A comparative characterization of the circulating miRNome in whole blood and serum of HCC patients

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miRNAs are considered promising non-invasive biomarkers. Serum represents the major source of biomarkers, being readily accessible for many analytical tests. Recently, whole blood has drawn increasing interest in biomarker studies due to the presence of cancer-interacting cells and circulating cancer cells. Although Hepatocellular Carcinoma (HCC) is the seventh most frequent cancer worldwide, fragmentated information exists regarding the miRNome characterization in blood and serum. We profiled the circulatory miRNome of paired serum and blood samples from 20 HCC patients, identifying 274 miRNA expressed in serum and 670 in blood, most of them still uncharacterized. 157 miRNA significantly differ between the two biofluids with 28 exclusively expressed in serum. Six miRNA clusters significantly characterize the two compartments, with the cluster containing miR-4484, miR-1281, miR-3178, miR-3613-3p, miR-4532, miR-4668-5p, miR-1825, miR-4487, miR-455-3p, miR-940 having the highest average expression in serum compared to blood. The ontological analysis revealed a role of these miRNAs in cancer progression, vascular invasion and cancer immune surveillance thought the regulation of DUSP1, PD-L1 and MUC1. Taken together, these results provide the most comprehensive contribution to date towards a complete miRNome profile of blood and serum for HCC patients. We show a consistent portion of circulatory miRNAs being still unknown.

Since their discovery in biofluids microRNAs (miRNAs) have drawn increasing interest in their use as circulatory biomarkers. These 21–23 short non-coding RNAs, responsible of the regulation gene expression in cells¹, represent a class of promising non-invasive biomarker candidates as relatively stable to repeated freeze-thaw cycles, temperature and pH variations and prolonged storage². More important, they are readily accessible in a wide range of biological samples, such as urine, saliva, blood, serum and other³.

Circulating miRNA profiles have been associated to different diseases⁴ but the most striking application regards several aspects of malignancies. Despite Hepatocellular Carcinoma (HCC) represents one of the most frequent cancers worldwide, ranking as the second cause of cancer-related death⁵, a comprehensive characterization of the circulatory microRNA profiling is still missing. Actually 2588 mature human miRNAs are annotated on miRBase 21 (http://www.mirbase.org); however only a small fraction has been studied and characterized in HCC. Most of the investigations of miRNA as biomarkers consider serum or plasma as the most readily available and promising reservoir of miRNAs. Recently, whole blood have been considered as an alternative source of miRNAs⁶ derived from blood cells such as neutrophils, monocytes, mature red blood cells⁷, regulatory T cells, recruited to the tumour site⁸, and cancer circulating cells. Since the role of these cells in cancer diseases, whole blood miRNAs became an attractive source of potential biomarkers during cancer pathogenesis.

Despite the increasing number of miRNA studies in HCC, a complete miRNome characterization of the biofluids is missing. In this study we performed an expression profiling of 1733 circulating miRNA from paired whole blood and serum samples. We believe that the fully characterization of these two compartments may provide important information on the miRNA species that characterize the two biofluids with relevant implications in the interpretation of future cancer biomarker studies.

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Materials and Methods

Patients. Twenty consecutive patients referring to the Liver Center between 2012 and 2017 with HCC according to the EASL criteria were enrolled for the study. Samples were collected at the time of HCC diagnosis, before any oncological treatment. The clinical and demographic features of the groups are shown in Table S1.

All the patients provided written informed consent and patient anonymity has been preserved. Investigation was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the regional ethical committee (Comitato Etico Regionale Unico FVG, No. 14/2012 ASUITS).

Sample collection. Fasting paired whole blood and serum samples were collected at the same time from each patient during clinical visits. Peripheral blood (3 ml) was collected in Tempus™ Blood RNA Tube containing 6 mL Stabilizing Reagent (Thermo Fischer Scientific, USA) and subsequently frozen at −80°C for long-term storage. Serum was collected in Vacuette® serum separating tubes (Greiner Bio-One GmbH, Austria). Samples were centrifuged at 3500 rpm for 10 minutes, aliquoted and stored at −80°C until further use.

