Bayesian network analysis of plasma microRNA sequencing data in patients with venous thrombosis

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MicroRNAs (miRNAs) are small regulatory RNAs participating to several biological processes and known to be involved in various pathologies. Measurable in body fluids, miRNAs have been proposed to serve as efficient biomarkers for diseases and/or associated traits. Here, we performed a next-generation-sequencing based profiling of plasma miRNAs in 344 patients with venous thrombosis (VT) and assessed the association of plasma miRNA levels with several haemostatic traits and the risk of VT recurrence. Among the most significant findings, we detected an association between hsa-miR-199b-3p and haematocrit levels ($P = 0.0016$), these two markers having both been independently reported to associate with VT risk. We also observed suggestive evidence for association of hsa-miR-370-3p ($P = 0.019$), hsa-miR-27b-3p ($P = 0.016$) and hsa-miR-222-3p ($P = 0.049$) with VT recurrence, the observations at the latter two miRNAs confirming the recent findings of Wang et al. Besides, by conducting Genome-Wide Association Studies on miRNA levels and meta-analyzing our results with some publicly available, we identified 21 new associations of single nucleotide polymorphisms with plasma miRNA levels at the statistical significance threshold of $P < 5 \times 10^{-8}$, some of these associations pertaining to thrombosis associated mechanisms. In conclusion, this study provides novel data about the impact of miRNAs’ variability in haemostasis and new arguments supporting the association of few miRNAs with the risk of recurrence in patients with venous thrombosis.

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Los micro-ARN (miARN) son pequeñas moléculas de ARN reguladoras que participan en varios procesos biológicos y están implicados en diversas patologías. Muestrables en los líquidos corporales, se ha planteado que los miARN pueden ser biomarcadores eficaces para el diagnóstico de enfermedades y/o características asociadas. Aquí hemos llevado a cabo un análisis de miARN plasmático con tecnología de secuenciación de última generación en 344 pacientes con trombosis venosa (TV) y hemos evaluado la asociación de los niveles de miARN con distintas características hemostáticas y el riesgo de recidiva de TV. Entre los hallazgos más significativos, hemos detectado una asociación entre hasa-miR-199b-3p y los niveles de hemocritos (p = 0,0016); dos marcadores que se habían asociado de forma independiente con el riesgo de sufrir TV. Asimismo, hemos observado una evidencia indicativa de asociación entre hasa-miR-370-3p (p = 0,019), hasa-miR-27b-3p (p = 0,016) y hasa-miR-222-3p (p = 0,049) y la recidiva de TV; los resultados los dos últimos miARN confirmaron los hallazgos recientes de Wang et al. (Clin Epigenetics, 2019). Además, al efectuar estudios de asociación del genoma completo sobre los niveles de miARN y al metaanализar nuestros resultados con otros disponibles públicamente, hemos identificado 21 asociaciones nuevas de polimorfismos de un solo nucleótido (PSN) con niveles de miARN plasmático con un umbral de significación estadística de p < 5 x 10^-4; algunas de estas asociaciones pertenecen a los mecanismos patogénicos de la trombosis.

Como conclusión, en este estudio se proporcionan nuevos datos sobre el impacto de la variabilidad de miARN en la hemostasia y nuevos argumentos que apoyan la asociación de algunas secuencias de miARN con el riesgo de recidiva en pacientes con trombosis venosa.
established biomarkers that serve these aims, even if D-dimers measurement has been proposed but lacks specificity. We here propose a comprehensive microRNA (miRNA) profiling from plasma samples of VT patients aimed at discovering miRNA-derived biomarkers discriminating between PE and DVT and associated with VT recurrence. MicroRNAs represent a class of small (~22 nucleotides) non-coding RNAs that participate in genes post-transcriptional regulation. It is now well-established that miRNAs are involved in the development of human diseases, in particular, cardiovascular ones. Several genes participating to thrombosis associated mechanisms have already been suspected to be subject to miRNA regulation.

So far, epidemiological studies looking for association of plasma miRNAs with VT outcomes are still scarce. Using plasma samples of 20 VT cases and 20 healthy individuals, Starikova et al. assessed the association of 97 miRNAs with VT risk among which 9 were found significantly (P < 0.05) associated with the outcome. As for Wang et al., by looking for the association of 110 miRNAs with the risk of VT recurrence in plasma samples of 39 cases and 39 controls, 12 miRNAs were identified. None of these observations, which were obtained on miRNA data profiled using RT-qPCR techniques, have yet been replicated.

Briefly, we here performed plasma miRNA profiling in 391 VT patients using a next-generation sequencing technology and assessed the association of identified miRNAs with several haemostatic traits and VT associated clinical outcomes. Association analyses were conducted using an original Bayesian network (BN) inference strategy aimed at identifying miRNAs with the highest abilities to serve as relevant biomarkers. In addition, we integrated genome-wide genotype data with miRNA expression levels in order to identify miRNAs that are under strong genetic control.

Methods

The MARTHA microRNA sequencing study

The MARseille Thrombosis Association project refers to a collection of VT patients recruited at the La Timone Hospital in Marseille, France, initially between 1994 and 2005 and further extended over the 2010–12 period. Detailed description of this collection has already been previously provided.

