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Effect of traditional or heat-not-burn cigarette smoking on circulating miRNAs in healthy subjects

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Abstract

Background: Traditional combustion cigarette (TCC) smoking is an established risk factor for several types of cancer and cardiovascular diseases. Circulating microRNAs (miRNAs) represent key molecules mediating pathogenetic mechanisms, and potential biomarkers for personalized risk assessment. TCC smoking globally changes the profile of circulating miRNAs. The use of heat-not-burn cigarettes (HNBCs) as alternative smoking devices is rising exponentially worldwide, and the circulating miRNA profile of chronic HNBC smokers is unknown. We aimed at defining the circulating miRNA profile of chronic exclusive HNBC smokers, and identifying potentially pathogenetic signatures.

Methods: Serum samples were obtained from 60 healthy young subjects, stratified in chronic HNBC smokers, TCC smokers and nonsmokers (20 subjects each). Three pooled samples per group were used for small RNA sequencing, and the fourth subgroup constituted the validation set.

Results: Differential expression analysis revealed 108 differentially expressed miRNAs; 72 exclusively in TCC, 10 exclusively in HNBC and 26 in both smoker

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groups. KEGG pathway analysis on target genes of the commonly modulated miRNAs returned cancer and cardiovascular disease associated pathways. Stringent abundance and fold-change criteria nailed down our functional bioinformatic analyses to a network where miR-25-3p and miR-221-3p are main hubs.

Conclusion: Our results define for the first time the miRNA profile in the serum of exclusive chronic HNBC smokers and suggest a significant impact of HNBCs on circulating miRNAs.

KEYWORDS

biomarker, cancer, cardiovascular, heated tobacco product, microRNA, smoking

1 | INTRODUCTION

A healthy lifestyle is nowadays regarded as the best action for disease prevention to decrease both chronic and acute disease incidence in the general population. Among the modifiable risk factors, traditional combustion cigarette (TCC) tobacco smoking is the leading one against which clinicians fight, with a demonstrated causative role in the establishment and progression of cancer, lung diseases, chronic obstructive pulmonary disease, diabetes and cardiovascular diseases (CVDs).[1–3]

Despite the efforts made worldwide in the last 15 years to reduce smoking through restrictions and higher taxation on tobacco, TCC use is still one of the main risk factors for premature mortality and morbidity, with the absolute number of smokers still showing an increasing trend in the world population.[4]

The mounting awareness of the harmful consequences of TCC smoking have pushed towards the development of alternative smoking devices, such as heat-not-burn cigarettes (HNBCs), which have been initially presented as safer than TCCs.[5] However, HNBCs still have harmful consequences on health, despite at a lower extent, which qualifies them as modified risk products. This definition is mostly due to the lack of combustion-related specific toxic compounds. Moreover, the number of HNBC users is rising exponentially, already involving over 6% of the adult population in Europe, with alarming projections for the next decade.[6,7]

TCC smoking can harm every organ of the body, and its pathogenetic consequences have been long studied using model systems and in vitro assays on specific cell types. TCC smoke has been shown to affect the profile of circulating molecules detected in serum as a consequence of chronic, as well as occasional exposure to cigarette smoke. These molecules include reactive oxygen species (ROS), growth factors and microRNAs (miRNAs) which can affect endothelial function, immune response and other mechanisms which can trigger disease establishment in multiple organs.[8–13]

MiRNAs are a family of short single-stranded non-coding RNA molecules that regulate post-transcriptional gene silencing through base-pair binding on their target mRNAs,[14] thereby regulating virtually all cellular and biological processes.[15]

Circulating miRNAs have been shown to play an active part in mediating both physiological and pathological mechanisms.[16] In addition, they can function as biomarkers for disease progression in cancer and CVDs, as well as for precision medicine applications and personalized risk assessment.[17,18]

Several transcriptomic studies have shown that TCC smoke exposure globally changes the profile of circulating miRNAs in both health and disease conditions.[12,19] Both chronic and acute exposure to cigarette smoke can affect the presence of specific miRNAs in the bloodstream which can induce consequences in multiple cell types, including endothelial cells,[11] or lung cells.[13] Changes in the circulating miRNAs can be reflected in specific organs, which show a modified miRNA expression profile after exposure to cigarette smoke.[13]

The effects of HNBCs on circulating miRNAs have not been thoroughly investigated yet. Given the relatively short time since their market launch, data on their long-term health effects (e.g. cancer or chronic diseases incidence) are still unavailable. Some evidence on the effects on miRNAs has been collected in one animal study revealing a global downregulation of miRNAs in rats exposed for 90 days to mainstream smoke from a modified risk smoking device[20] corresponding to the HNBCs used for our study.

In this paper, we aimed at profiling by next generation sequencing the circulating miRNAs in serum samples collected from young healthy individuals previously enrolled in the SUR-VAPES chronic clinical study, stratified based on chronic use of either TCCs or a specific HNBC device

(mean exclusive use of HNBC = 1.5 ± .5 years), and compared them to matched nonsmoker subjects.[21]

2 | METHODS

2.1 | Study approval

The samples used were collected for the SUR-VAPES Chronic clinical study. Subject recruitment, participant characteristics and experimental procedures were previously reported by Loffredo et al.[21] All the recruited subjects provided written informed consent to participate in the study which was granted approval by the Policlinico Umberto I Ethics Committee (protocol number 813/14) and conducted in compliance with the Declaration of Helsinki.

2.2 | Samples collection and experimental design

The serum samples had been previously collected from 60 healthy young subjects recruited for SUR-VAPES Chronic study, stratified in three experimental groups of 20: chronic HNBC smokers (mean exclusive use of HNBC = 1.5 ± .5 years), TCC smokers and nonsmokers (NS) (M/F ratio and average age: 55% – 28 years in NS, 50% – 27 years in TCC, and 60% – 33 years in HNBC smokers). All HNBC smokers had been smoking traditional cigarettes before switching to HNBC, and the exclusive use of either smoking device was self-reported by both TCC and HNBC smokers. The nonsmokers have never smoked using any device.

The SUR-VAPES CHRONIC study design included only young and disease-free subjects, in order to limit the confounding variables in the correlation analyses performed. In particular, the subjects enrolled in the study were considered healthy as per the absence of any chronic pathological condition (metabolic, inflammatory or organ disease), allergies, symptoms of cardiovascular alterations (alteration of blood pressure and heart rhythm), and had no fever or infection in the three months before blood drawing. In addition, the subjects did not take either food supplements or drugs in the 30 days preceding the tests. Finally, at the time of blood collection enrolled women were not menstruating. Blood drawing occurred at 8 AM in fasting condition and with smoke abstinence in both smoker groups for at least 12 h.

