

ORIGINAL ARTICLE

The non-coding RNome after splenectomy

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Funding information

NIH Clinical Center, Grant/Award Number: UH3TR00943-01; Center for Strategic Scientific Initiatives, National Cancer Institute, Grant/Award Number: 1R01CA182905-01, 1R01CA222007-01A1 and NIGMS 1R01GM122775-01; Ministry of National Education, CNCS-UEFISCDI, Grant/Award Number: PN-II-ID-PCE-2012-4-0018; The Program for Excellence in Research, Grant/Award Number: CEEEX 187/2006; Sister Institution Network Fund; Ladies Leukemia League grant, a Chronic Lymphocytic Leukemia Moonshot Flagship project

Abstract

Splenectomy is a common surgical procedure performed in millions of people worldwide. Epidemiologic data show that splenectomy is followed by infectious (sepsis) and non-infectious complications, with unknown mechanisms. In order to explore the role of the non-coding transcripts involved in these complications, we analysed a panel of circulating microRNAs (miRNAs), which were previously reported to be deregulated in sepsis, in the plasma of splenectomized patients. MiR-223 was overexpressed immediately and late after splenectomy, while miR-146a was overexpressed immediately after splenectomy, returning latter to basal levels; and miR-16, miR-93, miR-26a and miR-26b were overexpressed only late after splenectomy, suggesting similarities with sepsis. We also explored the non-coding (nc)RNome of circulating peripheral blood leucocytes by performing a ncRNA full genome profiling. We observed a reorganization of the ncRNoma after splenectomy, characterized by up-regulation of miRNAs and down-regulation of transcribed pyknons (T-PYKs). Pathway analysis revealed that deregulated miRNAs control pathways involved in immunity, cancer and endothelial growth. We checked the expression of the ncRNAs in 15 immune cell types from healthy donors and observed that plasma miRNAs, cellular miRNAs and T-PYKs have a cell-specific expression pattern and are abundant in different types of immune cells. These findings suggest that the ncRNAs potentially regulate the immune changes observed after splenectomy.

KEYWORDS

microRNA, non-coding RNA, OPSI, pyknon, sepsis, splenectomy

Dragomir and Tudor contributed equally to this article.

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1 | INTRODUCTION

The cause of asplenia or hyposplenism can be congenital, functional or surgical.¹ Splenectomy is a common elective surgical procedure for different haematological conditions: hereditary spherocytosis, immune thrombocytopenic purpura, sickle cell anaemia, myelofibrosis and also a part of more complex surgical approaches for malignant diseases where en bloc resection of the spleen is necessary.² Additionally, splenectomy is performed as an emergency surgery for trauma where the rupture of the organ needs to be quickly addressed.³ In the United States, there are over 1 million asplenic patients and 25 000 new splenectomies are performed yearly.¹ The spleen was regarded for decades as a dispensable organ, and its removal would not affect the morbidity and mortality of the patients.⁴ Only recently, numerous infectious and non-infectious complications were linked to splenectomy.⁵ The spleen is an organ abundant in innate immune cells, including macrophages essential for the elimination of encapsulated bacteria.⁶ Additionally, the spleen is the site where M immunoglobulins are produced by memory B cells, which have an important role in eradicating pathogens from blood.^{7,8} Therefore, the most feared complication of splenectomy is sepsis, referred to as overwhelming post-splenectomy infection (OPSI). The risk for sepsis is lifelong, and the infection has often a fulminant course.⁹

Despite intense research,^{10,11} the exact mechanisms that predispose asplenic/hyposplenic patients to OPSI and other complications remain elusive. In the last years, because of these complications, and the development of novel non-invasive therapies, elective splenectomy is avoided by haematologist despite known benefits and lesser economic impact.¹²

Herein, we propose a paradigm-shifting approach in which we explore the role of various categories of non-coding RNA (ncRNA) from plasma and immune cells of splenectomized patients in order to understand the non-coding RNome deregulations induced by splenectomy. NcRNAs comprise a broad range of RNA species including, among many others, long non-coding RNAs (lncRNAs),¹³ transcribed ultraconserved non-coding RNAs (T-UCRs),^{14,15} microRNAs (miRNAs),^{16,17} transcribed human/primate-specific pyknons (T-PYKs),¹⁸ small interfering RNAs (siRNAs)¹⁹ and piwi-interacting RNAs (piRNAs).²⁰ The ncRNome has been recently described as a regulator of the immune system,^{21,22} but its deregulation after splenectomy has not been studied.

In our previous studies, we detected by genome-wide screenings a panel of human and viral small non-coding miRNAs that are deregulated in peripheral blood mono-nuclear cells (PBMC) of septic patients.²³ We confirmed by RT-qPCR that several of these miRNAs are also deregulated in the plasma of septic patients.^{24,25} Next, we built miRNA networks for septic patients and healthy volunteers using the expression of these miRNAs and observed that in sepsis the miRNA network is fragmented, containing less edges and more isolated nodes.²⁶ Therefore, we hypothesized that the sepsis specific human and viral miRNAs could be deregulated in the circulating peripheral leucocytes and/or plasma of patients after splenectomy and could, at least in part, explain the predisposition towards infections in this patient group. We consider that (a) the ncRNA expression

after splenectomy could be used to detect the splenectomized patients at risk for sepsis and (b) the splenectomized patients at risk for OPSI could improve our understanding of sepsis—a disease for which clinicians are still in search for a cure.^{27,28}

2 | METHODS

2.1 | Clinical samples

The study subjects (both for the plasma study subjects, n = 27, Table S1 and peripheral blood leucocytes array profiling, n = 11, Table S2) were recruited prospectively between 2013 and 2015 from Fundeni Clinical Hospital (FCH), Bucharest, Romania. All clinical data and blood samples were obtained from participants who had given written informed consent, according to protocols approved by the FCH Ethics Committee. Blood was sampled from patients prior to elective splenectomy (D0), 7 days (D7), 30 days (D30), 90 days (D90), 180 days (D180) and 360 days after splenectomy (D360). All blood samples were collected in 10 mL EDTA tubes, plasma and peripheral blood leucocytes were processed, frozen in aliquots, and stored at -80°C after collection. All patients included in the study were vaccinated against *Haemophilus influenzae*, *Neisseria meningitidis* and *Streptococcus pneumoniae*.

2.2 | RNA extraction from plasma/peripheral blood leucocytes and reverse transcription (RT)

Total RNA was extracted and reverse transcribed as previously described.^{25,29} Briefly, RNA was obtained from 100 μL of plasma or peripheral blood leucocytes using the total RNA purification kit (NorgenBiotek, Cat. #37500) according to the manufacturer's protocol. RNA was eluted in 50 μL elution solution and RNA concentrations and quality were assessed using NanoDrop-1100. For the normalization of plasma sample-to-sample variation in the RNA isolation step, the *Caenorhabditis elegans*, cel-miR-39-3p and cel-miR-54-3p, (ThermoFisher SCIENTIFIC, Cat # A25576 and Cat #A25576), 25 fmol of each in a total volume of 1 μL , were used. For the normalization of sample-to-sample variation of RNA extracted from peripheral blood leucocytes, U6 was used as an endogenous normalizer. RNA was reverse transcribed using the TaqMan[®] miRNA Reverse Kit (Applied Biosystems, Cat. #4366596) in 10 μL RT reaction containing 10 ng of RNA, 0.1 μL of 100 mM dNTPs, 0.67 μL of Multiscribe reverse transcriptase, 1 μL of 10 \times RT buffer, 0.13 μL of RNase inhibitor and 1 μL of 5 \times miRNA-specific stem-loop RT primer (Applied Biosystems). Reverse transcription was performed in a Bio-Rad DNA engine with the following program: 16 $^{\circ}\text{C}$ for 30 minutes, 42 $^{\circ}\text{C}$ for 30 minutes, 85 $^{\circ}\text{C}$ for 5 minutes and then 4 $^{\circ}\text{C}$ on hold. The cDNA was diluted and stored at -20°C until analysis.

2.3 | Real-Time RT-qPCR profiling and normalization

The diluted cDNA (3 μL) was used as template in a quantitative PCR (qPCR) reaction with a total final volume of 5 μL . DNA amplification

was performed using TaqMan primers/probes specific for each miRNA (plasma: the 12 miRNAs previously detected by microarray to be deregulated in sepsis²⁴ and four additional miRNAs we used for the previously described sepsis miRNA network²⁶; peripheral blood leucocytes: miR-324 and miR-335) together with SsoFast™ Probes Supermix (Bio-Rad Laboratories, Cat. #172-5231). The reaction started with incubation for 3 minutes at 95°C followed by 40 cycles of 5 seconds at 95°C and 30 seconds at 60°C. All experiments were performed in triplicate. Ct values beyond the upper limit of the measuring system are imputed as 35. The raw Ct values, for the plasma samples, were normalized by Ct values of cel-miR-54-3p the exogenous normalizers ($\Delta Ct = Ct \text{ gene} - Ct \text{ cel-miR-54}$). We selected cel-miR-54-3p as normalizer, because it proved to be the most stable normalization method between the groups for the final analysis (smallest SD, no expression value over 30 cycles and no statistical difference between groups) (Figure S1A). For peripheral blood leucocytes samples, we used U6 as endogenous control ($\Delta Ct = Ct \text{ gene} - Ct \text{ U6}$). U6 proved to be a stable normalization method between the groups for the final analysis (Figure S1B). Finally, the relative expression of each miRNA was calculated using the equation $2^{-\Delta Ct}$.