The present study relies on a subsample of 391 VT patients that had been previously genotyped for genome-wide polymorphisms using dedicated genotyping array and with available plasma samples. For each sample, total RNA was extracted from 400 μL citrate plasma sample using miRNeasy Serum/Plasma kit from Qiagen. From 6 μL of total RNA, plasma miRNA libraries were then prepared with NEBNext Multiplex Small RNA Library Prep Set for Illumina. The manufacturer’s protocol was followed, with an optimized size selection method via Ampure XP beads, a specific dilution of adapters to 1/10, and 15 cycles of PCR amplification, with adapter sequences GATC GGAAGGCACACGGCTGAACTCCAGTCAC and CGACAGGTTCAG AGTTTCTACAGTGACGATC for 3’ and 5’ ends, respectively. Detailed characteristics of the experimental protocol for libraries preparation and sequencing have already been described.

MicroRNA alignment and quantification processes

Sequenced data were processed with the bioinformatic OptimiR pipeline in order to detect and quantify miRNAs. Briefly, OptimiR aligned miRNAs to a library composed of mature miRNA references sequences from miRBase. For miRNA integrating genetic variants in their sequence (called polymiRs), the reference library was upgraded by OptimiR with sequences integrating alternative alleles. Ambiguous alignments were resolved using a scoring algorithm that keeps only the most likely alignment while considering the frequent post-transcriptional modifications that miRNAs can undergo. Reads aligned on polymiRs were kept if they were consistent with the sample’s genotype, otherwise, they were discarded.

From the resulting miRNA abundances, we performed several quality assessments in order to discard unreliable data. First, samples that were poorly sequenced, i.e. with <100 000 reads aligned, were discarded (n = 33) as well as samples identified to be haemolyzed (n = 34). The degree of haemolysis was determined based on the optical density at 414 nm, and values exceeding 0.2 were defined as haemolyzed samples. Finally, in order to retain only highly expressed miRNAs, we kept only those with at least five counts in at least 75% of the remaining samples.

Abundances were then normalized using the rlog method from the DESeq2 R library. This normalization process takes into account differences in library sizes due to library preparation and sequencing protocols, and stabilize variance across miRNAs and samples to respect homoscedasticity constraints for further analysis. Principal component analysis (PCA) was applied to normalized abundances in order to identify individuals with outliers miRNA profiles. Individuals deviating by 3 SD from the centres of the first four PCAs (n = 10) were further excluded from downstream analyses, leaving 344 individuals for BN and association analyses.

Bayesian network analysis

A BN is a probabilistic directed acyclic graphical model that represents relationships among a large number of variables (here mainly miRNAs) with the aim of modelling the dependencies/interactions and conditional independencies between variables. Generally, any BN is defined by a directed acyclic graph structure G = (V, E) where V is the set of variables and E the set of edges representing the directional relationships between variables and P a joint probability distribution of the variables in the network. Three types of nodes can be identified in a given BN: the root nodes that are variables found to influence several other variables but are not themselves influenced by any other variables, the internal nodes that are both influenced by and modulate other variables, and finally terminal nodes that are variables that are not identified as influencing others (see Figure 1). Any variable influencing another variable in the network is referred to as a parental node for this later variable. In the following, we will mainly
focus on terminal nodes assuming that such nodes, as integrating the cumulative upstream effects of other variables, would serve as more relevant and powerful endophenotypes to be tested in relation to some outcomes of interest. In that context, BN analysis can also be viewed as a data reduction technique since, instead of testing the association of all initial variables with a given outcome, only the terminal nodes will be tested for association, reducing then the multiple testing burden. In this article, BNs will be constructed with the ‘bnlearn’ package that implements the relatively fast tabu search algorithm handling both discrete and continuous variables. In the current application, BNs will be created from all expressed miRNAs but also with the age and sex variables. These two latter variables have been shown to have strong influence on circulating miRNAs, and their integration in the BN analysis can then add information to more efficiently model the dependencies and conditional independence between some miRNAs.

Because tabu search is a greedy search algorithm, it may end up into a local optimum. To overcome such situation and to assess the stability of the BN analysis in identifying robust terminal nodes, we generated 2000 bootstrapped datasets composed of 95% of the initial samples and for each bootstrapped dataset, we randomly shuffled the way the input variables were ordered in the initial dataset. For each shuffled bootstrapped dataset, a BN was constructed and the terminal nodes identified. After 2000 bootstrap, we calculated the number of times a given variable was identified as a terminal node.

In order to assess whether the observed distribution of the number of terminal node’s occurrences deviates from the null hypothesis of no correlation structure between miRNAs, a permutation strategy was adopted. For each permutation, we randomly selected at least 40 variables whose values were permuted between individuals in order to break down the original data correlation structure. We generated 2000 of such permuted datasets and constructed a BN on each of them. From these permuted BNs, we counted the maximum number of times a given variable (that could be any miRNA, age, or sex) was identified as a terminal node and used this maximum value as a cut-off to identify robust terminal miRNAs in the unpermuted analysis above.

Association analysis with haemostatic traits and clinical outcomes
Identified terminal miRNAs were tested for association with several haemostatic traits available in MARTHA participants (see Table 1). Association analyses were performed using linear regression model and adjusted for age, sex, anticoagulant therapy, and combined plasma levels of hsa-let-7d-5p, hsa-let-7g-5p and let-7i-5p measured by qPCR, which serve as a control reference of miRNA levels. Individuals under anticoagulant therapy at the time of blood sampling were excluded for the analysis on protein C, protein S, and prothrombin time. For association testing, log-transformation was applied to the following variables: Activated Thrombin Generation Potential biomarkers (Endogenous Thrombin Potential, Lagtime), Partial Thromboplastin Time, Factor VIII, Homocysteine, Plasminogen Activator Inhibitor-1, Tissue Factor Principal Inhibitor, and von Willebrand Factor.