The sample size was estimated according to the measurements collected in the SUR-VAPES Chronic study, based on data from a previous study, considering a type I error probability $\alpha = .05$, power $1 - \beta = .90$, and a specific difference and standard deviation of the relevant parameters for the study.[21] The minimum estimated sample size for each group was $n = 17$ which was increased to $n = 20$.

Due to the lack of sufficient individual volumes of sera for a high-quality RNA extraction, we generated pooled samples following established guidelines in the literature.[22]

According to the sample size calculation method for RNAseq experiments,[23] given a type I error $\alpha = .05$, a power $1 - \beta = .80$ and an effect size of .4 (applicable for human RNAseq datasets), the analysis of three samples at a sequencing depth of 10 million reads per sample would allow accurate detection of a $\log_2$ fold change of at least 1.68. Thus, we pooled our sera in order to have three samples for sequencing and, and focused only on miRNAs showing a $\log_2$FC of two for the functional analyses and validation.

The subjects were subdivided in four subgroups of five each per experimental group using a simple randomization approach with the 'split' function in R. The anthropometric and available clinical parameters from the SUR-VAPES Chronic study were tested to be homogeneous within each group and checked against the whole population, for each subgroup to be representative of the whole group of subjects. This was achieved through statistical analysis of both continuous and categorical variables (see paragraph Section 2.5). The sera of subjects in each subgroup were pooled in identical volumes to generate the samples for RNA extraction. Five serum samples per group were left un-pooled: RNA from these samples was extracted separately to constitute the validation set.

2.3 | RNA extraction and miRNAs quantification

The pooled serum samples were centrifuged at 16,000 rcf in order to remove debris, platelets or cells, and RNA was extracted from 200 µL, using the Qiagen miRNeasy kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's instructions. Syn-cel-miR-39 spike-in synthetic RNA (Qiagen GmbH, Hilden, Germany) was added to monitor extraction efficiency. RNA quality was assessed using Agilent 2100 Bioanalyser (Agilent Technologies Inc, Santa Clara, CA, USA) and the Eukariote total RNA Pico kit (Agilent Technologies Inc, Santa Clara, CA, USA). RNA samples (6 µL for each pool) were used for library generation using the NEBNext Multiplex Small RNA Prep (New England Biolabs, Ipswich, MA, USA) as previously described by Galluzzo et al.,[24] and sequenced on a

single-read flow cell by bridge amplification and 75 cycles sequencing-by-synthesis on the Illumina NextSeq550 platform (Illumina Inc., San Diego, CA, USA). The average sequencing depth obtained was 9.57 million ± 2.15 raw reads.

The levels of miR-221-3p and miR-25-3p were quantified by absolute quantification using Qiagen LNA based SYBR green detection method (miRCURY LNA miRNA PCR assay – Qiagen). Briefly, 2 μL of total RNA was used to generate the cDNA according to the standard protocol by the manufacturer. The qPCR reaction was performed on the Applied Biosystems 7900HT machine, adding the relevant ROX concentration to the qPCR mix. For copy number determination, a standard curve was generated using cDNA derived from defined amounts of synthetic RNA for each miRNA. The miRNA amount was expressed as 'copy number per well' of the qPCR plate as per the standard curve.

2.4 | Bioinformatic analysis

The small RNA-sequencing analyses were performed according to Tarallo et al.[25] Briefly, reads were quality-controlled and trimmed of adapter sequences using Cutadapt v.3.7. Reads shorter than 14 nt were removed. Surviving reads were aligned on human miRBase v22.1 hairpin sequences using BWA v0.7.12. As described in Tarallo et al.,[25] the pipeline quantifies mature miRNA sequences annotated in miRBase as well as 'novel' mature miRNAs, identified based on the read mapping position within the hairpin sequence. Read count normalization and differential expression analysis was performed with DESeq2 v1.38.3. A miRNA was defined as differentially expressed (DE) if associated with a median number of reads >15 in at least one study group and a Benjamini–Hochberg (BH) adjusted p value (adj. p) < .05.

Functional enrichment analysis was performed using RBiomirGS v0.2.12 considering only validated miRNA-targets interactions from miRTarBase v7.0 and miRecords. The analysis was performed with respect to MSigDB gene sets (libraries: c2.cp.kegg.v7.4, c2.cp.reactome.v7.4, and c5.go.bp.v7.4). Only terms associated with an adjusted p < .05 in at least two gene targets were considered as significant. Semantic similarity analysis was performed using rrvgo v1.12 using the Resnik method and a similarity threshold of .85.

2.5 | Statistical analysis

Samples were randomly assigned to each of the four subgroups, and statistical difference in each variable described was assessed by either Fisher's exact test for categorical variables, or Wilcoxon rank-sum test for continuous variables. Correlation analyses were performed using the Spearman's method. These analyses were performed in R using the basic statistics package.[26] Real-time PCR data were analysed using ordinary one-way ANOVA and Fisher LSD post-test on Prism8 Software (GraphPad Software, Boston, MA, USA).

3 | RESULTS

3.1 | HNBCs change miRNA levels in substantial overlap with TCCs

Serum samples were collected from three groups of healthy young subjects, with homogeneous anthropometric features, and stratified according to smoking status (Table S1): Nonsmokers (NS), TCC smokers and exclusive HNBC smokers (mean exclusive use = 1.5 ± .5 years). All smokers were considered chronic as they had been using each device for at least 1 month. The abundance of miRNAs was detected in pooled sera samples, and data analysed using DESeq2 (Figure 1A). The principal component analysis showed a neat separation between NS and both smoker groups, and a less pronounced difference between TCC and HNBC smoker sera (Figure 1B). Head-to-head comparison of the detected miRNAs showed that a higher number of DE miRNAs had changed levels in sera from TCC as compared to HNBC smokers (Figure 2A,B), thus suggesting a globally lower impact of HNBCs on the profile of circulating miRNAs in healthy subjects, compared to TCC use.

Differential expression analysis identified 98 differentially expressed (DE) miRNAs in TCC and 36 DE miRNAs in HNBC smokers, with an overlap for 26 miRNAs (Table S2; Figure 2C). The comparison of the fold change of DE miRNAs in TCC and HNBC versus NS control shows a positive correlation (ρ = .61, p < .001), which indicates that exposure to TCC or HNBC smoke implicates changes in the circulating miRNA levels in the same direction, but possibly to different extents (Figure 2E).

