>
<td>19.48±5.62</td>
<td>28-80</td>
</tr>
</tbody>
</table>

1FIQ (Fibromyalgia Impact Questionnaire), MFI (Multi Fatigue Inventory) and SF-36 quality of life questionnaire. SD (standard deviation); SE (standard error); Range refers to the possible values in the studied group.

2.2. Overexpression of HERV sequences in FM

Based on the hypothesis that the hypomethylation patterns detected in ME/CFS and the altered miRNA levels in FM & ME/CFS may associate with aberrant activation of TEs [34], we proceeded to evaluate whether these patients present increased levels of HERVS, a subtype of TEs that can mimic infection.
Although RT-qPCR amplification of HERVs does not provide location specific information of the amplified sequences, we decided to take this overall estimation approach using sets of primers formerly described by Johnston et al., [40].

As shown in Figure 1, the FM patients participating in this study presented increased levels of HERV-H, HERV-K & HERV-W in their peripheral blood mononuclear cells (PBMCs), with respect to the levels registered in that same blood fraction of HCs.

![Figure 1. HERVs are overexpressed in FM. RT-qPCR amplification of HERV-H, HERV-K and HERV-W using total RNA from PBMCs is shown (N=14/group) (**p<0.001; *p<0.05). Primer sets are detailed in Table 2 and conditions used described in Methods. Relative expression levels were calculated as 2^-ΔΔCt values using GAPDH levels as reference. Group means and SEM values are shown.]

**2.3. Interferon overexpression and TNF-α underexpression in FM patients showing HERV activation**

In an effort to further understand the effects that the transcriptional activation of HERVs may have in the FM immune system and the reported inflammation in patients [4,11,41], we measured cellular interferon and tumour necrosis alpha (TNF-α) levels in PBMCs. Figure 2 shows that the patients with increased expression of HERVs, also presented higher levels of interferon (INF-β and INF-γ). Levels of TNF-α mRNAs, however, were not significantly affected although values presented higher variability across FM participants than in the HC group.

![Figure 2. INF-β and INF-γ levels are increased and TNF-α are unaffected in FM patients showing increased HERV levels. RT-qPCR amplification of INF-β and INF-γ and TNF-α are shown (N=14/group); (**p<0.001; **p<0.005); ns (non-significant). Primers sets used are detailed in Table 2 and conditions described in Methods. Relative expression was calculated as 2^-ΔΔCt values using GAPDH levels as reference. Group means and SEM values are shown.]

Although induction of interferon production was expected in response to the increased dsRNA levels deriving from activation of HERVs, the unchanged levels of TNF-α observed were somehow unexpected as inflammation often associates with increased TNF-α levels [42].

**2.4. tRNA decreased levels in FM patients overexpressing HERVs**
Interferon production activates endonucleases as part of the cellular response mechanisms to degrade invading virus, with the intervention of INF-stimulated genes (ISGs) [43-45].

Donovan et al., have recently shown that activation of the RNase L enzyme, a downstream target of INF signaling, leads to fragmentation of tRNAs (tsRNA or transfer RNA small fragments) for Histidine (His) and Proline (Pro), even before protein synthesis is shut down [46]. With the idea that these tsRNAs could therefore constitute surrogate markers of the activation status of RNase L, we proceeded to examine whether patient PBMCs presented differences in the content of these tRNAs with respect to those of HCs.

As shown in Figure 3 we, in fact, found reduced levels of tRNA-His and tRNA-Pro in PBMCs of the FM patients that presented increased HERV and INF levels with respect to HCs. Although detection threshold limits of the technique used did not allow detection of tsRNA fragments, total tRNA contents for these two tRNAs suggests that the INF induction (Figure 2) observed in these FM patients with activated HERV transcription (Figure 1) leads to tRNA degradation as predicted.

![Image of Figure 3](image-url)

Figure 3. Decreased tRNA levels in FM patients presenting increased HERV expression. Representative northern blot images of total RNA isolated from PBMCs (FM or HC, as indicated) with tRNA-His and tRNA-Pro and RNU6 specific probes, as detailed in Methods (A). Quantitation of tRNA levels in reference to RNU6 signals using Image J software are shown, (N=14/group) (**p<0.0002, ****p<0.0001) (B).

3. Discussion

The importance of the present study relies in that it shows, for the first time, that the activation of transposable elements, in particular some HERV sequences, is a mechanism linked to FM, potentially explaining the reasons for repeated failures in detecting exogenous infectious agents as etiologic triggers of the disease and for the flu-like symptoms patients experience [11-14]. Because of the reduced sample size and the limitation for identifying specific activated genomic loci with the method used, detection of molecular differences between FM patients suffering or not of comorbid ME/CFS was not possible. However, the results obtained by Rodrigues et al., [35] different to ours show activation only of HERV-K elements and no changes in HERV-W levels, potentially supporting differential TE activation across ME/CFS patient cohorts. It would be interesting to know whether the participating patients in Rodrigues study presented or not comorbid FM.