Terminal miRNAs were also tested for association with the DVT vs. PE outcome using a logistic regression model, while a Cox model was used to assess their association with VT recurrence whose information was available in 228 patients only. For the latter analysis, we applied the Cox survival model with left truncature and adjusted for age, sex, body mass index (BMI), and smoking. To address the multiple testing issue associated with the number of terminal miRNAs that will be tested for association with the phenotypes, we applied a Bonferroni correction based on the effective number of independent variables.

Genome-wide miR-eQTL analysis
As MARTHA participants have been typed for high-density genotyping arrays and imputed for common polymorphisms available in the 1000G reference panel, we performed genome-wide association study (GWAS) on each expressed miRNA for identifying miRNA expression quantitative trait loci (miR-eQTL) using the mach2QTL programme. Analyses were performed under the assumption of additive genetic effects and adjusting for the following covariates: sex, age of blood collection, anticoagulant prescription, RT-qPCR measured hsa-let-7 combination, and the four first principal genetic components retrieved from PCA analysis as previously described. GWAS results were filtered out for variants with minor allele frequency lower than 0.05 and with imputation criterion \( r^2 \) below 0.4. Finally, we combined the results of our miR-eQTL analysis with those previously described by Nikpay et al.

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**Figure 1** A Bayesian network example. In this illustrative BN example, variables V1, V2, and V3 are root nodes, V4 and V5 are internal nodes, and V6 and V7 are terminal nodes. V3 is also a parental node for V4 which is itself a parental node for V7.
available at https://zenodo.org/record/2560974 in order to identify additional single nucleotide polymorphism (SNP) × miRNA associations. For this, a random-effect model-based meta-analysis was adopted as implemented in the GWAMA software.\textsuperscript{32} SNP × miRNA associations were considered as cis effects when the SNP maps ± 1 Mb from the mature miRNA position. Otherwise, they were considered as trans. Any association with \( P \)-value < 3.2 \( 10^{-10} \) corresponding to the Bonferroni threshold corrected for the number of tested SNP × miRNA associations was considered as genome-wide significant. We also used a miRNA-wide threshold of \( P < 5 \times 10^{-8} \), the standard statistical threshold generally advocated in the context of a single GWAS, to identify additional suggestive associations.

**Results**

**The MARTHA microRNA cohort**

Detailed description of the clinical and biological characteristics of the 344 participants is shown in Table 1. Of note, 228 patients have been followed for the risk of recurrence for a mean time period of 11.4 ± 4.3 years. During this period, 41 patients experienced a new VT event.

After the application of the OptimIR workflow, 162 miRNAs were found expressed in the 344 MARTHA participants. Full miRNA data are provided in Supplementary material online, Table S1. The most expressed miRNA was the hsa-miR-122-5p (Supplementary material online, Figure S1), a miRNA known to be mainly expressed in liver and that was previously shown to be amongst the most abundant plasma miRNAs.\textsuperscript{33} Additional highly expressed miRNAs were hsa-miR-486-5p, hsa-miR-92a-3p, and hsa-miR-451a (Supplementary material online, Figure S1). Of note, the 25 most expressed miRNAs accounted for >90% of all sequenced reads that were aligned to miRNA mature sequences.

**BN analysis of microRNA data**

Under the null hypothesis of no specific structure in the miRNA data, all miRNAs were identified as a terminal node at least once and, on average, a miRNA was found as a

Table 1  Characteristics of the MARTHA miRNA cohort

| Variables                                      | N     | Mean ± SD\textsuperscript{a} |
|------------------------------------------------|-------|-------------------------------|
| Gender (male/female)                          | 344   | 144/200                       |
| Age (years)                                   | 344   | 52.1 ± 14.5                   |
| Smoking (yes/no)                              | 343   | 94/249                        |
| BMI (kg/m\(^2\))                              | 331   | 25.86 ± 4.62                  |
| Deep vein thrombosis/pulmonary embolism       | 344   | 259/85                        |
| Anticoagulant therapy (yes/no)                | 344   | 122/222                       |
| Antithrombin (IU/mL)                          | 313   | 102.41 ± 11.59                |
| Activated partial thromboplastin time (s)     | 341   | 33.42 ± 6.02                  |
| D-dimers (\( \mu \)g/mL)                     | 184   | 0.39 ± 0.33\textsuperscript{b} |
| FV (IU/mL)                                    | 150   | 109.21 ± 22.26                |
| FVIII (IU/dL)                                  | 294   | 135.07 ± 48.31                |
| FXI (IU/mL)                                   | 336   | 130.78 ± 31.99                |
| Fibrinogen (g/L)                              | 342   | 3.42 ± 0.66                   |
| Haemotocrit (L/L)                             | 343   | 0.42 ± 0.03                   |
| Homocysteine (\( \mu \)mol/L)                | 304   | 12.26 ± 6.55                  |
| Platelet count (G/L)                          | 344   | 7.90 ± 3.14                   |
| Mean platelet volume (fl)                     | 344   | 140.42 ± 13.19                |
| PAI-1 (UI/mL)                                 | 272   | 12.25 ± 13.44                 |
| Protein C (IU/mL)                             | 318   | 99.55 ± 40.36                 |
| Protein S (IU/mL)                             | 322   | 8.3 ± 7.29                    |
| TAFI (\( \mu \)g/mL)                          | 336   | 15.27 ± 4.72                  |
| TFPI (ng/mL)                                  | 336   | 14.17 ± 6.84                  |
| vWF (IU/dL)                                   | 308   | 154.34 ± 67.74                |
| Prothrombin time (%)                          | 344   | 87.63 ± 27.95                 |
| Thrombin generation                           | 193   |                               |
| Endogeneous thrombin potential (nM-min)       | 1761.44 ± 280.31 |
| Peak (nM)                                     | 341   | 340.35 ± 57.51                |
| Lagtime (min)                                 | 344   | 3.34 ± 1.17                   |
| VT recurrence during follow-up (yes/no)       | 228   | 41/187                        |