The profile of DE miRNAs revealed an overall downregulation of circulating miRNA abundance, with only 20 miRNAs upregulated in both groups of smokers versus NS (Figure 3A), consistently with previous studies.[19,27,28] Clustering analyses showed the similarity between the TCC and HNBC groups, with extensive overlap in the list of miRNAs commonly affected by both smoking types (Figure 3A). The detected level of DE miRNAs in HNBCs as compared to NS tends to an intermediate level between the NS and the TCC groups for both upregulated and downregulated miRNAs (Figure 3B).

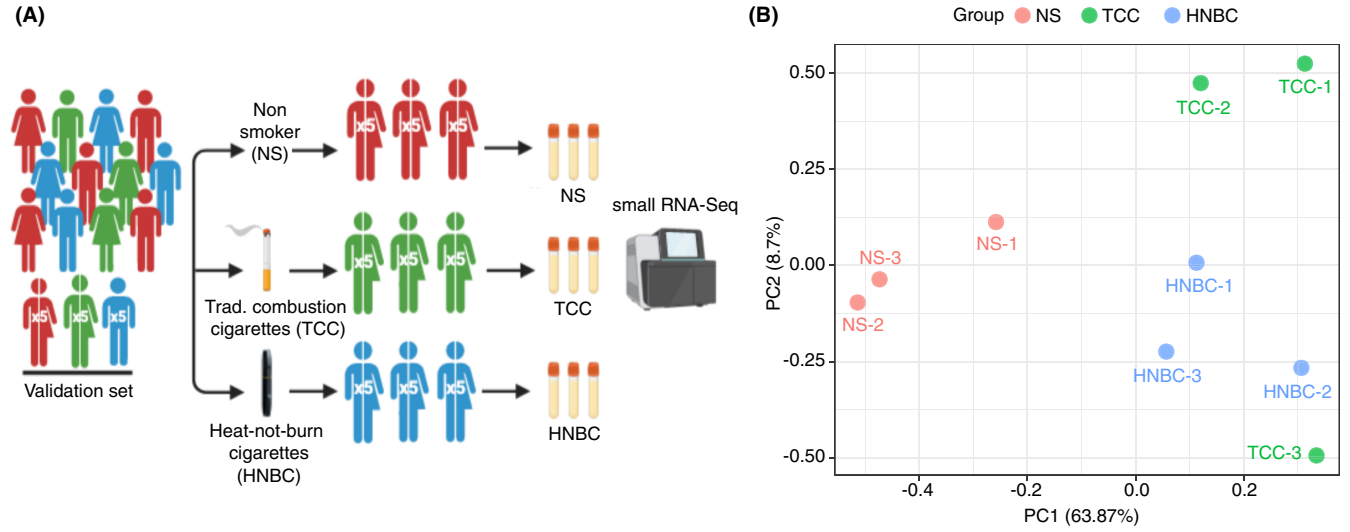

FIGURE 1 Analysis of circulating miRNAs in the serum of smokers. (A) Experimental design of the study. Three pools of serum composed of five subjects each were generated starting from a sample population of 20 subjects. The remainder five samples were not pooled and used as single subjects for validation of differentially expressed miRNAs. (B) Principal component analysis of the small RNAseq dataset. HNBC, heat-not-burn cigarette; NS, non-smokers; TCC, traditional combustion cigarette.

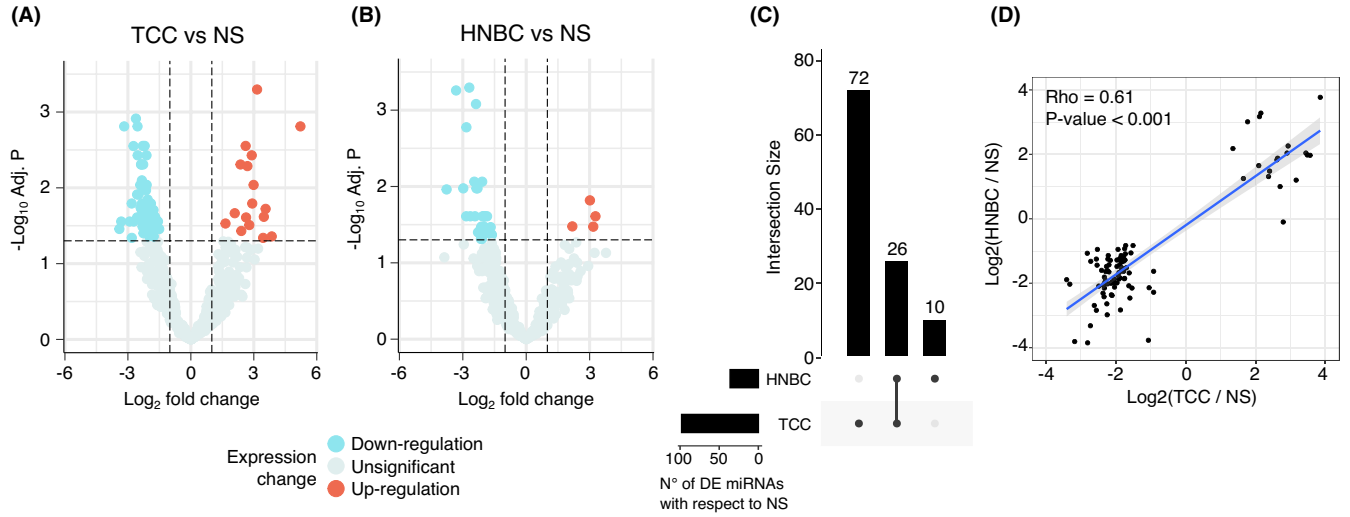

FIGURE 2 Analysis of differentially expressed circulating miRNAs. (A, B) Volcano plots showing the $\log_2$ fold change (FC) vs. $-\log_{10}$ p value of the differentially expressed miRNAs (DE miRNAs) in each group of smokers compared to nonsmokers. Dashed lines indicate the $\log_2$FC threshold set to 1 (vertical) and the $-\log_{10}$ p threshold, set to a value corresponding to the $-\log_{10}(.05)$ on the linear scale. (C) Upset plot showing the number of DE miRNAs in each group and the intersection, which constitutes the class of common DE miRNAs. (D) Correlation plot of the $\log_2$FC values in each comparison, showing the linear correlation of the changes observed in each group of smokers compared to the nonsmokers. HNBC, heat-not-burn cigarette; NS, non-smokers; TCC, traditional combusiton cigarette.