Although a genetic link to FM cannot be ruled out at present, evidence supporting a relevant role of environmental factors in FM and ME/CFS is growing [28, 30,47,48]. It should be mentioned at this point that some SNPs have been correlated with FM and ME/CFS [22,49] which, obviously, does not exclude the participation of epigenetic mechanisms in the pathophysiology of the disease.

Interestingly, the infection mimicry state possibly derived from the presence of complementary RNAs originated from transcription from either HERV end (HERV activation) in the studied subjects, correlates with higher INF-β and INF-γ levels and tRNA pool reductions, in the absence of the inflammatory marker TNF-α (Figures 1-3).
In relation with the findings of this study, the higher prevalence of musculoskeletal manifestations found in patients with malignant disease [50] and the increased prevalence of non-Hodgkin lymphoma in ME/CFS [51], we propose a model by which environmental factor mediated de-repression of TEs trigger INF response and cleavage of tRNAs. Patients with compromised RNase activity failing to degrade tRNAs could use intact tRNAs to retrotranscribe HERV RNA sequences, leading to genome instability. By contrast, the activated “cut and paste” TEs will not be retrotranscribed due to failure of fragmented tRNAs (tsRNAs) to prime [52], preserving genome integrity under a normalscenario (Figure 4).

**Figure 4.** Proposed mechanism linking overexpression of HERVs, INF induction and increased cancer susceptibility in patients with defective ribonuclease activity. ISF (Interferon stimulating factors); TLR3 (Toll-like receptor 3); MDA5 (Melanoma differentiation associated gene 5); RIG-I (Retinoic acid-inducible gene I); OAS (2'-5'-oligoadenylate synthetase); 2-5A (2’, 5’ oligomers of ATP); tsRNAs (transfer RNA small fragments); RT (reverse transcription); AGO (Argonaut).

It is therefore hypothesized that an increase in long dsRNA intracellular levels in FM and ME/CFS patients deriving from epigenetic changes, as described [20-31,34], could in fact constitute a trigger for the disease in the absence of viral or other type of infection. It is perhaps the particular type of activated elements what drives the individual phenotype towards autoimmunity (i.e. through the synthesis of antigenic peptides encoded by TEs), towards neurological defects, or other [33]. The fact that HERV-W immunopathogenic envelope protein (Env) associates with MS [53] and that HERV-E transcriptional activation in CD4+ T cells correlates with SLE [54] supports our proposed model (Figure 4).

The approach used in this study uses degenerate primers to RT-qPCR amplify a group of elements and it is therefore limited at providing information on the specific locations of the genome contributed to increased HERV levels. The recent report by Rodrigues et al., used different sets of primers and sets of samples, and yet, differential TE expression was detected supporting the robustness of this unfocussed overall approach. However, to nail down FM and ME/CFS-specific activated TEs, future studies should use more specific approaches, such as microarray-based HERV subtyping [55] or RNAseq, after differential methylation DIP (DNA immunoprecipitation), followed by repetitive sequence pipeline analysis [56]. A microarray approach that could help unveiling commonly activated HERVs in a particular disease is the HERV-V2 chip developed by Dr Mallet’s group at bioMÉrix, France, an array dedicated to a collection of 5,573 HERVs constructed with 23,583.
PBMC intracellular pools of tRNA-His and tRNA-Pro, probably could, as proposed by Donovan et al., constitute surrogate markers of RNase L activity and hence of TE activation. Although this possibility needs to be corroborated by complementary analysis of RNase L activity, the possibility of using this quantitative simple approach seems attractive. The method could even be improved, limitation wise, by the RtcB RT-qPCR method relying on the use of the RtcB ligase joining single stranded RNA with a 3’-phosphate or 2’,3’-cyclic phosphate to another RNA with a 5’-hydroxyl group prior to RT-qPCR amplification or RNAseq of RNase L cleavage products, as described [46,58]. This step would elevate the sensitivity of the assay used in our analysis (northern blot, Figure 3) for a low requirement of total RNA for the quantitation of tRNA fragments.

Although the levels of TNF-α have been reported increased in the blood of FM and ME/CFS patients by some authors, its association with disease remains controversial [41,59]. It should be highlighted that TNF-α levels are commonly assayed in serum while we used total RNA from PBMCs in our assays. Although TNF-α can be synthesized and released by some PBMC subpopulations such as CD4+ T lymphocytes and NK cells, macrophages & mast cells are the main producers or stores of this cytokine [60]. In this sense it is worth mentioning that Olsnes et al., noticed that following S. pyogenes bacterial stimulation only monocytes in transit to become macrophages secrete TNF-α, but not by peripheral blood monocytes [61]. Perhaps analysis of IL-10 levels may help clarify the potential involvement of compensatory anti-inflammatory pathway along with inflammatory cascade in FM in future HERV studies. Interestingly, it has been reported that IL-10 reduces pain perception by decreasing the level of IL-6 and TNF-α production by monocytes [62].