\textsuperscript{a}Count data are shown for categorical variables, other reported values were mean ± standard deviation.

\textsuperscript{b}In about 50% participants, D-dimers values were below the detection limit (0.22) and thus discarded. Mean and SD were then computed over all D-dimer values >0.22.
terminal node in 6.3% ± 3.5 of the permuted BNs, with a maximum of 18.3%. Using the latter threshold, the bootstrap BN analysis identified 15 terminal miRNAs and the number of times each of them was found as a terminal node in bootstrapped BNs is shown in Figure 2.

**Association of microRNAs’ levels with VT-associated biological and clinical traits**

The application of the Li and Ji multiple testing procedure estimated the number of effective independent terminal miRNAs as 14, leading to an adapted Bonferroni threshold of $3.6 \times 10^{-3}$. At this statistical level, only one association between terminal miRNAs and haemostatic traits was detected. Plasma levels of hsa-miR-199b-3p were negatively correlated ($\rho = -0.17$, $P = 0.0016$) with haematocrit levels. Interestingly, this miRNA has recently been reported to associate with VT risk whose association with haematocrit levels have already been described. The full results of the scan for association between miRNAs and haemostatic traits are given in Supplementary material online, Table S2.

Of note, the strongest association of terminal miRNAs with recurrence risk was observed for hsa-miR-370-3p [HR = 1.77 (1.09–2.88), $P = 0.019$], this miRNA being also the terminal miRNA that discriminated the most between DVT and PE [OR for PE = 0.72 (0.49–1.05), $P = 0.090$] (Table 2). Of interest, one of our terminal miRNAs, hsa-miR-197-3p, was reported to associate with VT recurrence in Wang et al. However, we did not observe here such trend for association [HR = 0.78 (0.35–1.76), $P = 0.55$]. Nevertheless, among the nine additional miRNAs reported in Wang et al. and also expressed in MARTHA, we found two with a suggestive association with VT recurrence: hsa-miR-27b-3p [HR = 0.4 (0.2–0.79), $P = 0.016$] and hsa-miR-222-3p [HR = 1.76 (1.01–3.08), $P = 0.049$] (Supplementary material online, Table S3).

**miR-eQTL analyses**

At the pre-specified genome-wide statistical level of $3.2 \times 10^{-10}$, three SNP × miRNA associations, all cis, were identified in the MARTHA study (Table 3). These were observed for rs12473206 with hsa-miR-4433b-3p ($P = 8.12 \times 10^{-15}$), rs2127870 with hsa-miR-625-3p ($P = 9.57 \times 10^{-14}$), and rs140930133 with hsa-miR-941 ($P = 5.07 \times 10^{-15}$). The latter two have already been observed in whole blood and adipose tissue. Using a more liberal miRNA-wide threshold of $P = 5 \times 10^{-8}$, 10 additional suggestive associations, 1 in cis and 9 in trans, were observed (Table 3). Regional association plots and boxplot summarizing the genotype × miRNA associations at these 13 main candidates are shown in Supplementary material online.

Of note, the most significant association was observed between hsa-miR-4433b-3p and rs12473206, a variant located within the mature miRNA sequence. It can be speculated that this variant impacts the maturation process of the miRNA or its target spectrum, and thus influences its plasma expression levels. In addition, two SNPs with cis effects on miRNA levels (thereafter referred to as cis SNPs) have been previously found to associate with levels of the protein encoded by the miRNA host gene. In whole blood, the miSNP rs2127870 was reported to
influence FUT8 levels, but FUT8 being the host gene for hsa-miR-625-3p. Similarly, the DNAJC5 rs2427555 that is in very strong linkage disequilibrium (LD) with the miSNP rs140930133, we here found associated with plasma hsa-miR-941 levels, has been reported to influence the expression of DNAJC5 in lymphoblastoid cells. These observations are supportive elements for the observed miSNP associations and would suggest a joint regulation of hsa-miR-625-3p and hsa-miR-941 expressions with those of their host genes as already documented for several miRNAs.