3.2 | Functional analysis of commonly deregulated miRNA targets points to cancer and CVD

A miRNA selection strategy was designed to be increasingly stringent and isolate a reliable subset of potentially bioactive miRNAs. The first selection has included a list of highly abundant DE miRNAs with a median normalized count >500 reads in at least one group: this list included the top 25% of the DE miRNAs in the analysis (Table 1). Based on the comparison in which the miRNA was identified as significantly DE, the list was divided in three classes: TCC-specific (TCC-var), HNBC-specific (HNBC-var) or common to both categories (COMMON). The clustering of samples using these miRNAs recapitulated that obtained with the whole DE miRNA list (Figure 4A). Of note, the HNBC-var class contained only one miRNA (miR-941); thus, the effect of HNBC on circulating miRNAs in our

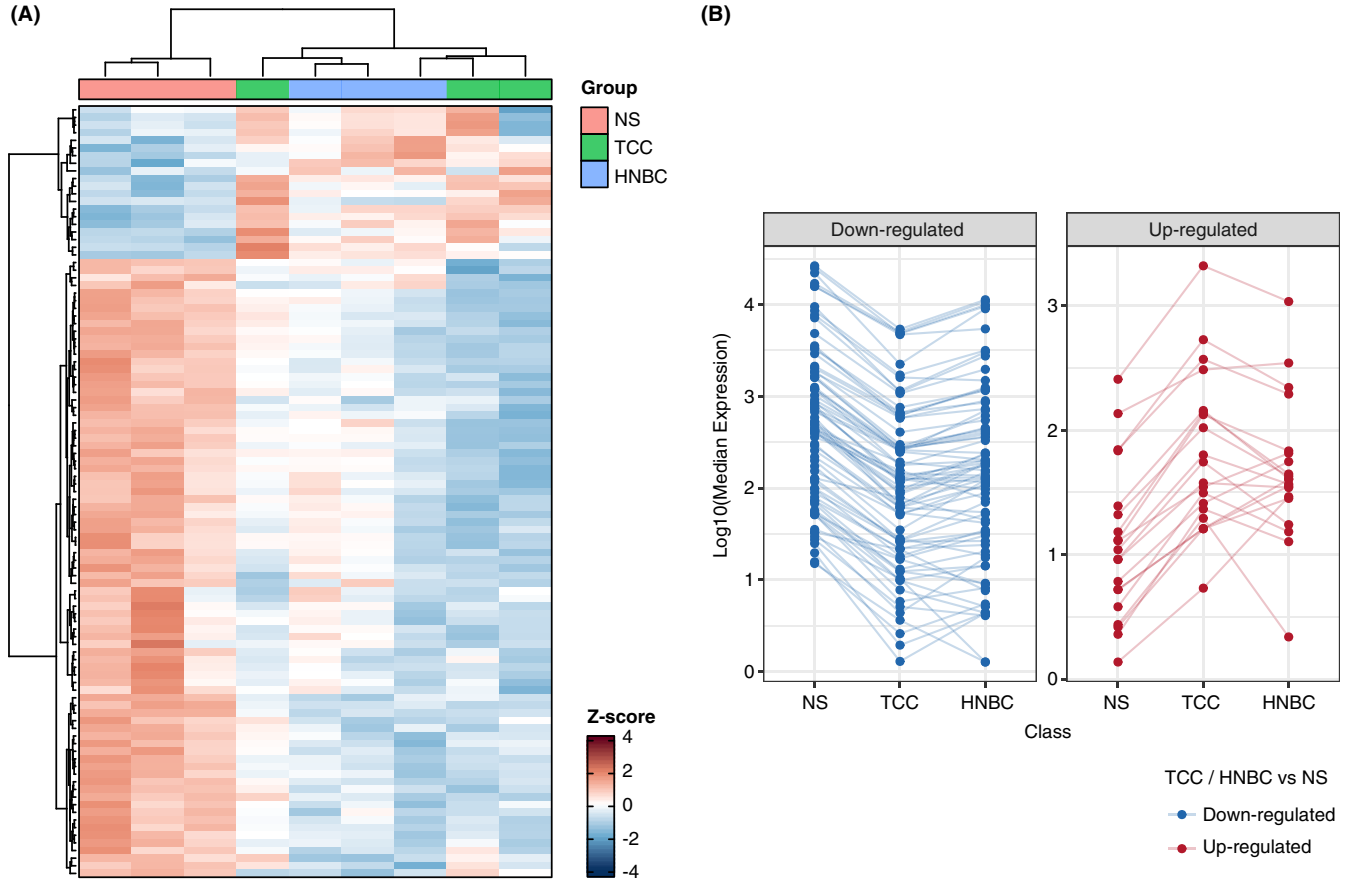

FIGURE 3 Clustering and global level of differentially expressed circulating miRNAs in traditional combustion cigarette (TCC) and heat-not-burn cigarette (HNBC) smokers. (A) Heatmap with hierarchical clustering showing the differentially expressed miRNA clustering in sera of nonsmokers (NS), TCC and HNBC smokers. (B) Line plot showing each single detected miRNA abundance in the three groups (Median expression value) for both downregulated and upregulated miRNAs. HNBC, heat-not-burn cigarette; NS, non-smokers, TCC, traditional combustion cigarette.

datasets is almost completely included in that of TCC. All the selected DE miRNAs were downregulated in smokers, except for miR-200a-3p and miR-200b-3p, which were upregulated in the TCC group (Figure 4A).

The global downregulation observed suggests a derepression of molecular pathways regulated by these miRNAs. The functional analysis was performed on the target genes of the DE miRNAs commonly deregulated in TCC and HNBC, only in one smoking group, and on a fourth group containing the whole list of TCC deregulated miRNAs (TCC-only = COMMON + TCC-var), to have a reference for the pathways potentially affected by TCC on its own. The Gene Ontology (GO) analysis highlighted 733 enriched biological processes that were clustered to 191 parent terms based on their semantic similarity (Tables S3 and S4). It also showed that the most significantly enriched terms refer to miRNAs deregulated in the COMMON class, which almost completely overlaps with the HNBC-var class (Figure S1), suggesting that the effects of both kinds of smoking on circulating miRNAs affect similar biological processes. The GO terms enriched in the targets of miRNAs in the COMMON category refer to cell differentiation, cell cycle control and immune response (Figure S1).