2.4 | Array design and data analysis

The arrays utilize nucleic acid hybridization of a 52 nt biotin-labelled cDNA target with DNA oligonucleotide probes attached to a gel matrix. The biotin-labelled cDNA targets are prepared by a reverse transcription into first strand cDNA. Total RNA is primed for reverse transcription by a random octamer conjugated with two biotins and a 52 nt long poly-A tail. This procedure results in an equal copy number of biotin cDNA targets to the ncRNA templates. The array contains a collection of probes for various types of ncRNAs: 18 009 probes corresponding to 1271 human pre-miRNAs, 8660 probes corresponding to 626 mouse pre-miRNAs (miRBase 21), 2745 probes corresponding to 479 ultraconserved elements, 16 314 probes corresponding to 1283 T-PYKs and 2197 probes corresponding to 97 lncRNAs. Some of the probes are designed from upstream or downstream regions of certain ncRNAs.

The arrays were analysed in R (version 3.5.1) (<http://www.r-project.org/>). Data pre-processing steps of background-correction, normalization and summarization were performed using functions in Limma library (<http://www.bioconductor.org/packages/devel/bioc/vignettes/limma/inst/doc/usersguide.pdf>). A threshold for positive spot selection for microarray data was calculated as the mean value of all the dark corner spots plus twice the standard deviation.³⁰ Differential expression of ncRNAs in a comparative analysis between pre- and post-splenectomy cases was determined by a fold change in absolute value equal or greater to 1.1 and a *P*-value obtained from the moderated *t*-statistic from LIMMA package <.05. Heatmaps displaying the most differentiated genes were generated using the heatmap.2 function of gplots library. We performed two independent ncRNA array profiling of peripheral blood leucocytes: 2017 training and 2019 validation. The data were submitted to GEO

and were assigned specific GEO accession numbers: GSE133588 for 2017 training cohort and GSE133589 for 2019 validation cohort.

2.5 | miRNA target prediction and pathway analysis

For miRNAs, we obtained experimentally confirmed miRNA-mRNA interactions from miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw>). Both weak and strong experimentally validated target mRNAs were selected for integrated pathway analysis with KEGG, Wikipathways, Reactome, BioCarta, Panther and NCI-Nature using Enrichr bioinformatics resources (<http://amp.pharm.mssm.edu/Enrichr/>). A *P*-value < .05 was considered significant.

2.6 | miRNA-T-PYK expression correlations

We searched for direct expression correlations between pairs of up-regulated miRNAs and up-regulated T-PYKs and pairs of down-regulated miRNAs and down-regulated T-PYKs; and for inverse expression correlations between pairs of up-regulated miRNAs and down-regulated T-PYKs and pairs of down-regulated miRNAs and up-regulated T-PYKs. We built a correlation matrix for deregulated miRNAs with deregulated T-PYKs. Significance was defined by a *P*-value from Spearman rank correlation test <.05.

2.7 | miRNA-T-PYK sequence complementarity and common transcription factors

For miRNA-T-PYK pairs inversely (negatively) correlated we downloaded and ran RNA22 version 2.0 (<https://cm.jefferson.edu/tools-and-downloads/>) to identify miRNA binding sites in the T-PYK sequence.³¹ The threshold used was a *P*-value (representing the likelihood that the target site loci is random) <.05.

For miRNA-T-PYK pairs directly (positively) correlated we retrieved from ENCODE Transcription Factor Targets Dataset the transcription factors (TFs) (from ChIP-Seq experiments³²) that are located up to 5000 bp upstream of the transcript of interest. For this step we used the GenomicRanges Bioconductor package from R.³³ We finally selected the TFs common to the miRNA-T-PYK pairs and displayed them using the OmicCircos package from (BioConductor <http://bioconductor.org/packages/release/bioc/html/OmicCircos.html>).

2.8 | RNA-Seq expression across different immune cells

For each gene of interest, we obtained the RNA-Seq expression across 13 immune cell types and two activated cell types from healthy donors from the Database of Immune Cell Expression, Expression quantitative trait loci (eQTLs) and Epigenomics (DICE) (<https://dice-database.org>).³⁴ Linear RNA-seq data were downloaded, and we performed further analysis for transcripts which were expressed at >1 average transcripts per million (TPM) across all the different immune cells.

2.9 | Statistical analysis

Statistical analyses were carried out with the GraphPad Prism 8 software. To determine whether the data followed a normal distribution, the Shapiro-Wilk normality test was performed. For the comparison between groups *P*-values were determined with an unpaired *t* test if the data were normally distributed, while the non-parametric Mann-Whitney-Wilcoxon test was applied on data with a non-normal distribution. All tests were two-sided, and a *P*-value < .05 was considered statistically significant.

3 | RESULTS

3.1 | The plasma miRNA signature differs between pre-splenectomy, early post-splenectomy and late post-splenectomy

During the time period 2013-2015, 27 patients (Table S1) were prospectively included in the study and blood was sampled for 25 patients before elective surgery (day D0) and 24 were sampled 7 days after surgery (day D7). Because not all patients returned for follow-up as the study was designed, we grouped together the patients that were sampled on day 30 and day 90 after surgery (days D30-90), totalling 28 blood sampled—eleven patients being sampled on both dates. We also grouped the patients sampled on day 180 and 360 (days D180-360) totalling 11 blood samples, two patients being sampled twice.

The design of the plasma study is presented in Figure 1A. Of all the analysed miRNAs only miR-223 was statistically up-regulated early (D7, *P* = .0150) and late (D30-90, *P* = .034 and D180-360, *P* = .0492) after splenectomy (Figure 1B). MiR-223 is well known to be overexpressed in systemic inflammatory response syndrome (SIRS) and down-regulated in sepsis.³⁵ The levels of miR-16, miR-93, miR-26a and miR-26b did not change early after splenectomy, but were up-regulated in the D30-90 (miR-16: *P* = .0007; miR-93: *P* = .0087; miR-26b: *P* = .0009 and miR-26a: *P* = .0014) and D180-360 (miR-16: *P* = .0131; miR-93: *P* = .0317; miR-26b: *P* = .0118 and miR-26a: *P* = .0047) groups compared to D0 (Figure 1C). Next, we observed that miR-146a is the only miRNA statistically up-regulated only immediately after splenectomy (D7, *P* = .0358; Figure 1D). Similarly, to miR-223, multiple studies reported miR-146a to be reduced in sepsis compared to SIRS.^{35,36} Very interesting, by excluding the three subtotal/partial splenectomized patients from the analysis, we obtained slightly better statistical results, regarding the six differently expressed miRNAs (Figure S2), but no changes regarding the other miRNA candidates (data not shown), indirectly supporting the idea that the removal of the spleen is the cause of miRNA changes in plasma.

We previously hypothesized that the immunosuppression caused by the removal of the spleen would induce the overexpression of

the Kaposi Sarcoma Herpes Virus (KSHV) miRNAs,⁵ similar to what we observed in sepsis.²⁵ The expression of the two sepsis specific KSHV-miRNAs: miR-k12-10b and miR-k12-12* did not change after splenectomy; moreover, miR-k12-12* was undetected in most of the patients before and after splenectomy. The expression level of miR-486, miR-342 and miR-150 was stable between the analysed groups. In addition, miR-23 showed very low expression levels before and after splenectomy, not being expressed in the plasma of multiple patients (Figure S3A).

In order to assess if the surgical trauma itself is the cause of the miRNA deregulation early after splenectomy we divided our cohort in minimally invasive treated patients (MI, *n* = 14) and open surgery patients (Open, *n* = 10). We compared the plasma miRNA expression of the two cohorts 7 days after surgery (D7). None of the 11 miRNA expressed in plasma showed any statistical difference between Open and MI (Figure S3B). Therefore, we concluded that the stress induced by the surgical intervention itself is not the cause of the deregulated miRNA expression.

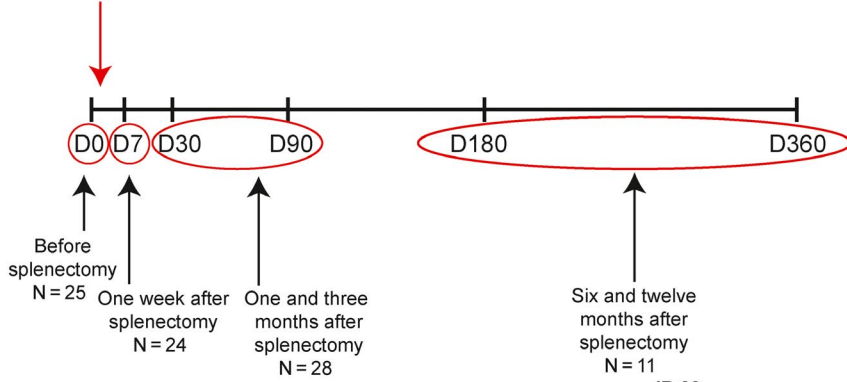
Finally, we correlated the plasma expression of the six differently expressed miRNAs with the level of total leucocytes, monocytes, lymphocytes, platelets (PLT) and haemoglobin (Hb). The level of miR-223, miR-16, miR-26b, miR-26a and miR-146a correlated with the abundance of PLT (Figure 1E and Figure S4A) and the expression of all six miRNAs moderately correlated with the abundance of lymphocytes (Figure 1F and Figure S4B). None of the six miRNAs correlated with the total leucocytes, monocytes or Hb level (data not shown). These results suggest that the deregulated plasma miRNAs could partially originate from PLT and lymphocytes.