One trans-eQTL located in the long non-coding RNA (lncRNA) LINC01849 was associated with hsa-miR-330-3p. The identified trans miSNP, rs1554362, is also an eQTL for

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**Table 2** Association of terminal miRNAs with VT outcomes in the MARTHA miRNA study

| miRNA        | VT recurrence | Pulmonary embolism vs. deep vein thrombosis |
|--------------|---------------|--------------------------------------------|
|              | HR (95% CI)   | OR (95% CI)                               |
| hsa-miR-370-3p | 1.77 (1.09–2.88) | 0.72 (0.49–1.05) |
| hsa-miR-184   | 0.53 (0.30–0.95) | 1.23 (0.92–1.66) |
| hsa-miR-4732-5p | 0.41 (0.18–0.92) | 0.70 (0.39–1.22) |
| hsa-miR-4433b-3p | 1.54 (1.04–2.29) | 1.01 (0.75–1.36) |
| hsa-miR-215-5p | 0.63 (0.37–1.09) | 1.11 (0.73–1.67) |
| hsa-miR-134-5p | 1.58 (0.85–2.91) | 0.89 (0.57–1.39) |
| hsa-miR-381-3p | 1.45 (0.83–2.56) | 0.81 (0.53–1.23) |
| hsa-miR-145-3p | 0.51 (0.15–1.76) | 0.62 (0.24–1.56) |
| hsa-miR-23a-3p | 0.67 (0.26–1.70) | 1.00 (0.51–1.93) |
| hsa-miR-197-3p | 0.78 (0.35–1.76) | 1.41 (0.79–2.56) |
| hsa-miR-150-3p | 1.23 (0.53–2.83) | 0.90 (0.49–1.66) |
| hsa-miR-484    | 1.20 (0.56–2.59) | 1.27 (0.69–2.38) |
| hsa-miR-199a-3p | 0.80 (0.22–2.86) | 1.17 (0.46–2.97) |
| hsa-miR-378d   | 0.81 (0.15–4.56) | 0.41 (0.10–1.46) |
| hsa-miR-20a-5p | 1.09 (0.40–2.95) | 0.74 (0.36–1.52) |

**Table 3** Significant associations at the 5 × 10<sup>−8</sup> statistical level between SNPs and plasma miRNA levels in the MARTHA miRNA study

| miRNA        | miRNA host gene | Top SNP    | MAF   | r<sup>2</sup> | Chr | Distance to 5' miRNA | Effect (SD) | P-value | SNP Genomic Context |
|--------------|-----------------|------------|-------|-------------|-----|----------------------|-------------|---------|-------------------|
| Cis associations                     |                   |           |       |             |     |                      |             |         |                   |
| hsa-miR-4433b-3p | Intergenic       | rs12473206 | 0.23  | 0.99       | 2   | −13                  | 0.979 (0.080) | 8.12 × 10<sup>−15</sup> | exonic_ncRNA (hsa-miR-4433b) |
| hsa-miR-625-3p | FUT8             | rs2127870  | 0.27  | 0.99 | 14   | 141025              | 0.533 (0.051) | 9.57 × 10<sup>−8</sup> | Intergenic (
DNAJC5) |
| hsa-miR-941   | DNAJC5           | rs140930133 | 0.19 | 0.97 | 20  | 8822                 | −0.349 (0.045) | 5.07 × 10<sup>−15</sup> | Intronic (DNAJC5) |
| hsa-miR-432-5p | RTL1             | rs201969986 | 0.29 | 0.95 | 14  | 177423              | −0.346 (0.063) | 3.31 × 10<sup>−8</sup> | Intergenic |
| Trans associations                   |                   |           |       |             |     |                      |             |         |                   |
| hsa-miR-184   |                   | rs144867605 | 0.07 | 0.82 | 11  | 75957983             | 0.804 (0.134) | 2.02 × 10<sup>−9</sup> | Intergenic |
| hsa-miR-654-5p |                   | rs11109171 | 0.44 | 0.99 | 12  | 98098091            | −0.246 (0.042) | 3.28 × 10<sup>−9</sup> | Intergenic |
| hsa-miR-320c  |                   | rs10151482 | 0.06 | 0.93 | 14  | 41934917            | 0.427 (0.074) | 6.47 × 10<sup>−9</sup> | Intergenic |
| hsa-miR-184   |                   | rs143007764 | 0.06 | 0.65 | 3   | 142899139           | 0.916 (0.161) | 1.14 × 10<sup>−8</sup> | Intergenic |
| hsa-miR-1-3p  |                   | rs73245753 | 0.12 | 0.79 | 4   | 26292392            | 0.589 (0.105) | 2.31 × 10<sup>−8</sup> | Intergenic |
| hsa-miR-330-3p |                   | rs1554362  | 0.45 | 0.82 | 2   | 101221457            | −0.227 (0.041) | 2.81 × 10<sup>−8</sup> | Intronic (LINC01849) |
| hsa-miR-582-3p |                   | rs4522365  | 0.13 | 0.83 | 15  | 29964742             | 0.314 (0.057) | 2.91 × 10<sup>−8</sup> | Intergenic |
| hsa-miR-4446-3p |                   | chr12:95274192:1 | 0.09 | 0.61 | 12  | 95274192            | −0.492 (0.089) | 3.07 × 10<sup>−8</sup> | Intergenic |
| hsa-miR-320d  |                   | rs12800249 | 0.05 | 0.63 | 11  | 21240436            | 0.481 (0.088) | 4.33 × 10<sup>−8</sup> | Intronic (NELL1) |

MAF, minor allele frequency; r<sup>2</sup>, imputation quality criterion.
the PDC3L transcript levels in different tissues according to the GTEx database. Another intronic miSNP located in the NELL1 gene was associated with hsa-miR-320d levels. The seven other trans eQTL are located in intergenic regions.

We sought to in silico replicate these miSNP associations using the results from Nikpay et al. who scanned for genetic polymorphisms associated with miRNA levels in 710 plasma samples. Unfortunately, as the Nikpay et al. study relied on a genotyping array focusing mainly on coding regions and used a very stringent imputation quality criterion ($r^2 > 0.9$), it was not possible to assess all our candidate associations. Only four were testable (hsa-miR-941 × rs140930133, hsa-miR-432-5p × rs201969986, hsa-miR-654-5p × rs11109171, hsa-miR-320c × rs10151482) among which only the association of rs140930133 with hsa-miR-941 levels replicated ($P = 3.3 \times 10^{-11}$).