The molecular pathways regulated by the affected miRNAs were further investigated using a KEGG analysis approach, which retrieved a relevant number of cancer-associated pathways enriched in targets of DE miRNAs, followed by signalling and CVD pathways (Figure 4B; Table S3). The comparison of KEGG terms between the TCC-only and COMMON/HNBC-var categories showed that the terms in the latter group are still enriched in cancer-related pathways. This observation points to a deregulation of circulating miRNAs in chronic HNBC smokers targeting pathways known to be altered in cancer and overlapping with the deregulation observed in TCC smokers. The KEGG analysis was in line with the GO analysis where the enriched terms were related to neoplastic transformation of cells and CVD emergence (Figure S1).

As a further selection criterion, the miRNAs with at least two-fold change in the COMMON category (HNBC and TCC) were filtered, among those with a minimum of

TABLE 1 Selection of differentially expressed microRNAs.

	Median levels			log_2FC			Adjusted *p* value			
ID	**NS**	**TCC**	**HNBC**	**TCC vs. NS**	**HNBC vs. NS**	**TCC vs. HNBC**	**TCC vs. NS**	**HNBC vs. NS**	**TCC vs. HNBC**	**Class**
miR-223-3p	532	61	54	−3.18	−3.81	.64	**.002**	**.000**	1.00	Common
miR-221-3p	1191	179	198	−2.62	−2.69	.08	**.001**	**.001**	1.00	Common
miR-199a-5p	1256	249	203	−2.37	−2.31	−.07	**.005**	**.011**	1.00	Common
miR-191-5p	7681	1606	1490	−2.11	−2.38	.26	**.004**	**.001**	1.00	Common
miR-25-3p	9499	1111	2752	−2.33	−2.15	−.18	**.016**	**.035**	1.00	Common
miR-106b-3p	798	115	231	−2.33	−2	−.33	**.008**	**.034**	1.00	Common
miR-363-3p	525	119	174	−2.14	−2	−.14	**.011**	**.025**	1.00	Common
miR-30e-5p	1874	408	547	−2.11	−1.96	−.14	**.009**	**.025**	1.00	Common
miR-1307-3p	3552	578	897	−2.06	−1.99	−.07	**.014**	**.025**	1.00	Common
miR-127-3p	1663	298	425	−1.9	−1.84	−.06	**.023**	**.040**	1.00	Common
miR-186-5p	677	259	242	−1.62	−1.68	.06	**.027**	**.034**	1.00	Common
miR-941	732	135	189	−1.54	−2.15	.61	.122	**.037**	1.00	HNBC-var
miR-222-3p	3278	759	1155	−2.24	−2.02	−.22	**.028**	.071	1.00	TCC-var
miR-151a-3p	22,146	2240	9010	−2.41	−1.6	−.81	**.009**	.110	1.00	TCC-var
let-7d-5p	1024	274	357	−2.05	−1.87	−.18	**.030**	.076	1.00	TCC-var
miR-4433b-3p	588	65	186	−2.56	−1.25	−1.31	**.012**	.264	1.00	TCC-var
miR-361-3p	756	127	202	−1.91	−1.87	−.04	**.035**	.064	1.00	TCC-var
miR-16-2-3p	858	178	330	−2.27	−1.48	−.8	**.005**	.099	1.00	TCC-var
miR-146a-5p	4844	1076	1969	−2.2	−1.43	−.77	**.003**	.074	1.00	TCC-var
miR-532-5p	840	270	364	−1.91	−1.67	−.24	**.022**	.065	1.00	TCC-var
miR-409-3p	3369	656	1174	−2.16	−1.29	−.87	**.019**	.184	1.00	TCC-var
let-7i-5p	15,833	4712	5438	−1.84	−1.61	−.23	**.023**	.066	1.00	TCC-var
miR-140-3p	1610	275	617	−1.97	−1.48	−.49	**.028**	.128	1.00	TCC-var
miR-28-3p	1604	270	452	−1.82	−1.62	−.19	**.028**	.076	1.00	TCC-var
miR-425-5p	1195	277	405	−1.78	−1.63	−.15	**.023**	.055	1.00	TCC-var
miR-146b-5p	2129	667	857	−1.86	−1.49	−.37	**.044**	.136	1.00	TCC-var
miR-30d-5p	24,944	4818	9573	−1.9	−1.44	−.46	**.016**	.088	1.00	TCC-var
miR-423-3p	8589	1708	3164	−1.79	−1.55	−.24	**.035**	.099	1.00	TCC-var
let-7b-5p	26,456	5397	11,296	−1.85	−1.35	−.5	**.030**	.152	1.00	TCC-var
miR-24-3p	2916	620	1245	−1.85	−1.32	−.53	**.022**	.128	1.00	TCC-var

(Continues)

TABLE 1 (Continued)

ID	Median levels			log_2FC			Adjusted *p* value			Class
	NS	TCC	HNBC	TCC vs. NS	HNBC vs. NS	TCC vs. HNBC	TCC vs. NS	HNBC vs. NS	TCC vs. HNBC	
miR-451b-3p	1207	246	701	−1.89	−1.24	−.65	**.046**	.236	1.00	TCC-var
miR-451b	15,782	4884	9914	−1.79	−1.32	−.46	**.044**	.168	1.00	TCC-var
miR-128-3p	7175	1149	3040	−1.87	−1.23	−.64	**.023**	.168	1.00	TCC-var
miR-451a	16,998	5033	10,824	−1.77	−1.31	−.46	**.044**	.168	1.00	TCC-var
miR-142-5p	1812	584	728	−1.74	−1.28	−.46	**.025**	.128	1.00	TCC-var
miR-543	915	269	446	−1.75	−.93	−.82	**.025**	.290	1.00	TCC-var
miR-148b-3p	737	264	388	−1.57	−1.06	−.5	**.028**	.170	1.00	TCC-var
miR-328-3p	2033	621	1213	−1.51	−.83	−.68	**.035**	.311	1.00	TCC-var
miR-200a-3p	68	535	221	3.48	1.98	1.5	**.024**	.248	1.00	TCC-var
miR-200b-3p	257	2101	1085	3.44	2.04	1.41	**.046**	.292	1.00	TCC-var

Note: Table showing the list of miRNAs selected according to abundance in serum (counts >500). Significant adjusted *p* values are reported in bold. Bold character in the ID column shows the differentially expressed miRNAs with a fold change greater than two in the common class.