3.2 | Splenectomy induces a reorganization of the non-coding transcriptome in peripheral blood leucocytes

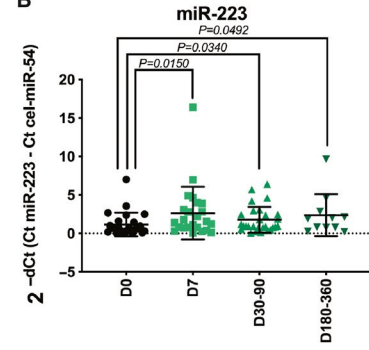
In order to explore the changes in ncRNome from circulating blood leucocytes, we performed two independent ncRNA array profiling (2017 training cohort and 2019 validation cohort) on the RNA extracted from peripheral blood leucocytes collected before and 7 to 30 days after splenectomy. In the 2017 training cohort 10 matched samples, from five patients sampled at D0, D7 - three samples and D30 - the other two samples (D7 and D30 were grouped together as post-splenectomy), were included. Patients' characteristics are listed in Table S2. The patients clustered in pre- and post-splenectomy based on the different miRNA expression. We found 274 up-regulated and 139 down-regulated miRNAs (Figure 2A). In the 2019 validation cohort 12 samples from six patients, five samples from D0, five samples from D7 and two samples from D30 (D7 and D30 were grouped together as post-splenectomy), were included. Patients'

FIGURE 1 MiRNA expression in plasma before and after splenectomy. A, Design of the study: patients were sampled before splenectomy (D0), 7 d after splenectomy (D7), 30 d after splenectomy (D30), 90 d after splenectomy (D90), 180 d after splenectomy (D180) and 1 y after splenectomy (D360). B, MiR-223 expression in plasma after splenectomy; C, miR-16, miR-93 miR-26b and miR-26a expression after splenectomy and D, miR-146a expression after splenectomy. *P*-value was calculated using Mann-Whitney test. Correlation between the expression of miR-223, miR-16 and miR-26b and E, PLT level and F, lymphocytes abundance

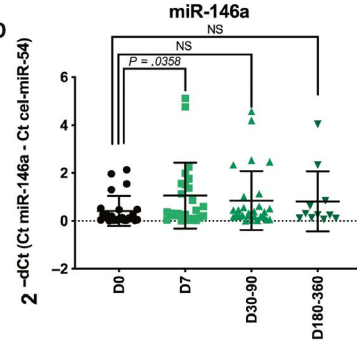
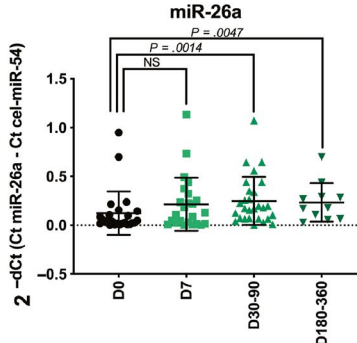
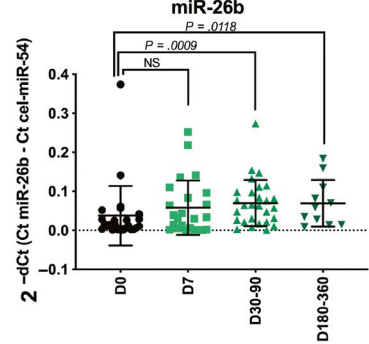
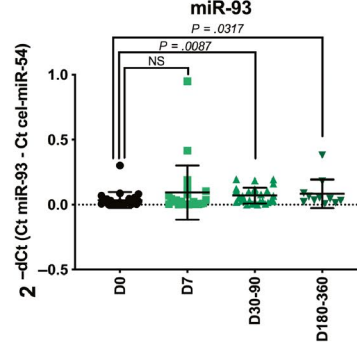
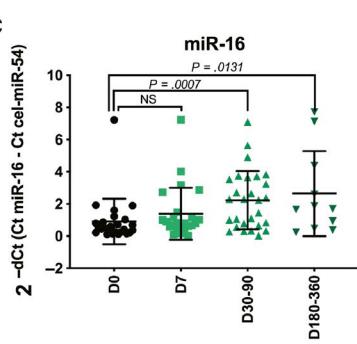
A Splenectomy



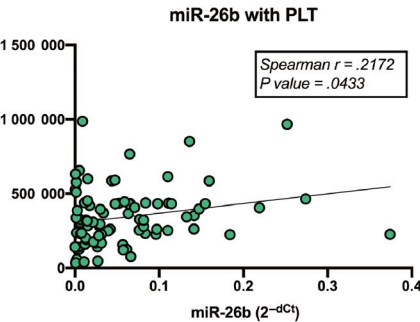
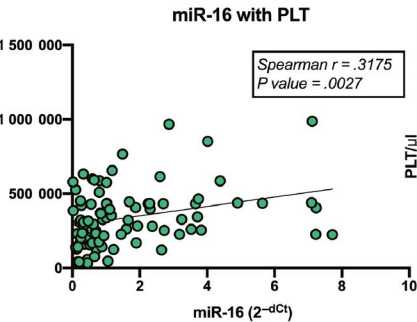
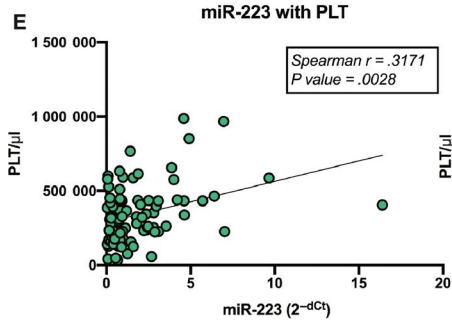
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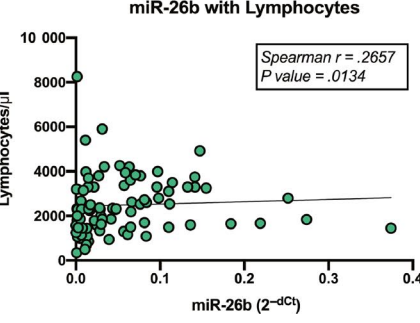
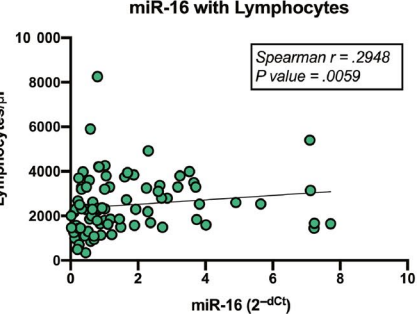
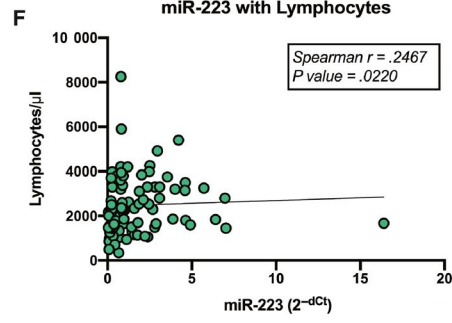
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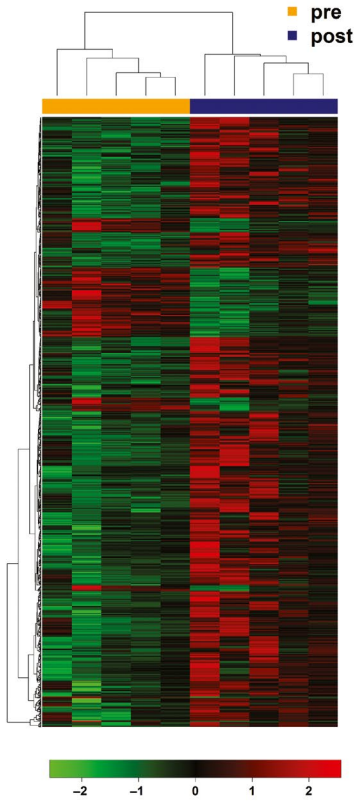
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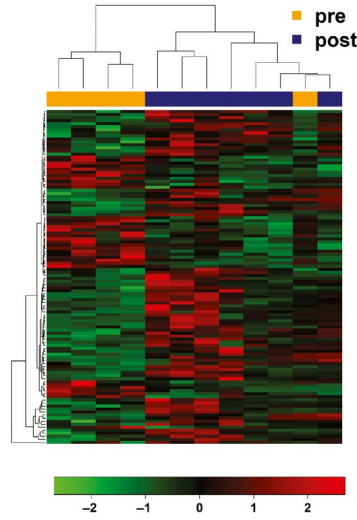
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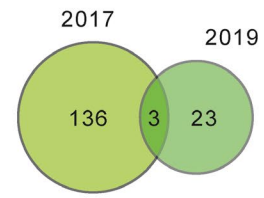
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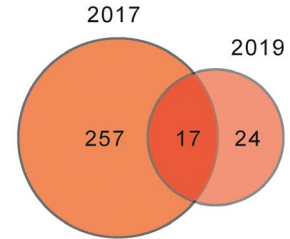
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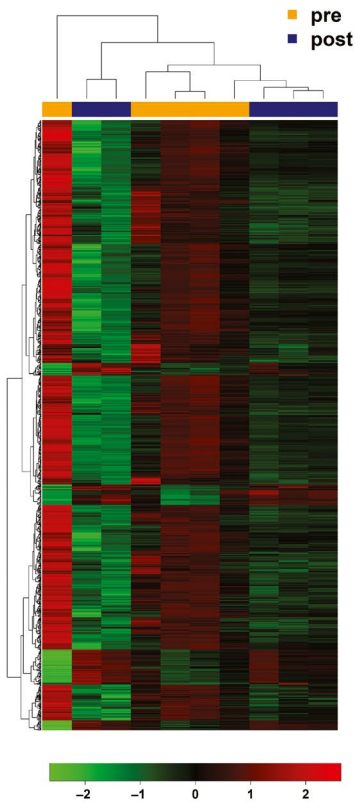
C Common down-regulated miRNA