Moreover, tRNA fragments, as well as other small RNA products of RNase L activity may interfere with the miRNA processing machinery, by their capacity of binding the Drosha and Argonaut (AGO) components of the microRNA biogenesis pathway [63], explaining, at least partly, the reported differential miRNA patterns (miRNomes) in FM and ME/CFS [23-28] and in additional neuroimmune diseases where transcriptional activation of HERVs has been reported [64]. miRNomes, therefore, may be also informative of the activated state of TEs. Particular miRs preferentially associating to RNase L activity, TE activity and disease status remain to be identified.

Finally, it should be highlighted that the participants of this study enrolled only after a minimum 12h period of medication withdrawal prior to blood draw (please see the Methods section for details). As documented in our recent publication [28] and recommended by the NINDS ME/CFS Common Data Elements initiative [65], molecular biomarker research of FM and ME/CFS should restrict participation when possible or count with careful medication registry to minimize and control drug-associated biases.

4. Materials and Methods

4.1. Participating individuals & associated data

This study was approved by the Public Health Research Ethics Committee DGSP-CSISP of Valencia, núm.20190301/12, Valencia, Spain. Patients were invited to participate by advertising the study at local patient associations. HCs were invited at the Umivale mutual health insurance company, Valencia, Spain, during their routine annual checkup visit, to avoid additional phlebotomies. HCs were matched by age (±5 years) to participating patients.

Patients, all female, underwent a thorough clinical interview and medical examination to assess clinical criteria for FM, using the 2011 American College of Rheumatology (ACR) criteria [2,3] and
ME/CFS comorbidity according to Canadian [7] and/or International Consensus [8] criteria. Patients with health problems other than FM and ME/CFS were excluded. Patient health status was also evaluated with the use of standardized questionnaires, including the FM Impact Questionnaire (FIQ) case report form [36,37], the Multi-fatigue inventory (MFI) questionnaire [38], and the quality of life SF-36 instrument [39]. Participating patients agreed to withdraw medication at least 12 h prior to blood draw. Participating HCs were included only if not having a medical history of chronic pain and/or fatigue, or serious health complications. Medicated HCs were also excluded. A single 10-20 mls sample was provided per participant.

4.2. PBMC isolation and total RNA extractions

For the isolation of PBMCs, 10 mls of blood were collected in K2EDTA tubes (Becton Dickinson) and processed within 2 h by dilution at 1:1 (v/v) ratio in phosphate-buffered saline solution (PBS), layering over 1 volume of Ficoll-Paque Premium (GE Healthcare) and separation by density centrifugation at 500x g for 30 min (20°C, brakes off). The PBMC layer was washed with PBS and resuspended in red blood cell lysis buffer (155 mM NH4Cl, 10 mM NaHCO3, 0.1 mM EDTA, and pH 7.4), kept on ice for 5 min, and centrifuged (20°C at 500x g for 10 min), to remove contaminating erythrocytes. The washed pellets were adjusted to a final concentration of 10^7 cells/ml in freezing medium (90 % FBS, 10 % DMSO), aliquoted and deeply frozen in liquid nitrogen until use. Total RNA was extracted with RNAzol (Molecular Research Center) according to the manufacturer’s instructions. RNA quality was assessed using Agilent TapeStation 4200 (Agilent Technologies). Only RNA samples with RNA Integrity numbers (RIN) above 7 were further analyzed.

4.3. RT-qPCR amplification

Reverse-transcription was performed with the High-Capacity cDNA reverse Transcription kit (Applied Biosystems, cat. 4308228), using 1 µg of total RNA according to manufacturer’s guidelines. cDNAs were used for Real time PCR using the PowerUP Sybr Green Master Mix (Applied Biosystems, cat. 100029283) and a Lightcycler LC480 instrument (Roche). Standard amplification conditions were used, including a single hotstart polymerase preactivation cycle at 94°C for 15 min, and up to 45 amplification cycles, each one consisting of 3 steps: denaturation at 95°C for 15s, annealing at 50-60°C for 30s and extension at 70°C for 30s. Sequences of specific primers used are detailed in Table 2. GAPDH levels were used for the relative quantification of the RNAs amplified, 2^(-\Delta\Delta Ct) analysis to calculate fold-change was applied.