Conversely, we looked into the MARTHA results to replicate the 223 miSNP associations that were significantly ($P < 5 \times 10^{-8}$) detected in the Nikpay et al. study. We were able to test 92 of them among which 37 replicated at the nominal level of $P = 0.05$ in MARTHA (Table 4). These involved 29 cis and 8 trans miSNP associations.

### Table 4: Association of SNPs with plasma miRNA levels in 710 VT patients

| miRNA   | SNP   | Chr | Position(bp) | EA  | EAF  | $\beta$ | SE  | $P$            |
|---------|-------|-----|--------------|-----|------|---------|-----|----------------|
| miR-197-3p | rs7350703 | 1   | 110129740 T  | 0.16 | -0.078 | 0.011 | 0.123 | $10^{-12}$ |
| miR-26b-5p | rs12623740 | 2   | 219665715 A  | 0.49 | -0.060 | 0.007 | 3.37 | $10^{-18}$ |
| miR-152-3p | rs9910516 | 16  | 46183160 A  | 0.23 | 0.093 | 0.016 | 1.52 | $10^{-08}$ |
| miR-27b-3p | rs10993381 | 9   | 97639463 T  | 0.07 | 0.170 | 0.014 | 3.10 | $10^{-19}$ |
| miR-182-5p | rs26973783 | 7   | 129431977 G  | 0.32 | 0.115 | 0.020 | 2.36 | $10^{-08}$ |
| miR-191a-5p | rs1443282 | 16  | 199010721 C  | 0.27 | 0.211 | 0.022 | 9.03 | $10^{-21}$ |
| miR-191a-5p | rs12125200 | 1   | 198992043 A  | 0.27 | 0.340 | 0.013 | 1.13 | $10^{-11}$ |
| miR-192a-5p | rs4147470 | 5   | 148258107 T  | 0.49 | -0.131 | 0.014 | 7.71 | $10^{-20}$ |
| miR-26b-5p | rs833083  | 2   | 219336959 T  | 0.41 | -0.076 | 0.006 | 3.96 | $10^{-08}$ |
| miR-181a-5p | rs878254  | 1   | 192957141 A  | 0.48 | -0.122 | 0.015 | 3.54 | $10^{-05}$ |
| miR-236a-5p | rs2360961 | 1   | 19900277 C  | 0.40 | -0.151 | 0.016 | 4.39 | $10^{-20}$ |
| miR-30d-5p | rs13282464 | 10  | 153705729 T  | 0.15 | 0.092 | 0.007 | 2.02 | $10^{-03}$ |
| miR-443b-5p | rs6740438 | 2   | 64528046 C  | 0.13 | 0.163 | 0.029 | 1.78 | $10^{-08}$ |
| miR-30d-5p | rs13268530 | 8   | 135727196 T  | 0.15 | 0.095 | 0.007 | 1.68 | $10^{-05}$ |
| miR-21-5p | rs2665392 | 17  | 57809453 A  | 0.16 | 0.059 | 0.011 | 3.59 | $10^{-08}$ |
| miR-435b-5p | rs35530140 | 2  | 64539015 C  | 0.21 | -0.130 | 0.022 | 9.86 | $10^{-09}$ |
| miR-584-5p | rs9325212 | 5   | 148248818 A  | 0.39 | -0.085 | 0.015 | 7.62 | $10^{-09}$ |
| miR-181a-5p | rs1861924 | 19  | 199121330 A  | 0.18 | 0.137 | 0.020 | 2.06 | $10^{-11}$ |
| miR-1908-5p | rs174561  | 11  | 61582708 C  | 0.30 | 0.151 | 0.012 | 4.76 | $10^{-06}$ |
| miR-139-3p | rs1098849 | 11  | 72269302 T  | 0.25 | 0.124 | 0.022 | 3.30 | $10^{-08}$ |
| let-7i-5p | rs6581454 | 12  | 62934442 G  | 0.47 | 0.039 | 0.006 | 3.04 | $10^{-04}$ |

| Trans associations |
|---------------------|
| miR-222-3p | rs11070216 | 15  | 39817245 T  | 0.19 | -0.067 | 0.012 | 4.87 | $10^{-08}$ |
| miR-222-3p | rs970208  | 15  | 39864403 G  | 0.32 | -0.064 | 0.010 | 8.79 | $10^{-10}$ |
| miR-143-3p | rs4734879 | 8   | 106583124 G  | 0.28 | 0.239 | 0.031 | 2.88 | $10^{-14}$ |
| miR-1-3p | rs11906462 | 20  | 61185952 T  | 0.20 | 0.310 | 0.033 | 6.28 | $10^{-20}$ |
| miR-320a | rs1443651 | 2   | 68569316 G  | 0.45 | -0.036 | 0.006 | 7.12 | $10^{-10}$ |
| miR-16-5p | rs137214 | 22  | 35288857 T  | 0.28 | 0.041 | 0.007 | 1.76 | $10^{-08}$ |
| miR-326-3p | rs600038 | 9    | 136151806 C  | 0.21 | 0.055 | 0.009 | 5.95 | $10^{-09}$ |
| miR-320c | rs1443651 | 2   | 68569316 G  | 0.45 | -0.031 | 0.005 | 2.77 | $10^{-10}$ |

*One-sided test $P$-value.