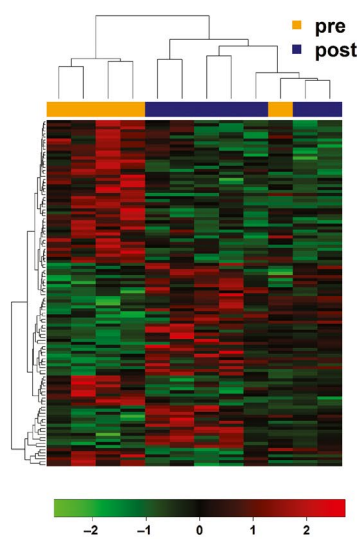
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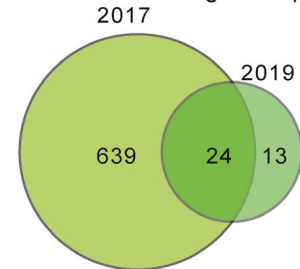
E Pyknon expression training cohort



F Pyknon expression validation cohort



G Common down-regulated pyknons



H Common up-regulated pyknons

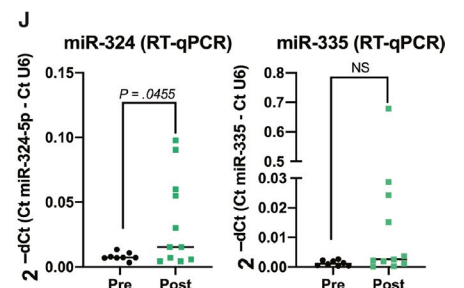
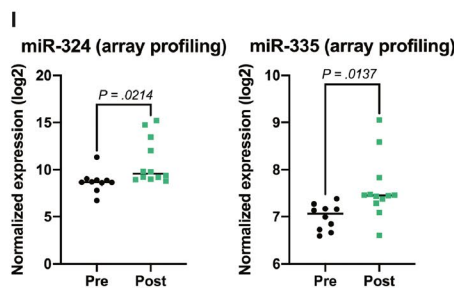
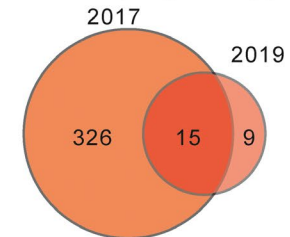


FIGURE 2 ncRNome array of peripheral blood leucocytes. A, miRNA expression in peripheral blood leucocytes before and after splenectomy in training cohort. B, miRNA expression in peripheral blood leucocytes before and after splenectomy in validation cohort. C, Venn diagram of common down-regulated miRNA. D, Venn diagram of common up-regulated miRNAs. E, T-PYKs expression in peripheral blood leucocytes before and after splenectomy in training cohort. F, T-PYKs expression in peripheral blood leucocytes before and after splenectomy in validation cohort. G, Venn diagram of common down-regulated T-PYKs. H, Venn diagram of common up-regulated T-PYKs. I, MiR-324 and miR-335 expression in the array profiling and J, miR-324 and miR-335 expression using RT-qPCR. *P*-value was calculated using unpaired *t* test

characteristics are listed in Table S2. The patients clustered in pre- and post-splenectomy (with one outlier) based on the miRNA expression. We observed 41 up-regulated and 26 down-regulated miRNAs (Figure 2B). We detected a clear tendency of having more up-regulated miRNAs than down-regulated after splenectomy (Figure S5A and S5B). We looked for common miRNAs between the two cohorts and found 3 common down-regulated miRNAs (Figure 2C and Table 1) and 17 up-regulated miRNAs (Figure 2D and Table 1).

Except miRNAs, the other notably deregulated component of the ncRNA transcriptome after splenectomy in peripheral leucocytes were the human/primate-specific T-PYKs (Figure S5A and S5B). Pyknons (PYKs) are non-random patterns of minimum 16 bases in length that repeat at least 40 times in the intergenic portion of the genome.³⁷ Many of the PYKs are located in genomic regions which are transcribed (therefore termed T-PYKs). In the 2017 training cohort the patients clustered relatively well in pre- and post-splenectomy based on the differently expressed T-PYKs (two post-splenectomy samples clustered in the pre-splenectomy group). We found 341 up-regulated and 663 down-regulated T-PYKs after splenectomy (Figure 2E). We validated these findings using the 2019 cohort. The patients were clustered in pre- and post-splenectomy (with one outlier—one pre-splenectomy patient clustered with the post-splenectomy ones)

based on the differently expressed T-PYKs. We found 24 up-regulated and 37 down-regulated T-PYKs after splenectomy (Figure 2F). Interestingly, opposite to miRNAs, which showed a tendency to be up-regulated after splenectomy, more T-PYKs were down-regulated after splenectomy (Figure S5A and S5B). Next, we looked for common deregulated T-PYKs between the 2 cohorts and found 24 common down-regulated (Figure 2G and Table 2) and 15 up-regulated T-PYKs (Figure 2H and Table 2). We checked the genomic location of these T-PYKs and seven down-regulated and seven up-regulated were mapping onto introns or exons of known genes (Table 2).

Regarding the other components of the ncRNome, lncRNAs and T-UCRs, we observed only minimal changes (Figure S5A and S5B). After splenectomy 35 lncRNAs were up-regulated and 1 down-regulated according to the 2017 training cohort and 6 lncRNAs up-regulated and 3 down-regulated according to the 2019 validation cohort. Of these, none of the down-regulated lncRNAs were common between cohorts, but 6 lncRNAs were up-regulated in both cohorts (Figure S5C and S5D and Table S3). T-UCRs showed only negligible differences, the splenectomy induced the up-regulation of 63 T-UCRs and down-regulation of 3 according to the 2017 training array and 1 T-UCRs was up-regulated and 1 down-regulated according to the 2019 validation array. None of the deregulated T-UCRs were

Name	Direction	FCH 2017	<i>P</i> -value 2017	FCH 2019	<i>P</i> -value 2019
hsa-mir-1183	Down	-1.27393	.03113	-1.18910	.03371
hsa-mir-4482	Down	-1.20112	.02403	-1.18134	.00547
hsa-mir-4776-1	Down	-1.16736	.03171	-1.19098	.04709
hsa-mir-675	Up	2.560167	.03462	3.128122	.00577
hsa-mir-324	Up	1.457542	.02040	2.997813	.02767
hsa-mir-3685	Up	2.032401	.03613	1.760359	.03325
hsa-mir-652	Up	1.956314	.01214	1.697622	.02557
hsa-mir-939	Up	1.745731	.01544	1.713752	.04477
hsa-mir-409	Up	1.668098	.01710	1.739345	.03260
hsa-mir-4326	Up	1.794196	.03021	1.428883	.03194
hsa-mir-1257	Up	1.632484	.02338	1.550926	.04808
hsa-mir-4436b-2	Up	1.722482	.01916	1.369651	.02399
hsa-mir-4437	Up	1.369138	.02573	1.599208	.01717
hsa-mir-4688	Up	1.440179	.02481	1.392915	.03300
hsa-mir-335	Up	1.333544	.02235	1.372287	.03255
hsa-mir-550b-1	Up	1.446671	.04414	1.257944	.02142
hsa-mir-4652	Up	1.355628	.01477	1.259334	.02772
hsa-mir-3610	Up	1.362889	.01384	1.229792	.03763
hsa-mir-340	Up	1.236528	.02906	1.158496	.02546
hsa-mir-555	Up	1.174054	.04957	1.193683	.03226