Assessment of hemolysis. The hemoglobin in serum was assessed with Beckman Coulter® DU® 730 spectrophotometer using the Harboe Direct spectrophotometric method with Allen correction: Hb(g/l) = (167.2 × A415 − 83.6 × A580 − 83.6 × A380) / 1/1000 × 1/dilution in H2O. The considered cut-off for serum was 0.020 g/L.

RNA isolation and quality assessment. Total RNA was isolated from Tempus™ Blood Tubes using the MagMAX™ for Stabilized Blood Tubes RNA Isolation Kit (Thermo Fischer Scientific, USA) following the manufacturer’s protocol. Small RNAs were isolated from 300 μL of serum using the miRCURY™ RNA Isolation Kits (Exiqon, Vedbaek Denmark) and quantified with the Qubit microRNA Assay Kit (Thermo Fischer Scientific, USA). The quality of the total RNA and miRNAs extracted was assessed with the Agilent RNA 6000 Nano Kit (Agilent Technologies, USA) and Agilent Small RNA kit (Agilent Technologies, USA) respectively, by using the 2100 Bioanalyzer Instrument (Agilent Technologies, USA). Samples with RIN less than 6 were discarded from the subsequent profiling experiments.

Microarray profiling. Small RNAs were extracted from whole blood and serum and independently profiled on the Affymetrix microRNA arrays containing 1,734 human mature miRNA probe sets. 130 ng of RNA were detected in blood (Table 1).

In silico data analysis. We search for the miRNA expression patterns in the miRmine database (http://guanalab.cmb.med.universitaetieidelberg.de/mirmine/index.html) that collects the expression profiles from different publicly available miRNA-seq datasets. Validated miRNA targets were downloaded from http://mirtarbase.mbc.nctu.edu.tw/php/index.php. ORiilla (http://cbl-gorilla.cs.technion.ac.il), was used to determine pathway and GO terms enrichment of the miRNA validated targets. The search was relative to a background set of approximately 14,800 human genes that could be associated with any miRNA from MiRTarBase. Enrichment results were visualized as “TreeMaps” generated in REVIGO (http://revigo.irb.hr/). MiRNA target networks were built by using Tidyverse (version 1.2.1) and Igraph (version 1.1.2) packages in R environment (RStudio Team (2015). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA (http://www.rstudio.com/)). The StarBase v.3.0 database, a Clip-Seq based miRNA expression dataset, generated by The Cancer Genome Atlas (TCGA) repository was used to search for differently expressed miRNAs in HCC tissues.

Data analysis and statistical methods. Gene expression differences between paired blood and serum were calculated with the Affymetrix transcriptome analysis control suite analyzed using paired one-way analysis of variance (ANOVA) test. Multiple testing correction was performed with the Benjamini-Hochberg method and false discovery rate (FDR), corrected p-values were calculated. The relationship between expression levels of each miRNA within the family was analyzed using Pearson correlation. Correlation matrices were generated with R studio software by using and the Hmisc (version 4.1-1) Performance Analytics (version 1.4.3541) and Corrplot (version 0.84) packages.

Results and Discussion

Profiling of total blood and serum identifies different patterns of microRNAs. We profiled the miRNome of 20 paired total blood and non-hemolized serum samples from HCC patients though the Affymetrix Genechip mirna 3.0 array build on miRBase release 20.

According to the Absent/Present calling of the Affymetrix algorithm, we divided the circulatory miRNome in five quantiles based on their presence in the analyzed population. We considered the miRNAs in the first quantile as not expressed miRNAs, since they were detectable in less than five subjects. We found 1459 out of the 1734 mature miRNA not expressed in serum, 99 were expressed in the 2nd quantile of the population (considered as rare miRNAs), 53 and 43 in the 3rd and 4th quantiles, respectively, and 79 in the 5th quantile (Table 1).