EA, effect allele; EAF, effect allele frequency.
Among these eight trans miSNP associations, three deserve to be highlighted. First, plasma levels of hsa-miR-143-3p were influenced by the intronic ZFPM2 rs4734879, ZFPM2 being a locus reported to associate with venous thrombosis risk and platelet function. In MARTHAl, plasma levels of hsa-miR-143-3p were negatively significantly correlated with BMI ($\rho = -0.24$, $P = 3.6 \times 10^{-4}$) and borderline significant with PAI-1 activity levels ($\rho = -0.21$, $P = 5.3 \times 10^{-3}$) (Supplementary material online, Table S2). Second, hsa-miR-126-3p plasma levels were associated with the rs600038 located in the promoter region of the ABO gene. This polymorphism is in strong LD with several other ABO polymorphisms that are known to associate with VT risk, including the rs579459 ($\rho^2 = 0.99$) tagging for the A1 ABO blood group. In MARTHAl, plasma levels of hsa-miR-126-3p were strongly and positively correlated ($\rho = 0.20$) with red cells ($P = 1.73 \times 10^{-5}$), lymphocytes ($P = 2.5 \times 10^{-4}$), platelets ($P = 5.9 \times 10^{-4}$), and polymorphs ($P = 6.0 \times 10^{-4}$) (Supplementary material online, Table S2). Third, polymorphisms (rs970280, rs11070216) in the promoter region of the THBS1 gene were found associated with plasma levels of hsa-miR-222-3p. This miRNA has been previously reported to associate with the risk of VT recurrence and has a suggestive association ($P = 0.049$) in our study (Supplementary material online, Table S3), where it positively correlated with antithrombin levels ($\rho = 0.21$, $P = 8.8 \times 10^{-4}$) (Supplementary material online, Table S2). THBS1 encodes Thrombospondin-1 and is known to be involved in angiogenesis and platelet aggregation.

Finally, we performed a random-effect meta-analysis of both datasets in order to discover additional miSNPs. At the $5 \times 10^{-8}$ statistical threshold, we identified seven new cis and five new trans miSNP associations (Table 5). None of these miSNP associations appeared to involve loci with documented link with thrombosis related traits.

**Discussion and conclusion**

In this study, we reported the largest investigation to date of miRNA plasma profiling in a cohort of VT patients. Capitalizing on the application of a next-generation sequencing technology, known to be more efficient and sensitive to detect and quantify miRNAs compared with microarray or RT-qPCR techniques, we were able to detect 162 highly expressed miRNAs. These miRNAs were then tested for association with several VT-related phenotypes including 38 haematological traits and VT recurrence. In order to deal with the correlation between miRNA levels and reduce the multiple testing burden associated with the number of tested miRNAs, we deployed an original BN analysis aimed at identifying miRNAs that could serve as more powerful biomarkers for the investigated traits. In addition, as our studied VT patients had been previously typed for genome-wide genotypes, we were able to perform GWAS on each of the 162 miRNAs, and combined our results with some previously obtained in disease-free individuals in order to identify novel associations of common SNPs with plasma miRNA levels.

Several conclusions could be derived from this work. First, we did not identify any miRNA that significantly associated with the risk of VT recurrence. In our study, the miRNA that discriminated the most between patients with or without recurrence, but also between DVT vs. PE patients, was the hsa-miR-370-3p. Several works have already reported the involvement of hsa-miR-370-3p in lipids metabolism and one of the most robust target gene for hsa-miR-370-3p is CPT1A whose role in lipid metabolism is also very documented. Hsa-miR-370-3p is also predicted to target drug-metabolism genes, such as CYP2D6 and VKORC1L1, that are related to the warfarin anticoagulant pharmacotherapy. Aside this miRNA, we observed a trend of association with VT recurrence for the hsa-mir-Z7b-3p and hsa-miR-222-3p that has been previously identified in Wang et al. but these associations ($P = 0.016$ and $P = 0.0495$, respectively) did not survive any multiple testing correction (Supplementary material online, Table S3). Larger studies would be mandatory to confirm these observations and increase our chance to identify other miRNAs associated with the risk of recurrence in VT patients. Second, we observed several significant associations of miRNAs with haematological traits that deserve further replication in independent studies. One can highlight the significant correlation between haematocrit levels and plasma levels of hsa-miR-199b-3p, a miRNA that has been reported to be associated with VT risk. Third, our miR-QTL study identified about 25 significant ($P < 5 \times 10^{-8}$) associations of SNPs with plasma miRNA levels, of which, to the best of our knowledge, 21 have never been reported, including a dozen of trans associations. These associations could help deciphering the genomic architecture of complex diseases where miRNAs are involved. For example, plasma levels of hsa-miR-143-3p were found to be associated with the rs4734879 mapping to ZFPM2, a gene known to associate with platelet function and VT risk. We also observed a strong association of rs12473206 with plasma levels of hsa-miR-4433b-3p, a miRNA whose serum levels have recently shown to be associated with stroke. The impact of this SNP on stroke risk deserves to be further and deeply investigated. The results of our GWAS on miRNA levels were combined with those obtained by Nikpay et al. and freely available at https://zenodo.org/. However, only SNPs with imputation quality greater than 0.90 are available at this resource, which has hampered our ability to replicate some of the main associations observed in the MARTHAl miRNA study. To facilitate future studies aimed at disentangling the genetic regulation of miRNAs, the results of the 162 GWAS performed on miRNA levels in MARTHAl will be available for download at https://zenodo.org/.