TABLE 1 Common up- and down-regulated miRNAs in training and validation cohorts

TABLE 2 Common up- and down-regulated T-PYKs in training and validation cohorts

Name	Direction	FCH 2017	P-value 2017	FCH 2019	P-value 2019	Location	Distance and features
PYK1_r_109421341	Down	-3.157	.01829	-1.499	.03217	TAF13	1194
PYK1_r_153234356	Down	-1.580	.03266	-1.476	.04810	LENEP	941
PYK10_f_135005869	Down	-2.829	.00914	-1.208	.04615	PRAP1	0 (intron)
PYK14_r_99513962	Down	-1.355	.02172	-1.149	.04171	EVL	0 (intron)
PYK16_f_14539607	Down	-1.490	.01995	-1.288	.01039	PARN	0 (intron)
PYK17_r_18227787	Down	-2.083	.00729	-1.360	.02385	EVPLL	0 (intron)
PYK19_f_10908960	Down	-1.253	.01311	-1.2383	.02454	TIMM29	7044
PYK19_f_11054965	Down	-2.016	.02183	-1.252	.04332	LDLR	5858
PYK19_f_14035273	Down	-1.912	.02366	-1.412	.02138	PALM3	4302
PYK19_f_54644916	Down	-3.061	.01896	-1.254	.03844	PIH1D1	0 (intron)
PYK19_f_59043367	Down	-1.937	.01422	-1.329	.03617	<i>lnc-MYADM-1</i>	16 246
PYK19_r_10905877	Down	-2.166	.02455	-1.332	.02717	TIMM29	3961
PYK2_f_136313238	Down	-2.490	.01405	-1.211	.04872	MCM6	212
PYK20_r_61758560	Down	-1.178	.01054	-1.171	.04750	RTEL1-TNFRSF6B	832
PYK22_f_18663875	Down	-1.528	.02084	-1.258	.03184	DGCR6L	17 671
PYK22_f_26666993	Down	-2.197	.00055	-1.192	.04307	TTC28-AS1	0 (intron)
PYK22_r_26390402	Down	-1.219	.04227	-1.254	.02712	<i>lnc-CRYBA4-10</i>	9451
PYK22_r_41255282	Down	-3.290	.01750	-1.905	.01668	RRP7A	9509
PYK3_f_37853846	Down	-2.107	.02052	-1.218	.03221	ITGA9	17 561
PYK8_f_141724439	Down	-2.400	.00018	-1.280	.01000	ERICD	6726
PYK8_f_23188562	Down	-2.530	.02127	-1.344	.02493	R3HCC1	12 779
PYK8_r_24458777	Down	-1.614	.01696	-1.204	.03533	ADAM7	18 404
PYK8_r_25973252	Down	-1.598	.03446	-1.639	.04874	PPP2R2A	0 (intron)
PYK9_r_124739822	Down	-2.541	.00692	-1.363	.01822	<i>lnc-RABGAP1-1</i>	0 (exon)
PYK9_f_66967215	Up	2.041	.02159	1.837	.03182	AQP7P1	0 (intron)
PYK9_f_121549067	Up	2.630	.02737	1.228	.04395	<i>lnc-B3GNT10-1</i>	222 843
PYK17_f_18215890	Up	2.377	.00675	1.293	.03002	EVPLL	5695
PYK17_f_6818539	Up	1.963	.04250	1.191	.02373	ALOX12-AS1	10 408
PYK8_r_9983280	Up	1.549	.01375	1.574	.04551	MSRA	0 (intron)
PYK8_r_26201845	Up	1.785	.00736	1.292	.03956	PPP2R2A	0 (intron)
PYK7_r_26015368	Up	1.681	.01638	1.336	.02603	<i>lnc-HNRNPA2B1-2</i>	48 377
PYK11_f_58077988	Up	1.474	.03587	1.449	.02559	LPXN	0 (intron exon)
PYK22_r_40724325	Up	1.372	.01544	1.388	.04438	WBP2NL	135
PYK22_r_40972545	Up	1.272	.03374	1.445	.02897	OGFRP1	22 943
PYK19_f_14256192	Up	1.398	.01243	1.178	.02292	LINC01841	35 580
PYK9_f_121106135	Up	1.230	.02768	1.329	.02078	BRINP1	0 (intron)
PYK4_f_8090605	Up	1.209	.02987	1.330	.02806	ABLIM2	0 (intron)
PYK19_f_14462821	Up	1.286	.02914	1.240	.04980	GIPC1	0 (intron)
PYK8_r_33519285	Up	1.283	.02138	1.220	.04989	RNF122	5314

Gene in bold = the pyknon is located inside the genomic sequence of the gene (intron or exon).

common between the two arrays (Figure S5E and S5F). Hence, we can conclude that splenectomy induces mostly a rearrangement of the miRNA and T-PYK component of the non-coding transcriptome, characterized by opposite tendencies: miRNAs up-regulation and T-PYKs down-regulation.

We used a second method to confirm in the same patient cohort (Table S2), two of the top up-regulated miRNAs in both array profiling (miR-324: $P = .0214$ and miR-335: $P = .0137$; Figure 2I). We selected miR-324 and miR-335 also because these miRNAs were reported to play functional roles in inflammation and sepsis, respectively.^{38,39}

By using RT-qPCR, we observed that miR-324 is statistically overexpressed post-splenectomy in peripheral blood leucocytes ($P = .0455$) and miR-335 followed the same trend like in the array profiling but did not reach statistical significance because of the heterogeneous expression among the patients (Figure 2J).

3.3 | Deregulated miRNAs and T-PYKs are part of a complex transcriptional network

To obtain further insights into the mechanism of the deregulated ncRNA transcripts we analysed the downstream pathways controlled by differently expressed cellular miRNAs and checked the expression correlations between deregulated miRNAs and T-PYKs. We retrieved all the experimentally confirmed targets of the common up- and down-regulated miRNAs from peripheral blood leucocytes (Table S4) and performed pathway analysis. We found 233 up-regulated pathways and 767 down-regulated pathways (Table S5); the top 15 down- and up-regulated pathways are presented in Figure 3A. Four of the up-regulated pathways are linked to the immune response: herpes simplex virus 1 infection, interferon type I signalling pathway, interleukin-11 (IL-11) signalling pathway and T-cell antigen receptor signalling pathway. IL-11 was proposed recently as a possible new therapy for sepsis.⁴⁰ Many of the top down-regulated pathways were linked to cancer (p53 effectors, breast cancer, breast cancer pathway, transcriptional misregulation in cancer, proteoglycans in cancer, signalling by Wnt, and pathways in cancer) connecting splenectomy with cancer, a long-term complication of this surgical procedure.⁴¹ Curiously, VEGFA-VEGFR2 pathway was simultaneously up and down-regulated implying a complex regulation by the differently expressed miRNAs after splenectomy. Moreover, another up-regulated pathway was VEGFR3 signalling in lymphatic endothelium. The effect of the deregulated miRNAs on VEGFA-VEGFR pathways can be interpreted in the context of vascular complications associated with splenectomy: myocardial infarction,^{42,43} stroke,⁴⁴ venous thromboembolism^{42,45} and pulmonary hypertension.⁴²

Next, we performed miRNA-T-PYK expression correlation analysis in both validation and training cohort. We selected only correlations confirmed in both groups and displayed the average of the correlation coefficients using a corrplot library. We detected a high degree of correlation between miRNAs and T-PYKs, 273 significant correlation pairs of miRNA-T-PYKs out of 780 possible (35%) were observed. All miRNAs correlated significantly with multiple common T-PYKs. Some miRNAs (miR-4437, miR-652, miR-675 and miR-3685) correlated with twenty or more T-PYKs, being central elements of the miRNA-T-PYK interaction. Similarly, two T-PYKs: PYK17_f_18215890 (located 5695 nucleotides upstream of *EVPLL* coding gene) and PYK16_f_14539607 (located in the intron of *PARN* coding gene) correlated with 16 and 15 miRNAs, respectively (Figure 3B). PYK22_f_18663875 (located 17 671 nucleotides upstream of *DGCR6L* coding gene) and PYK8_r_33519285 (located 5314 nucleotides upstream of *RNF122* coding gene) were the only T-PYKs which did not correlate with any miRNA.

In order to understand the mechanism that controls this synchronized transcription we checked for genomic colocalization of the miRNA-T-PYK and only 10 out of 273 (3.66%) pairs shared the same chromosome, but were not closely located (Table S6). We also checked for TFs experimentally confirmed by ChIP-seq data which are common for miRNA-T-PYK pairs that positively correlated. Out of 123 pairs, 62 (50.4%) had experimentally confirmed common TFs, therefore partially explaining the positive correlations between miRNAs and T-PYKs (Figure 4A and Table S7).

Regarding the miRNA-T-PYK pairs, which showed an inverse correlation, we looked for complementary sequences between miRNA and T-PYK. Complementarity between the transcripts implies a potential direct interaction and could partially explain the inverse correlation.⁴⁶ We found 19 complementary sequences between miRNA and T-PYK (Figure S6 and Table S8). Two miRNAs showed more than one interaction with a T-PYK and two T-PYK were targeted by multiple miRNAs, hence the 19 complementary sequences represented 16 pairs out of the maximum total of 153 (10.26%). These 19 interactions form a complex network that to some extent explains the inverse correlations (Figure 4B).

Overall, these data show that deregulated miRNAs control pathways involved in immunity, cancer and endothelial growth and that a common mechanism exists that controls the expression of miRNAs and T-PYKs after splenectomy resulting in a complex and highly connected molecular network.

3.4 | Plasma miRNAs, cellular miRNAs and cellular T-PYKs have a cell-specific expression pattern in healthy individuals

In order to explore the function of these transcribed ncRNAs we assessed their expression in 15 different immune cell types (13 immune cells and 2 activated cell types) from 91 healthy donors from DICE database.³⁴ Five out of six plasma miRNAs which we found to be up-regulated after splenectomy are highly expressed in immune cells of healthy individuals. MiR-223 showed a very interesting expression pattern, having high expression in classical monocytes (57.53 TPM) and in non-classical monocyte (17.12 TPM), implying a role in innate immunity. Similarly, miR-26b showed high expression only in classical monocytes (5.27 TPM), being almost unexpressed in the other subtypes of immune cells. MiR-146a showed a specific expression in naïve activated CD4 and CD8 positive T cells (17.55 and 9.12 TPM, respectively) and in memory TREG CD4 and CD8 positive T cells (17.94 and 12.04 TPM, respectively), implying a role in regulating the adaptive immune system. Similarly, miR-93 was highly expressed in naïve activated CD4 and CD8 positive T cells (8.21 and 11.24 TPM, respectively), but also in naïve B cells (8.71 TPM). MiR-26a showed a moderate expression across all the different cell types, with the highest in T-helper 17 cells (2.56 TPM; Figure 5A). The only miRNA with very low expression in immune cells of healthy donors was miR-16, suggesting that it may originate from non-immune cells.