In blood 1064 miRNAs were undetectable, 102 were in the 2nd quantile while 382 were present in the 5th quantile of the population (Table 1). Among the four quantiles of expressed miRNAs in the two biofluids, 28 serum miRNAs were not expressed in blood, suggesting a pure extra-blood origin; most of them belong to the 2nd quantile representing the rarely detected miRNAs. MiR-4258, miR-3197 and miR-3162-3p are abundantly expressed only in serum of HCC patients. Seventeen miRNAs expressed in serum were rarely detected in blood (2nd blood quantile), while 1035 were undetectable in both biofluids. On the contrary, 424 miRNAs were uniquely detected in blood (Table 1).
| 2nd quantile | miR-4490, miR-4423-3p, miR-4436-3p, miR-3152-3p, miR-4657, miR-200b-3p, miR-2277-5p, miR-1972, miR-335-5p, miR-4797-5p, miR-3927-3p |
| 3rd quantile | miR-4725-5p, miR-32-3p, miR-344a-
| 4th quantile | miR-548a |
| 5th quantile | None |

| SERUM |
|---|
| miR-100-3p, miR-101-3p, miR-124b-5p, miR-1257, miR-1278, miR-1284, miR-1286-3p, miR-140-5p, miR-146b-3p, miR-154-3p, miR-188-3p, miR-214-3p, miR-21-3p, miR-299-3p, miR-29b-3p, miR-29b-1-5p, miR-29c-3p, miR-301a-3p, miR-30a-3p, miR-30c-3p, miR-3129-5p, miR-3140-3p, miR-3146, miR-3150a-5p, miR-3151p, miR-3156-3p, miR-3173-5p, miR-325, miR-338-3p, miR-34c-3p, miR-3607-5p, miR-3617-5p, miR-3622b-3p, miR-3650, miR-3667-3p, miR-3675-3p, miR-3689c, miR-370-3p, miR-373-5p, miR-374a-5p, miR-374b-5p, miR-379-5p, miR-3918, miR-3925-5p, miR-3934-5p, miR-4257, miR-4272, miR-4294, miR-4326, miR-4428, miR-4433, miR-4435, miR-4444, miR-4465, miR-4478, miR-4511, miR-4540, miR-4653-5p, miR-4659a-3p, miR-4660, miR-4662a-3p, miR-4698, miR-4703-5p, miR-4705, miR-4720-3p, miR-4721, miR-4725-3p, miR-4763-5p, miR-4774-5p, miR-4778-5p, miR-4795-5p, miR-485-5p, miR-493-5p, miR-509-3p, miR-548a, miR-554, miR-557, miR-595, miR-598-3p, miR-616-5p, miR-623, miR-765, miR-922 |

| BLOOD |
|---|
| let-7a-5p, miR-1225-5p, miR-1276, miR-1307-3p, miR-130a-3p, miR-134-5p, miR-136-3p, miR-150-3p, miR-151a-3p, miR-155-5p, miR-157a-5p, miR-157b-3p, miR-181b-5p, miR-18a-5p, miR-1909-5p, miR-20b-3p, miR-222-3p, miR-22c, miR-26a-5p, miR-27a-3p, miR-29a-3p, miR-30a-3p, miR-3162-5p, miR-342-3p, miR-361-5p, miR-364, miR-3678-3p, miR-3776, miR-378f, miR-378w, miR-3835, miR-4286, miR-4306, miR-4430, miR-4441, miR-4492, miR-4495, miR-4749-5p, miR-4791, miR-4800-3p, miR-486-3p, miR-491, 5p, miR-502-5p, miR-503-5p, miR-532-3p, miR-550-3p, miR-574-3p, miR-629-3p, miR-885-3p, miR-885-5p, miR-939-5p |

| 4th quantile | let-7c-5p, let-7i-5p, let-7i-5p, let-7j-5p, let-7j-5p, let-7j-5p, miR-103a-3p, miR-107, miR-1224-5p, miR-126-5p, miR-126b-5p, miR-1275, miR-130b-3p, miR-140-3p, miR-146a-5p, miR-17-5p, miR-181a-5p, miR-191-5p, miR-191-10p, miR-191a-5p, miR-197-3p, miR-20a-5p, miR-221-3p, miR-223-3p, miR-22b-3p-5p, miR-24-5p, miR-30d-5p, miR-3124-5p, miR-3135b, miR-320e, miR-3621, miR-3646, miR-371b-5p, miR-409-3p, miR-4253, miR-4459, miR-4486, miR-4741, miR-574-3p, |