500 normalized counts in at least one group, thus restricting to nine DE miRNAs (Table 1—miRNAs in bold character). This approach, aimed at identifying genes functionally affected by the observed miRNA deregulation in blood and possibly target organs, and restricted the analysis to 10% of all the DE miRNAs in the dataset (Table 1; Figure S2). For these selected miRNAs, a network was designed including only miRNA-gene interactions experimentally validated (Table S5). This set of stringent parameters identified a core network composed of nine miRNAs and 203 genes organized in two main hubs (Figure 5A) where the genes targeted by the most-connected miRNAs (i.e. miR-25-3p and miR-221-3p) are involved in cancer and CVD-related pathways. Specifically, miR-25-3p was connected with cancer- and CVD-related genes, while miR-221-3p prevalently with cancer-related genes (Figure 4A). The expression of these two miRNA hubs was tested in the independent validation set of five subjects per group through quantitative real-time PCR, and data confirmed the expression levels observed in the small RNA-Seq dataset (Figure 5B).

4 | DISCUSSION

The use of extracellular miRNAs as biomarkers has been proven effective as well as minimally invasive for the detection and follow-up of many diseases. Indeed, changes in circulating miRNAs have been associated with many pathological conditions, such as (but not limited to) type 2 diabetes, obesity, CVD and cancer. Therefore, alterations occurring in healthy individuals due to specific habits such as smoking should be well described and considered when assessing the reliability of specific miRNAs as disease biomarkers. The miRNAs identified here as correlated with the chronic and exclusive use of HNBC may be biomarkers of exposure to this specific alternative smoking device, and may regulate gene expression in target organs.

The identification of a signature of miRNAs altered by chronic smoking of both TCC and HNBC in healthy subjects is relevant in the use of miRNAs as disease biomarkers. For example, it has been reported that TCC smoking can affect the reliable identification of circulating miRNAs, including miR-25-3p, as diagnostic biomarkers in lung cancer[29]; hence, the smoking status, including the use of modified risk products such as HNBCs, might interfere with the monitoring of the disease. The analysis of circulating miRNAs may also serve as an indicator of the physiological status of the subjects, and changes in the circulating miRNAs have been correlated with differences in lifestyle activities, such as exercise or smoking, in otherwise healthy people,[19,30] as well as in physiological ageing.[31] In this unique and homogeneous group of

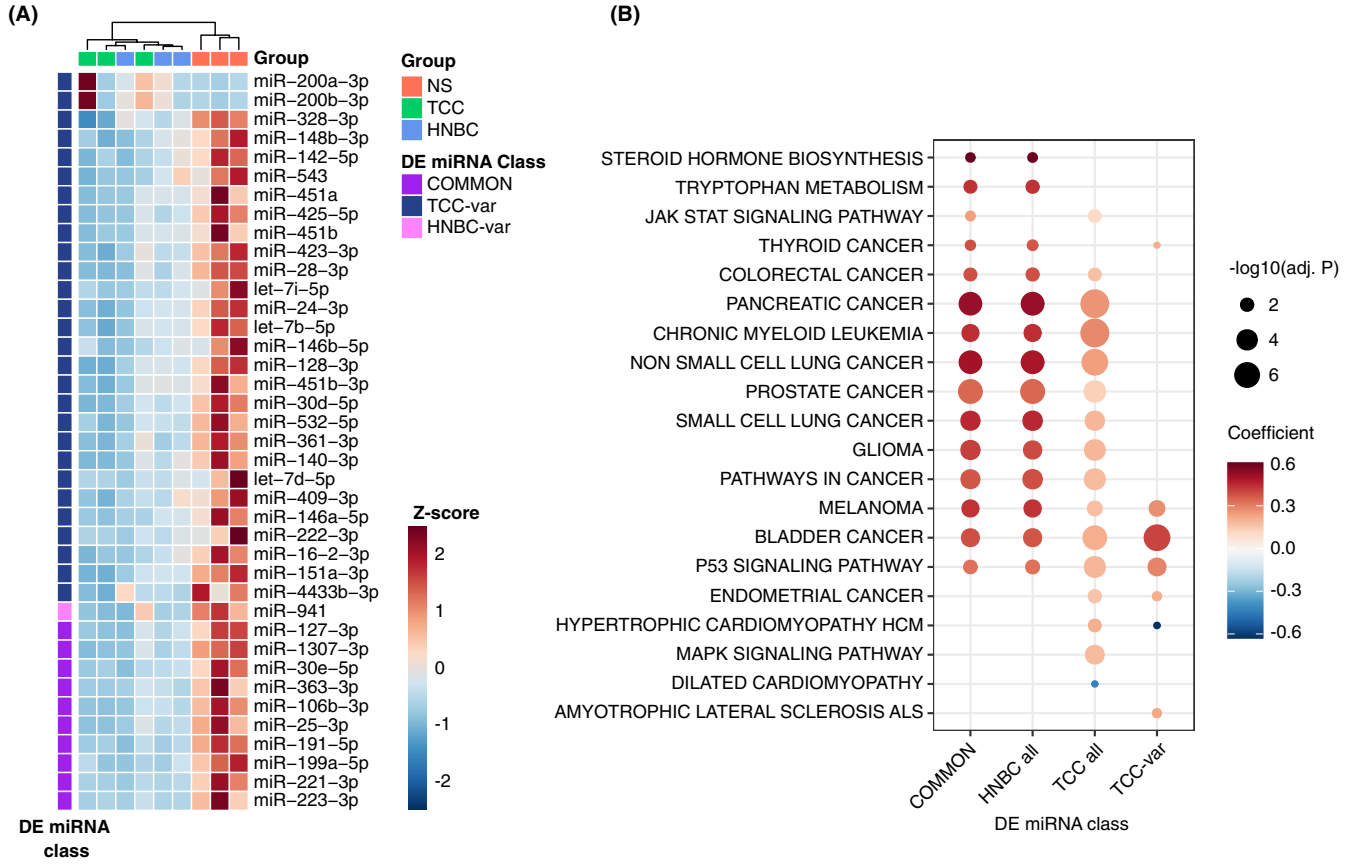

FIGURE 4 Highly abundant circulating miRNAs and the functional pathways affected. (A) Heatmap with hierarchical clustering showing the relative fold-change of the sub-list of differentially expressed miRNAs (DE miRNAs) selected according to abundance in serum (counts >500). The code colour in the legend shows the sample groups, as well as the classification according to whether the miRNA level changes are detected in either both types of smokers (Common) or only in one of the two (TCC_var and HNBC_var). (B) Bubble graph showing the enriched terms in KEGG functional analysis of the targets of the DE miRNAs changing in both groups (Common), in the HNBC group including the class Common (HNBC all), in the TCC group including the class Common (TCC all) and only in TCC group (TCC-var). Bubble size is linearly correlated with the significance of the enrichment ($-\log_{10}$ adj. p value) and bubble colour is related to the coefficient, which is the predicted up- (red) or down-regulation (blue) of each listed pathway. HNBC, heat-not-burn cigarette; NS, nonsmokers; TCC, traditional combustion cigarette.