Only one out of the three cellular miRNAs confirmed in both cohorts to be down-regulated showed a high expression level in immune cells

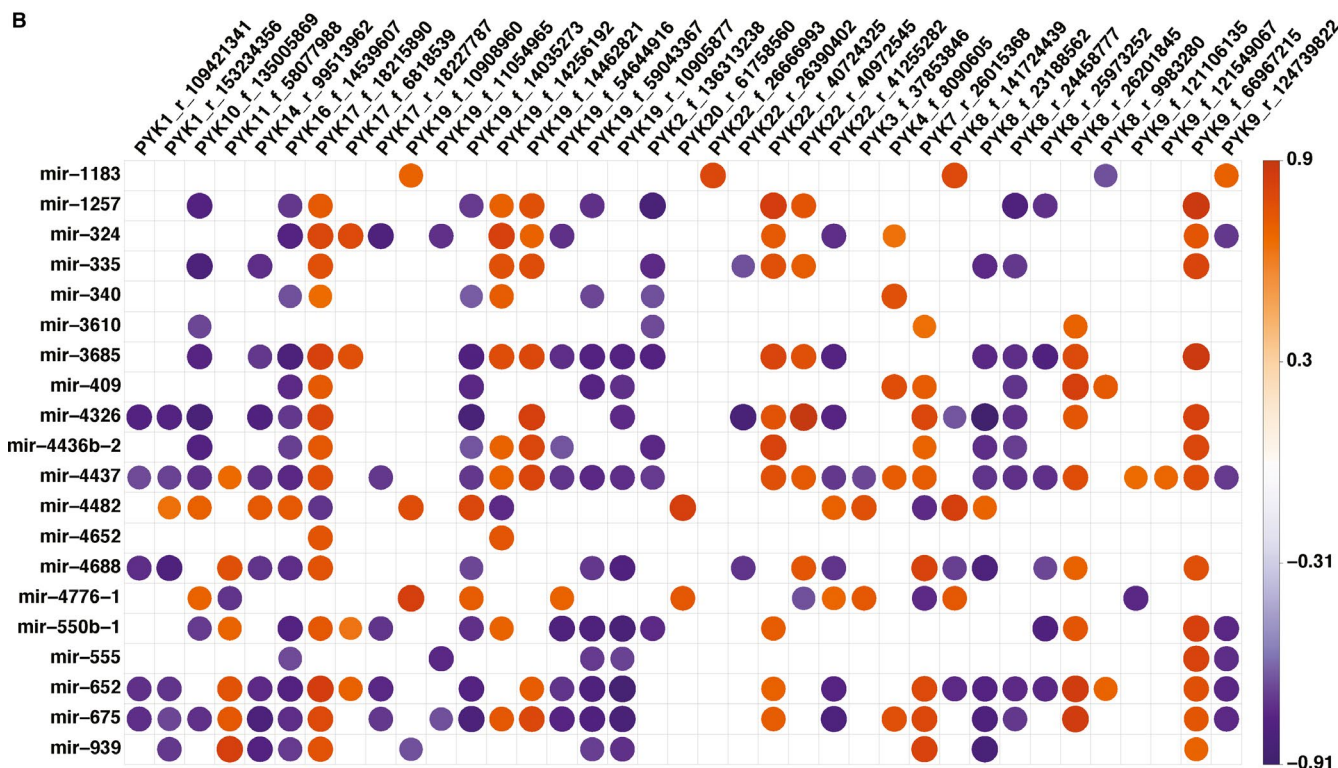
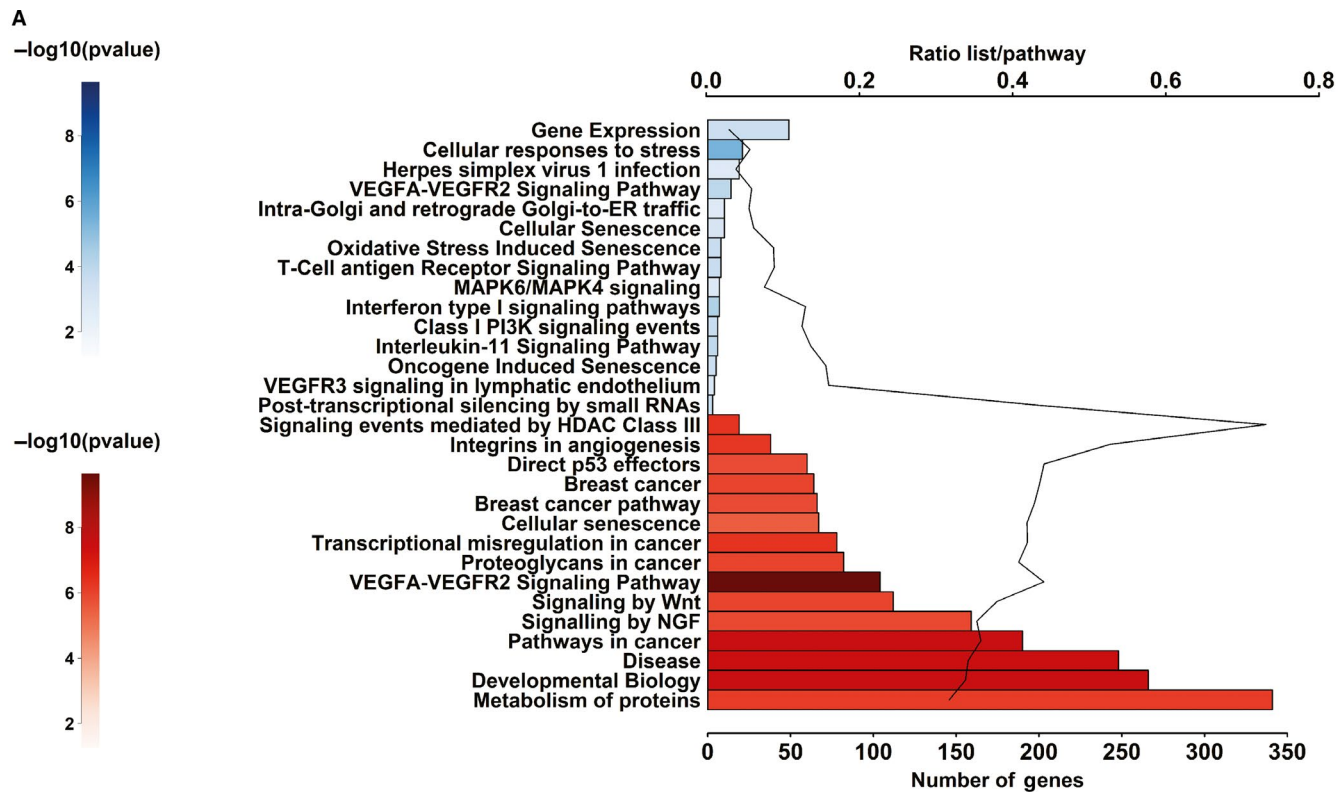


FIGURE 3 Pathways controlled by the common up- and down-regulated miRNAs. A, Top up- and down-regulated pathways by common miRNAs. Upper x-axis represents ratio of genes targeted by common miRNAs to whole number of genes from pathway. Lower x-axis represents total number of genes controlled by common miRNAs (blue—pathways controlled by down-regulated miRNAs and red—pathways regulated by up-regulated miRNAs). B, Corplot library of miRNAs and T-PYKs correlation, (red—directly correlated pairs and blue—inversely correlated pairs)