Table 2. Sequences of primers used in qPCR amplifications

<table>
<thead>
<tr>
<th>Primer</th>
<th>Reference</th>
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<tbody>
<tr>
<td>HERV-W F</td>
<td>5’-GGCCAGGCATCAGCCCAAGACTTG-3’ [40]</td>
</tr>
<tr>
<td>HERV-W R</td>
<td>5’-CTTTAGGGGCTGGAAAGCCACT-3’</td>
</tr>
<tr>
<td>HERV-H F</td>
<td>5’-CCTTTATTACCCAAATCTGCTCCCGAYAT-3’ [40]</td>
</tr>
<tr>
<td>HERV-H R</td>
<td>5’-TTTAGTTGGGACAGTCTCTTTCATCCARTG-3’</td>
</tr>
<tr>
<td>Interferon-β-F</td>
<td>5’-ACCTCCGAAACTGAGATCTCCTCA-3’ [66]</td>
</tr>
<tr>
<td>Interferon-β-R</td>
<td>5’-TGCTGTTGAAAGATGCTTGA-3’</td>
</tr>
<tr>
<td>Interferon-γ-F</td>
<td>5’-GTGGAGACCATCAAGGAAGACA-3’ self-designed</td>
</tr>
<tr>
<td>Interferon-γ-R</td>
<td>5’-TGCTTCTGCTGACAGATCCTGA-3’</td>
</tr>
<tr>
<td>HERV-K env-F</td>
<td>5’-CACAATAGAAGAAGTGCACG-3’ [67]</td>
</tr>
<tr>
<td>HERV-K env-R</td>
<td>5’-CATAGGCCAAGCTTGTATAG-3’</td>
</tr>
<tr>
<td>TNF-α F</td>
<td>5’-AAAGCTGTAGCAGCCATCTGAG-3’ [40]</td>
</tr>
<tr>
<td>TNF-α R</td>
<td>5’-GCCCTCACCATGTACTCTCCACC-3’</td>
</tr>
<tr>
<td>GAPDG F</td>
<td>5’-TGAAGGCTGGAGTCAAACGGAT-3’</td>
</tr>
<tr>
<td>GAPDG R</td>
<td>5’-TTCTACCCCTGGACGGTGCCA-3’ [68]</td>
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</tbody>
</table>

4.4. Small RNA northern blot analysis

3 µg of total RNA were separated in 15% denaturant polyacrylamide gels (urea 7M) and run 1 hour at 300V, as previously described [69]; transferred to Hybond-N+ nylon membrane (GE
Healthcare, USA) with transfer buffer (trisodium citrate 6mM, Sodium phosphate dibasic 8mM) for 2 hours at 350 mA at 4ºC. Then, membranes were cross-linked with ultraviolet light (UV) at 1200 µjoules during 1 minute and hybridized with 5’ biotin-labeled probes (Integrated DNA technologies) specific to tRNA-His (5´-CAG AGT ACT AAC CAC TAT ACG ATC ACG GCC-3´), to tRNA-Pro (5´-CCG AGA ATC ATA CCC CTA GAC CAA CGA GCC-3´) or to RNU6 (5´- CGA ATT TGC GTG TCA TCC TTG-3´) for normalization, as described by Donovan et al., [40]. Hybridization proceeded after membrane blocking, with 50–100 pmol/ml overnight at 40ºC with shaking, in hybridization oven. After three washes (10–15 minutes at RT with washing buffer 1X SSC, 0.1% SDS), the membrane was developed with streptavidin-horseradish peroxidase (HRP) conjugate (ThermoFisher Scientific), at final concentration of 125 pg/ml in pre-hybridization buffer and ECL™ Prime Western Blotting System in an ImageQuant LAS 4000 Mini (GE Healthcare). Signals obtained were quantified with the Image J software [70].

4.5. Statistical analysis & plotting
Continuous data are expressed as means ± SD, as indicated. Statistical differences were determined using two-tailed unpaired t-tests. Differences between groups were considered significant when p < 0.05. Analysis were conducted with the SPSS package 13.0 (SPSS Inc, Chicago, IL, USA). Plots were drawn using the GraphPad Prism 5.0 program.

5. Conclusions
To our knowledge this is the first study to report increased expression of HERV-K, HERV-H, HERV-W and INFs correlating with decreased tRNA levels, in the immune system of FM patients.

Although the levels of these molecules may serve as biomarkers of FM and/or ME/CFS and/or biosensors of TE activation, the RT-qPCR overall estimation approach used in this pilot study may turn unspecific, broadly associating TE activity with neurological and inflammatory processes. Thus, future efforts evaluating activation of particular HERVs or TE chromosomal sites should more precisely define disease-specific mechanistic information.

Importantly, the model proposed here linking disease-specific epigenome modifications, TE activation, inflammation and an increased risk of cancer in individuals with compromised RNase activity (Figure 4) might be applicable not only to FM and ME/CFS patients but to any individuals with similar molecular disorders.

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Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>HERV</td>
<td>Human Endogenous Retro Virus</td>
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<tr>
<td>TE</td>
<td>Transposable Element</td>
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References