healthy young individuals smoking exclusively either TCCs or HNBCs, enrolled in the SUR-VAPES Chronic study, the effects of TCC smoke on circulating miRNAs seem to be extensively recapitulated by the modified risk product namely HNBCs. The changes detected in circulating miRNAs of young healthy smokers using TCC as well as HNBC exclusively might predispose these subjects to the development of diseases, or to have a weaker response to organ injury or damage.[32]

Interestingly, we observed a common trend for the abundance of circulating miRNAs modulated in HNBC smokers to be positioned at intermediate values between the NS and the TCC smokers. Despite with some limitations due to the pooling of samples and the lack of a matched control group of former smokers (many of the HNBC smokers in our study are in fact former TCC smokers, with abstinence from TCC since at least 1.5 years[21]), our data cannot rule out a partial contribution of a wash-out effect in the HNBC group. Nonetheless, it has been reported that quitting smoking brings the profile of most plasma miRNAs back to levels comparable to nonsmokers in 1 month,[19] and the subjects enrolled in the present study in the HNBC group have been exclusively smoking HNBCs for 1.5 years on average. Therefore, our data can indeed point out that some fundamental alterations on circulating miRNAs are maintained when switching from TCC to chronic exclusive HNBC smoke, albeit to a lower global extent compared to chronic TCC use.

Many GO terms and pathways identified in our study from the list of altered miRNAs are linked with leukocyte biology and differentiation. One might speculate that potential systemic effects of the observed circulating miRNA depletion might contribute to disease development also via altered inflammatory responses.[33]

Our final stringent network analysis evidenced two main hubs, with interesting interactions and targets from

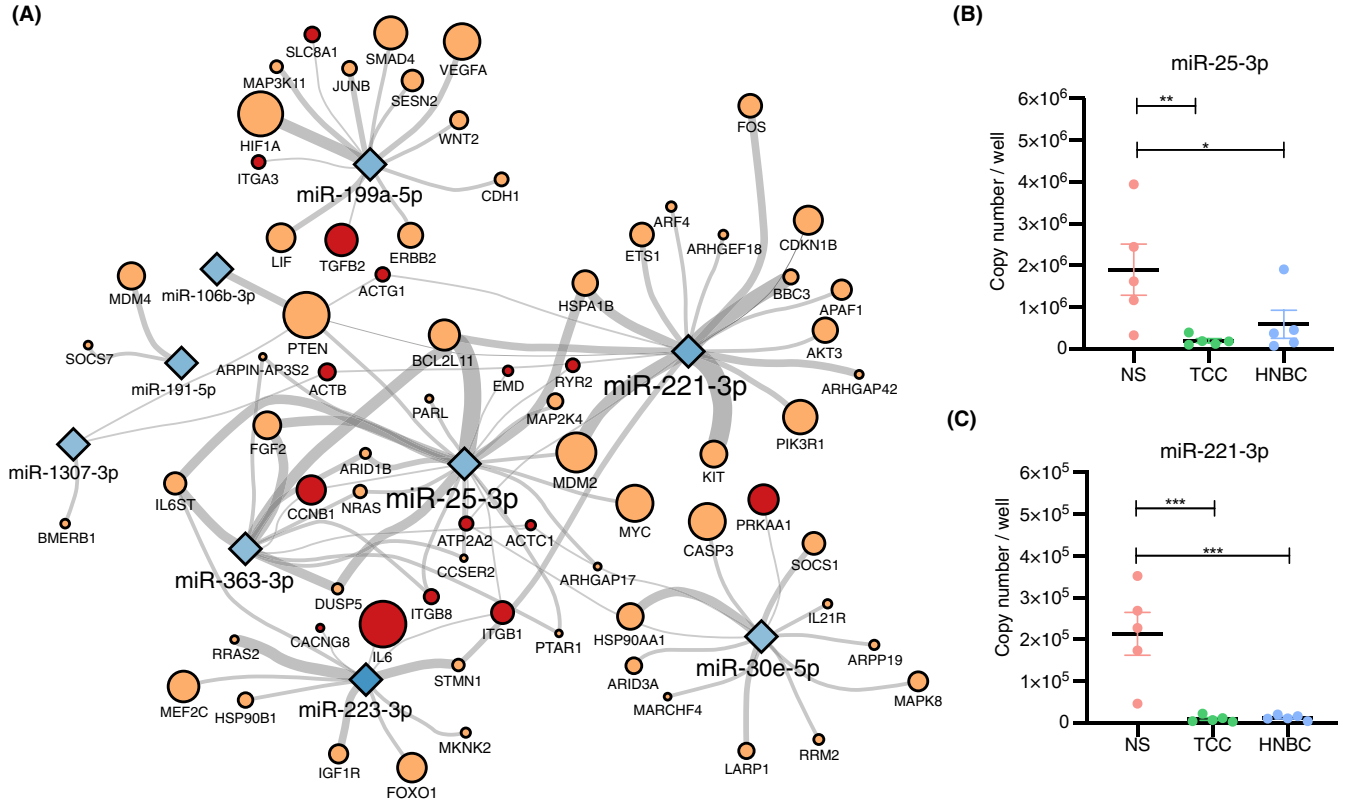

FIGURE 5 Network for target analysis of selected DE miRNAs. (A) Network of the miR/mRNA connections of the most abundant and highly changing miRNAs in both TCC and HNBC smokers (counts >500 and fold-change >2) compared to NS. The size of each gene is proportional to the number of times the term occurs within the enriched pathways in our functional analysis. The edge thickness is proportional to the number of evidence supporting the miR-mRNA interaction. The colouring of genes refers to the gene being present in cardiovascular disease- (red) or cancer- (orange) related pathways. (B) Dot plot showing the copy number obtained by real-time qPCR absolute quantification of the two hub miRNAs identified, that is miR-25-3p and miR-221-3p. Each dot shows the copy number/well in the PCR reaction as obtained by the standard curve method. Error bars show SEM statistical analysis was performed using two-way ANOVA with multiple comparison testing. *$p < .05$, **$p < .01$, ***$p < .001$. HNBC, heat-not-burn cigarette; NS, nonsmokers; TCC, traditional combustion cigarette.