Altogether, this study produced a rich source of information relating to plasma miRNAs and biological/clinical traits associated with VT that could be of great use to generate and/or validate new hypothesis.

**Supplementary material**

Supplementary material is available at *European Heart Journal-Supplement* online.
Table 5  Significant \((P < 5 \times 10^{-8})\) associations of miSNP with miRNA plasma levels derived from the MARTHA miRNA and Nikpay et al.\textsuperscript{31} meta-analysis

| miRNA   | chr | Position (bp) | SNP    | EA    | EAF  | \(r^2\) | \(\beta\) | SE   | \(P\)    | EAF   | \(\beta\) | SE   | \(P\)   | \(P^a\) | \(\beta\) | SE   | \(P^b\) |
|---------|-----|---------------|--------|-------|------|--------|--------|------|---------|-------|--------|------|---------|-------|--------|------|---------|
| **Cis associations** | | | | | | | | | | | | | | | | | |
| miR-181b-5p | 1   | 199257141     | rs878254 | A     | 0.485 | 0.90  | -0.054 | 0.032 | 0.0916 | 0.480 | -0.071 | 0.013 | 1.64 \(10^{-7}\) | 0.61 | -0.069 | 0.012 | 3.18 \(10^{-8}\) |
| miR-148a-3p | 7   | 25991977      | rs9639523 | T     | 0.375 | 0.87  | -0.081 | 0.034 | 0.0191 | 0.344 | -0.072 | 0.013 | 2.03 \(10^{-7}\) | 0.80 | -0.073 | 0.013 | 8.41 \(10^{-9}\) |
| let-7a-5p | 9   | 96916230      | rs10512230 | T    | 0.287 | 1.00  | 0.040  | 0.031 | 0.1934 | 0.315 | 0.026  | 0.004 | 6.49 \(10^{-8}\) | 0.67 | 0.027  | 0.005 | 2.19 \(10^{-8}\) |
| let-7d-5p | 9   | 97229465      | rs4497033 | T     | 0.492 | 0.99  | -0.061 | 0.036 | 0.0895 | 0.463 | -0.028 | 0.005 | 1.50 \(10^{-7}\) | 0.36 | -0.029 | 0.005 | 3.85 \(10^{-8}\) |
| miR-2110 | 10  | 115933905     | rs17091403 | T   | 0.091 | 1.00  | -0.141 | 0.043 | 1.13 \(10^{-3}\) | 0.074 | -0.103 | 0.023 | 9.90 \(10^{-6}\) | 0.44 | -0.112 | 0.020 | 4.34 \(10^{-8}\) |
| miR-342-3p | 14  | 100256449     | rs8011282 | C     | 0.474 | 0.99  | 0.095  | 0.030 | 1.39 \(10^{-3}\) | 0.487 | 0.067  | 0.014 | 5.65 \(10^{-6}\) | 0.41 | 0.073  | 0.013 | 3.68 \(10^{-8}\) |
| miR-99b-5p | 19  | 52160843      | rs11084100 | C    | 0.392 | 1.00  | -0.067 | 0.024 | 5.17 \(10^{-3}\) | 0.419 | -0.065 | 0.012 | 1.12 \(10^{-7}\) | 0.94 | -0.066 | 0.011 | 1.50 \(10^{-8}\) |
| **Trans associations** | | | | | | | | | | | | | | | | | |
| miR-215-5p | 2   | 171402733     | rs724806 | C     | 0.252 | 0.97  | 0.091  | 0.057 | 0.1123 | 0.326 | 0.143  | 0.027 | 1.44 \(10^{-7}\) | 0.40 | 0.134  | 0.024 | 4.09 \(10^{-8}\) |
| miR-10b-5p | 7   | 13236107      | rs6948643 | G   | 0.264 | 1.00  | -0.071 | 0.040 | 0.0766 | 0.285 | -0.09  | 0.017 | 2.84 \(10^{-7}\) | 0.66 | -0.087 | 0.016 | 4.62 \(10^{-8}\) |
| let-7d-3p | 11  | 2611449       | rs1024164 | A    | 0.133 | 0.87  | -0.083 | 0.034 | 0.0147 | 0.092 | -0.065 | 0.013 | 7.78 \(10^{-7}\) | 0.63 | -0.068 | 0.012 | 3.18 \(10^{-8}\) |
| miR-378a-3p | 11  | 133763476     | rs10894759 | A  | 0.317 | 0.99  | 0.066  | 0.028 | 0.0206 | 0.296 | 0.059  | 0.011 | 7.86 \(10^{-7}\) | 0.82 | 0.060  | 0.011 | 3.58 \(10^{-8}\) |
| miR-7-5p | 15  | 41614621      | rs7163989 | G    | 0.293 | 0.99  | -0.112 | 0.041 | 6.68 \(10^{-3}\) | 0.278 | -0.089 | 0.016 | 1.48 \(10^{-7}\) | 0.61 | -0.093 | 0.016 | 2.70 \(10^{-9}\) |

EAF, estimated allele frequency; \(r^2\), imputation quality criterion; \(\beta\), allele effect.

\(P^a\)-value of the test for heterogeneity between the MARTHA and Nikpay studies.

\(P^b\)-value of the combined effect obtained through a random-effect meta-analysis of the results of both studies.
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