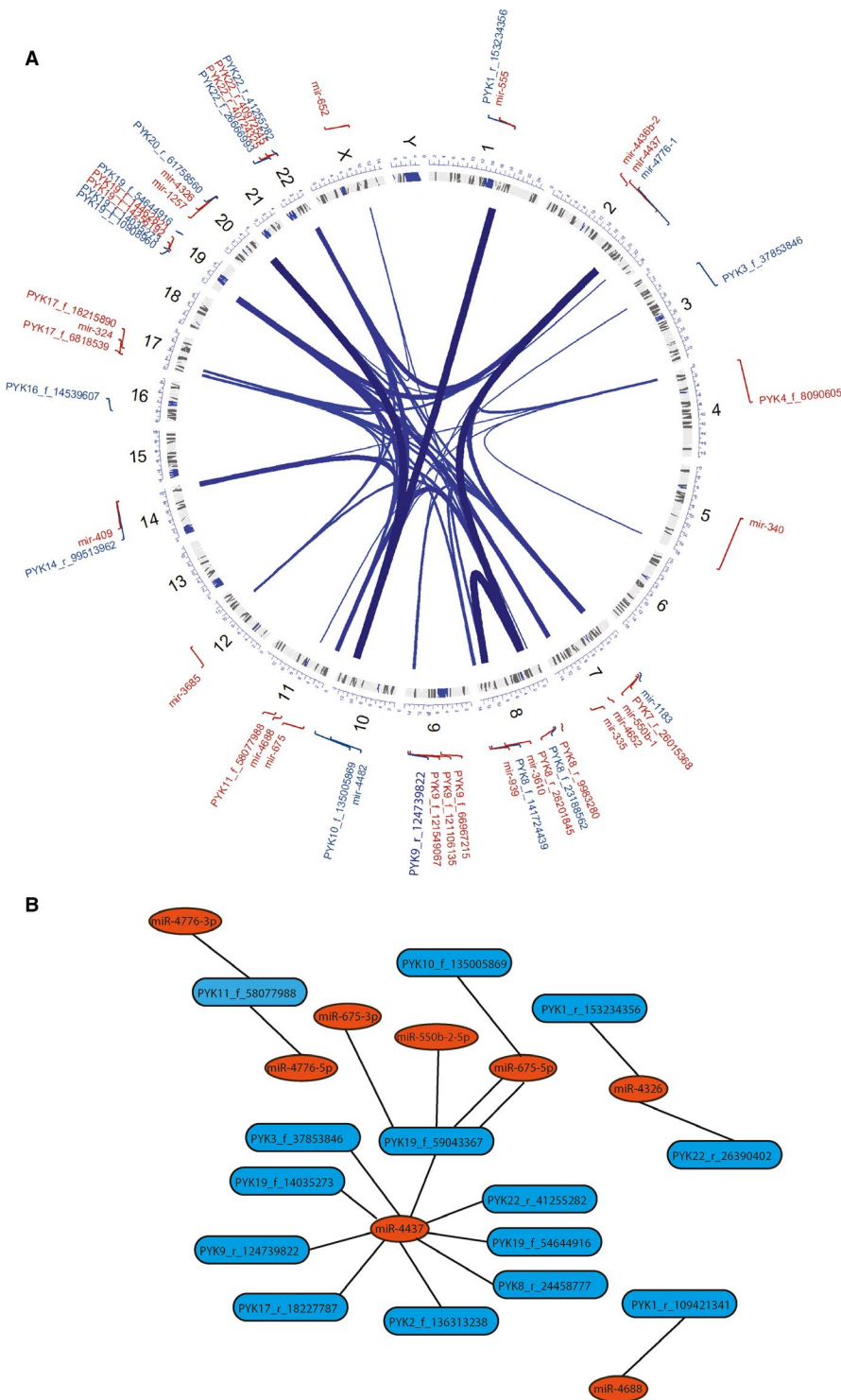


FIGURE 4 MiRNA-T-PYK interaction. A, TF regulation for miRNA-T-PYK pairs with same type of change and directly correlated (B) Network of miRNA and T-PYK pairs inversely correlated and with sequence complementarity

of healthy donors: miR-4482. MiR-4482 is overexpressed in activated CD4 and CD8 positive T cells (23.21 and 15.55 TPM, respectively), implying a role in adaptive immunity (Figure 5B). Of the 17 miRNAs found to be down-regulated after splenectomy, five miRNAs were expressed in the immune cells of healthy donors. MiR-324 showed an overall high expression in all the 15 types of immune cells, with the highest in CD16 positive Natural Killer cells (NK) (35.53 TPM) and in non-classical monocytes (31.93 TPM), implying a role in the innate immune system. MiR-3685 and miR-4688 showed a low to medium expression level.

MiR-3685 had the highest expression in CD4 positive TREG T cells (4.25 TPM) and miR-4688 in NK cells (3.6 TPM) (Figure 5C). MiR-4326 and miR-555 showed an average low expression in all the different immune cell lines with no cell-specific expression pattern (Figure S7).

Next, we checked for the expression of the host genes of the T-PYKs. From the group of down-regulated T-PYKs 8 out of 24 mapped known annotated genes and from the group of up-regulated T-PYKs 7 out of 15 mapped known annotated genes (Table 2). Hence, we were able to assess the expression in immune cells only

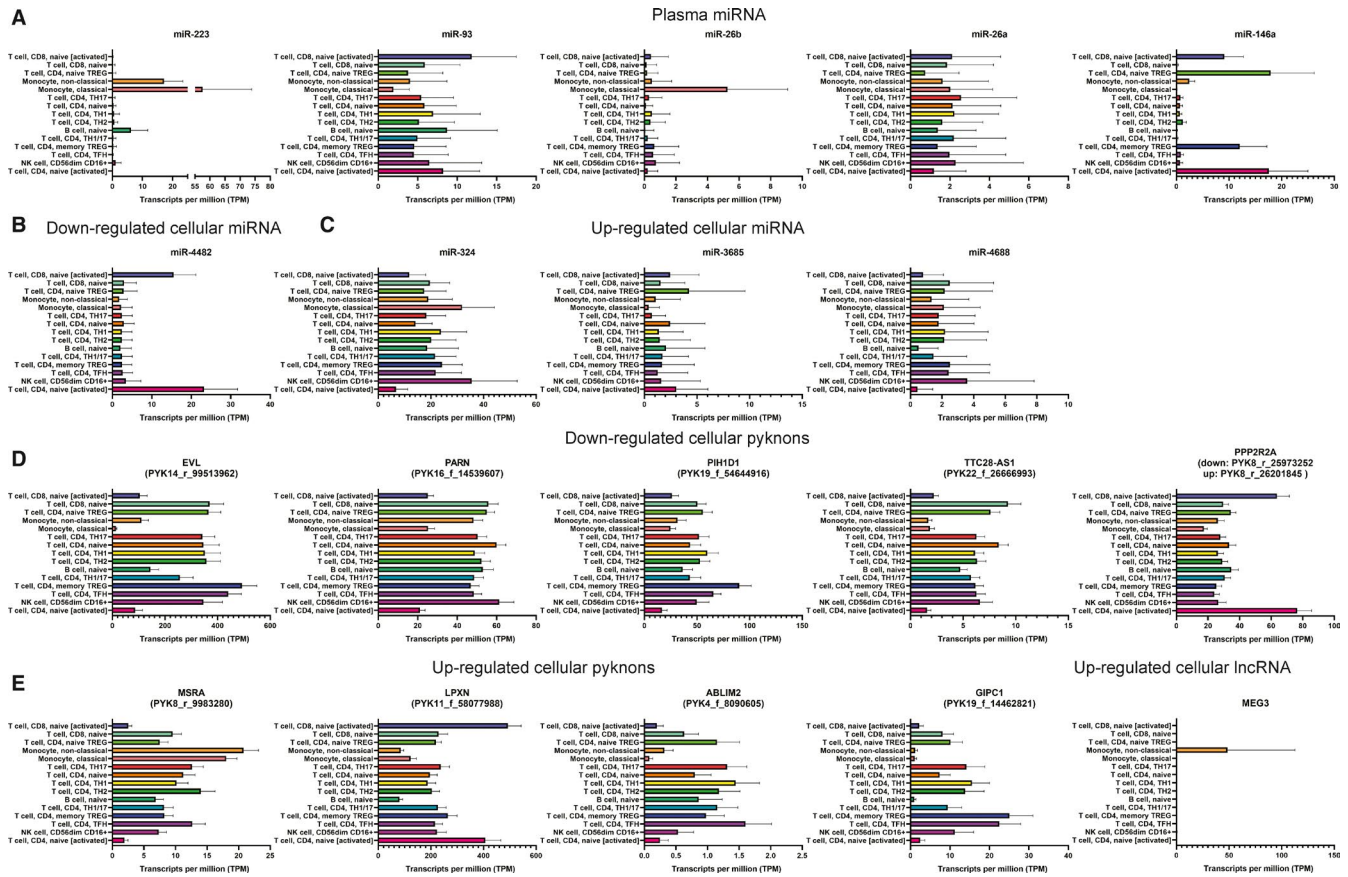


FIGURE 5 ncRNA expression in 13 immune cell types and 2 activated cell types from 91 healthy donors. A, Plasma miRNA, B, common down-regulated cellular miRNAs, C, common up-regulated cellular miRNAs, D, common down-regulated cellular T-PYKs, E, common up-regulated cellular T-PYKs and F, common up-regulated lncRNA expression in circulating immune cells

for these T-PYKs. Five out of eight host genes of the down-regulated T-PYKs were high to very high expressed (>100 TPM) in immune cells from healthy individuals. *EVL* (Enah/Vasp-like) host gene of PYK14_r_99513962 showed a very high expression, especially in memory TREG cells (493 TPM) and in follicular helper T cells (TFH) (441.17 TPM). *EVL* encodes for a protein which is highly expressed in lymph nodes and spleen and was recently linked to transendothelial diapedesis of T cells.⁴⁷ *PARN* (poly(A)-specific ribonuclease) host gene of PYK16_f_14539607 showed a high expression in most of the immune cells, with the highest in NKs (61.29 TPM). *PIH1D1* (PIH1 domain containing 1) host gene of PYK19_f_54644916 showed a specific high expression in memory TREG cells (90.13 TPM) and the lncRNA *TTC28-AS1*, the host gene of PYK22_f_26666993 was high in naïve CD4 and CD8 positive T cells (8.37 and 9.37 TPM, respectively). Intriguingly, *PPP2R2A* (protein phosphatase 2 regulatory subunit B α) contained simultaneously a down- and an up-regulated T-PYK: PYK8_r_25973252 and PYK8_r_26201845, proving the fact that transcriptional regulation of T-PYKs is complex and potentially independent from host genes. *PPP2R2A* showed a highly cell-specific expression pattern with very high levels in naïve activated CD4 and CD8 positive T cells (76.56 and 63.76 TPM, respectively). The B55 α subunit of *PPP2R2A* was reported as regulator of multiple miRNAs in acute myeloid leukaemia (AML),⁴⁸ partially explaining the

synchronized T-PYK-miRNA deregulation observed after splenectomy (Figure 5D).