a pathological perspective. The precursor of miR-25-3p is part of a genomic cluster, highly conserved in vertebrates, which includes miR-106b and miR-93 pre-miRNA. Three mature miRNAs of this cluster show downregulation in the serum of smokers, with miR-25-3p being the most abundant among the three members of the cluster (9499 normalized reads for miR-25-3p, compared to 396 of miR-93-5p, and 797 of miR-106b-3p, as per the normalized expression values in DE analysis, in the NS group). Deranged expression of miR-25-3p has been associated with multiple cardiovascular and respiratory insults and diseases. This miRNA was shown to be regulated in a time-dependent way in cardiac hypertrophy, fibrosis and heart failure.[34] In many systems and after different pathological stimuli, miR-25-3p decrease determines an increase in oxidative stress. This effect is mediated by the de-repression of *NOX4*, as demonstrated in cardiac injury, as well as in a kidney model of streptozotocin-induced type II diabetes.[35,36] This is also consistent with the general increase in ROS and oxidative stress markers previously described in our samples in the SUR-VAPES Chronic study.[21] Thus, a lower level of circulating miR-25-3p could predispose the organism to oxidative stress in multiple organs. Moreover, the miR-25 cluster has shown protective effects against TGF-beta-induced epithelial-to-mesenchymal transition, which is responsible for tissue fibrosis in different organs, including the heart.[37–39] MiR-25-3p has also a protective role against apoptosis in both heart and brain ischemia/reperfusion injury, as demonstrated in rat ventricular myocytes and in neuronal cell models.[40,41] Finally, atherosclerosis development with intimal hyperplasia was also linked to miR-25-3p repression in vascular smooth muscle cells subjected to thrombospondin treatment.[42] In addition to CVD, miR-25-3p was demonstrated to be repressed in human tracheal smooth muscle cells exposed to pro-inflammatory cytokines, as a model of bronchial asthma. The repression of miR-25-3p resulted in the increase of its target gene Krüppel-like factor 4 (*KLF4*), a well-known mediator of inflammation.[43] Tobacco smoking from both TCC and HNBC is associated with asthma symptoms and

with a faster development of lung disfunction.[44] Overall, miR-25-3p could mediate disease mechanisms associated with smoke-induced increase in airway epithelia inflammation.

MiR-221-3p is the second main hub identified by our network analysis (Figure 4A), and it has been described as both an oncogene and a tumour suppressor, depending on the cellular context. In particular, miR-221-3p was found to: (i) inhibit cell proliferation and induce apoptosis in various cancers, such as erythroleukemia and gastrointestinal stromal tumour pathogenesis[45,46]; (ii) inhibit the proliferation and migration of epithelial ovarian cancer cells through downregulation of *ARF4*[47]; and (iii) to promote cell cycle arrest and apoptosis, and inhibit proliferation in medulloblastoma cells.[48] Therefore, reduced miR-221-3p levels point to several tumour-promoting pathways. The circulating levels of miR-221-3p are also downregulated in atherosclerosis and stroke, where they can be used as a biomarker.[49,50] Interestingly, the molecular mechanism whereby low levels of miR-221-3p might accelerate atherosclerosis has been linked with oxidative stress increase. In fact, miR-221-3p was found to inhibit ox-LDL-induced foam cell formation, to suppress ox-LDL-induced oxidative stress and apoptosis in monocytes,[51] thus opposing to atherosclerotic plaque formation and progression. Notably, the subjects included in the present study have been previously assessed for vascular function, oxidative stress and platelet activation, all of which were found impaired in the HNBC cohort of the SUR-VAPES Chronic study.[21] The increase in oxidative stress and platelet activation was assessed through the measurement of different soluble markers; interestingly, the levels H_2O_2 sNOX2-dp (soluble NOX2-derived peptide) both markers of oxidative stress, and sCD40L (soluble CD40 Ligand) a marker of platelet activation, were inversely correlated with those of circulating miR-221-3p, with sCD40L also correlating with miR-25-3p levels, in the 15 individual samples from the validation set (Figure S3). It is worth noticing that both miRNAs significantly correlated to the cotinine levels in this same validation set.

We acknowledge several limitations of our study. The number of subjects and the amount of available sample were limited, and the sample pooling has not allowed correlation analyses on the available blood parameters or vascular function for all the subjects. This has not allowed a thorough assessment of the validity of the detected DE miRNAs as markers for exposure to smoke from TCCs or HNBC devices. Also, working with human samples from healthy subjects, we could not provide insights on the correlation between circulating miRNA levels and their expression in organs and tissues. Nevertheless, to the best of our knowledge the data collected here are the first evidence of the effects of chronic exclusive HNBC smoking on the circulating miRNAs in young healthy individuals. Despite a wider and multi-cohort analysis being needed, the results of this pilot study showed a promising role of circulating miRNAs as biomarkers of an early molecular alteration that might occur in HNBC users.

In conclusion, our findings will need to be further developed in future studies to provide insights on the efficacy of circulating miRNAs as biomarkers for early-stage diseases linked with smoke exposure from both sources, as well as on the molecular mechanisms leading to cellular dysfunction through impaired miRNAs signalling due to smoke exposure.

AUTHOR CONTRIBUTIONS

VP and FP conducted experiments and analysed data; GFe designed and performed bioinformatic analyses; BP and ST conducted experiments and provided reagents; CC and EF conducted experiments; XD analysed data; CN, LL, RC and GFr acquired clinical data and provided the serum samples; GBZ statistical analysis; IC and FP designed the study and wrote the manuscript.

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CONFLICT OF INTEREST STATEMENT

Giuseppe Biondi-Zoccai has consulted for Amarin, Balmed, Cardionovum, Crannmedical, Endocore Lab, Eukon, Guidotti, Innovheart, Meditrial, Microport, Opsens Medical, Terumo and Translumina, outside the present work. All other authors report no conflict of interest.

DATA AVAILABILITY STATEMENT

The raw and processed small RNA-Seq data were deposited in Gene Expression Omnibus with the identifier GSE233237.

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SUPPORTING INFORMATION

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

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