From the group of up-regulated T-PYKs, five mapped onto genes expressed in immune cells, including PYK8_r_26201845 which is located on *PPP2R2A* gene. *MSRA* (methionine sulfoxide reductase A) the host gene of PYK8_r_9983280 was highly expressed in classical and non-classical monocytes (18.04 and 20.79 TPM, respectively) and recently it has been shown that *MSRA* protects against LPS induced septic shock.⁴⁹ *LPXN* (leupaxin), the host gene of PYK11_f_58077988, showed a particular expression in immune cells, with very high levels in naïve activated CD4 and CD8 positive T cells (407.03 and 493.3 TPM, respectively). *LPXN* has been reported to form fusion transcripts in AML by translocation and induce leukemogenesis.^{50,51} *ABLIM2* (actin binding LIM protein family member 2) the host gene of PYK4_f_8090605 had an overall low expression level, with the highest expression in TFH cells (1.6 TPM). *GIPC1* (GIPC PDZ domain containing family member 1) the host gene of PYK19_f_14462821 showed a high expression, with the highest levels in CD4 positive TREG cells (25.1 TPM) (Figure 5E).

Finally, we checked the expression of the six common up-regulated lncRNAs in immune cells. Four out of the six lncRNAs were not available in DICE database, *XIST* was not expressed and *MEG3* showed a highly cell-specific expression, reaching high levels only in non-classical

monocytes (48.49 TPM) (Figure 5F). MEG3 was recently reported to bind miR-138 and derepress IL-1 β , intensifying the reaction of macrophages to bacterial infections of the lung and preventing sepsis.⁵²

Overall, these findings emphasize that plasma miRNAs, cellular miRNAs and cellular T-PYKs have a cell-specific expression pattern and are transcribed in all the different types of immune cells, potentially regulating the complex immune changes observed after splenectomy.

4 | DISCUSSION

This study is an initial step in understanding the complexity of the infectious and non-infectious complications that follow splenectomy by studying the ncRNome. Our data prove that splenectomy induces extensive deregulations of miRNAs similar to sepsis in plasma, and a synchronized reorganization of the ncRNome in circulating immune cells.

Firstly, we show that splenectomy is followed by up-regulation of circulating miRNAs similar to sepsis. MiR-146a is up-regulated at 7 days after splenectomy and returns to a basal level at 1 month; miR-16, miR-93, miR-26a and miR-26b are up-regulated 1-3 months after splenectomy and remain up-regulated at 6-12 months; and miR-223 is up-regulated from day 7 and the high levels persist at 1 year after splenectomy. MiR-223 is a known modulator of the immune system and was previously reported to be up-regulated in multiple inflammatory diseases including rheumatoid arthritis, HIV and type 2 diabetes.³⁰ Very interesting, Wang et al³⁵ showed that serum miR-223 is up-regulated in SIRS, but decreases in sepsis, being able to differentiate SIRS from sepsis. Hence, we can envision that the down-regulation of miR-223 in splenectomized patients can be a potential marker for the onset of sepsis. In our previous study, we showed that the same group of miRNAs comprising miR-16, miR-26a, miR-26b and miR-93 are up-regulated in plasma of non-surgical septic patients.²⁵ Recent data showed that in a murine sepsis model, mice treated with miR-146a have less apoptotic splenic macrophages and splenectomy diminishes the therapeutic effect of miR-146a.⁵³ This similarity between splenectomy and sepsis could be an initial explanation for the higher risk of sepsis after the removal of the spleen. Close follow-up of the splenectomized patient can lead to a better understanding of sepsis and to the development of new RNA based therapies. Because splenectomy induces an immunosuppressive state, we hypothesized that it should be followed by an up-regulation of viral miRNAs, similarly to sepsis.^{5,25} Curiously, the removal of the spleen did not induce any deregulation of the viral miRNAs (miR-k12-10b and miR-k12-12*), but such data should be followed by an overall viral miRNAs screen.

Secondly, splenectomy induces a major reorganization of the non-coding transcriptome in peripheral blood leucocytes. In order to understand the intracellular ncRNome we performed a array profiling on the ncRNA from leucocytes from pre- and post-splenectomy patients. The plasma deregulated miRNAs (miR-223 and miR-26b) could be found among the miRNAs deregulated in peripheral blood leucocytes by the 2017 array profiling, suggesting that some of the plasma miRNAs could originate from blood leucocytes. Interestingly, we observed a major reorganization of the ncRNome

at the cellular level, characterized mainly by miRNA up-regulation and T-PYKs down-regulation. The reorganization of the ncRNome is analogous to the observations made by Xiao et al, who coined the term "genomic storm" after studying the transcriptome (only mRNA) of critically injured humans (including healthy individuals stimulated with endotoxin). The authors observed that over 80% of mRNAs are significantly changed after severe trauma, burn injury or endotoxemia.⁵⁴ Our array profiling data suggest that splenectomy induces a reprioritization of the non-coding component of the transcriptome in circulating leucocytes and most probably during sepsis (or other types of systemic inflammation) the amplitude of this deregulation is even higher. The changes we observed at the ncRNA level in peripheral leucocytes could partially explain why splenectomized patients are susceptible to infections and supplementary mechanistic studies are necessary to confirm this. Additionally, our data prove that not only the coding transcriptome should be studied to understand the physiology and pathology of the immune system, but also the non-coding component, which shows similar degrees of dynamics.

Thirdly, we observed that the cellular deregulated miRNAs control pathways with roles in immunity, endothelial growth and cancer, linking our data with the most common complications of splenectomized population. Moreover, pairwise analysis of deregulated transcripts proved that the expression of miRNAs and T-PYKs is significantly correlated implying a synchronized deregulation of the ncRNome after splenectomy.

Finally, we assessed the expression of the circulating plasma miRNAs, cellular miRNAs, T-PYKs and lncRNAs in 13 different types of immune cells and 2 activated cell types. Most of the up-regulated plasma miRNAs and common cellular non-coding transcripts are expressed in various immune cells and have highly cell-specific expression patterns, implying a broad role in immune regulation.

A first limitation of our study is the fact that, except splenectomy, a second intervention carried on all included patients was vaccination against *Haemophilus influenzae*, *Neisseria meningitidis* and *Streptococcus pneumoniae* and it is well known that vaccination stimulates the adaptive immune system.⁵⁵ Therefore, it is impossible to determine, using clinical samples, if any of the observed changes in the ncRNA expression are induced by vaccination (adaptive immune system) or by splenectomy (which affects mainly the innate immune system). Additionally, in a recent publication it was observed that the levels of miR-451a changes after influenza vaccination and anti-correlates with pro-inflammatory cytokines.⁵⁶ Interventional in vivo studies are necessary to clarify these details.

A second limitation of the study is the fact that we do not know the precise origin of the circulating miRNAs. It seems that these miRNAs are not originating solely from the circulating immune cells (we did not find the same miRNAs in plasma and circulating immune cells, but we found a positive correlation between the expression of the miRNAs and lymphocytes and PLT, respectively). The spleen coordinates the systemic immunity and splenectomy is followed by changes of multiple types of immune cells⁵⁷ and for instance it is well known that miR-223 regulates the biology of peritoneal macrophage.⁵⁸ We could speculate that immune cells from other lymphoid

and non-lymphoid organs are also involved in the deregulated plasma miRNA expression, after splenectomy, proving that miRNA transfer is an important cell-to-cell communication mechanism.⁵⁹

A third limitation is the heterogeneous and small population (n = 27, circulating miRNA study and n = 11, array profiling) used for this study. We included in this study a population of electively splenectomized patients, most of the patients suffered from benign haematological pathologies or benign pathologies of the spleen, moreover three patients underwent partial/subtotal splenectomy. It would be interesting to check the ncRNome expression in subgroups of patients with specific pathologies and to determine which of the transcripts are specific to a given pathology or to splenectomy per se.

Certainly, such data should be confirmed in additional larger sets of patients analysed by independent groups and further followed by functional studies to understand the specific mechanisms related to splenectomy long-term complications. Overall, our exploratory study introduces the idea that splenectomy leads to changes at the ncRNA level, which could lead to a better understanding of the infectious and non-infectious complications that follow splenectomy.

ACKNOWLEDGEMENTS

The work of Dr Vasilescu and Dr Tudor was supported in part by grant CEEEX 187/2006 (The Program for Excellence in Research) and by Ministry of National Education, CNCS-UEFISCDI project number 22 from 28/08/2013 (PN-II-ID-PCE-2012-4-0018). Dr Calin is the Felix L. Endowed Professor in Basic Science. Work in Dr Calin's laboratory is supported by National Institutes of Health (NIH/NCATS) grant UH3TR00943-01 through the NIH Common Fund, Office of Strategic Coordination (OSC), the NCI grants 1R01 CA182905-01 and 1R01CA222007-01A1, an NIGMS 1R01GM122775-01 grant, a U54 grant #CA096297/CA096300 - UPR/MDACC Partnership for Excellence in Cancer Research 2016 Pilot Project, a Team DOD (CA160445P1) grant, a Ladies Leukemia League grant, a Chronic Lymphocytic Leukemia Moonshot Flagship project, a Sister Institution Network Fund (SINF) 2017 grant, and the Estate of C. G. Johnson Jr

CONFLICT OF INTEREST

The authors confirm that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

MPD, ST, GAC and CV designed the study. MPD, ST, GAC and CV drafted the manuscript; all authors edited and approved the final version. ST and CV recruited human samples. MPD, KO, ST, MS, DEG performed experiments. MS, MPD, CI, MC, WRH performed the array and computational analysis.

DATA AVAILABILITY STATEMENT

The array data that support the findings of this study are openly available in GEO at <https://www.ncbi.nlm.nih.gov/geo/>, accession

numbers: GSE133588 for 2017 training cohort and GSE133589 for 2019 validation cohort.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Dragomir MP, Tudor S, Okubo K, et al. The non-coding RNome after splenectomy. *J Cell Mol Med.* 2019;23:7844-7858. <https://doi.org/10.1111/jcmm.14664>