| 5th quantile | let-7d-5p, let-7e-5p, let-7f-5p, let-7g-3p, let-7g-5p, let-7h-3p, let-7i-3p, let-7j-3p, let-7j-5p, let-7j-5p, let-7j-5p, miR-103a-3p, miR-107, miR-1224-5p, miR-126-5p, miR-126b-5p, miR-1275, miR-130b-3p, miR-140-3p, miR-146a-5p, miR-17-5p, miR-181a-5p, miR-191-5p, miR-191-10p, miR-191a-5p, miR-197-3p, miR-20a-5p, miR-221-3p, miR-223-3p, miR-22b-3p-5p, miR-24-5p, miR-30d-5p, miR-3124-5p, miR-3135b, miR-320e, miR-3621, miR-3646, miR-371b-5p, miR-409-3p, miR-4253, miR-4459, miR-4486, miR-4741, miR-574-3p, |

| Not expressed | miR-202-5p, miR-2116-3p, miR-3121-3p, miR-3153, miR-323b-3p, miR-337-3p, miR-3663-3p, miR-3942-3p, miR-4290, miR-4466-3p, miR-4652-3p, miR-4701-5p, miR-4722-3p, miR-4789-5p, miR-4793-3p, miR-548a, miR-559, miR-570-3p, miR-602, miR-665, miR-877-5p, miR-1234-3p, miR-4452, miR-4505-5p, miR-563, miR-4258, miR-3197, miR-3162-3p |

| None |

All the remaining miRNAs listed are not expressed.

Table 1. miRNA expression in blood and serum of HCC patients.
Considering the miRNAs log2 average signal intensities the 5th serum group contains the miRNAs with the highest expression in serum and the higher expression compared to blood, with the exception of mir-486-5p, -92a-3p, -185-5p, -320d, -let7b-3p, -4429, -451a; -4505, -4443, -4530, -1587, -877-5p, -4710. Mir-4689 has the same expression in both biofluids (Fig. 1a).

The serum 4th quantile has a more skewed distribution of the miRNA expression levels. The 50% of the miRNAs have a higher expression in blood, mir-let7c-5p, -106a-5p, -378-3p, -151a-3p and miR-584-5p have the highest blood expression compared to serum with 8.64 -14.16 (min-max) average expression in blood vs 1.59-2.37 (min-max) average expression in serum (Fig. 1b). MiR-4258, miR-3197 and miR-3162-3p were not detected in blood but have a high expression level in serum suggesting a pure extra-blood origin. Interestingly, very limited data existing link these miRNAs to cancer10,11. Mir-4258 was associated to chemo-resistant breast cancer cell lines and FFPE tissues10, while mir-3197 was up-regulated in colorectal liver metastases11. No or limited evidence exists about the expression of these miRNAs in HCC, offering new opportunities for future explorative studies in clinical specimens. Twelve out of fifty-three miRNAs have a higher expression in serum compared to blood in the third serum group. MiR-4452, -1234-3p, -563, and miR-450b-5p were undetectable in blood. In this group, 29 miRNAs have an average log2 signal intensity less than 2. Mir-24-3p, -1234-3p, -3621, and miR-4720 -5p are the only miRNAs with an average log2 signal intensity greater than 2 and with the higher expression in serum (Fig. 2a).

The miRNAs in the 2nd quantile have the lowest average log2 signal intensities in serum. We defined them as rare miRNAs since they are present in less than the 40% of the screened population. Twenty-one of them were not expressed in blood. Mir-200b-3p, -297, -335-5p, -1972, -2277-5p, -3152-3p, -3927-3p, -4439, -4490, -4657, -4797-5p are also low expressed and rare miRNA in blood with the only exception of miR-2277-5p that has an average expression of 5.84 (Fig. 2b).

Fifty-two highly blood expressed miRNAs belonging to 5th blood quantile are also present in this group, with the exception of miR-23c, -1273f, -4791, that have a low average expression.

Differentially expressed miRNAs in whole blood and serum. We then compared the expression of the shared miRNA between the two biofluids, identifying 157 miRNA significantly differently expressed between total blood and serum (Anova p < 0.05). We filtered the miRNAs, identifying a total of 28 highly expressed miRNAs in serum, with a ≥10-fold higher levels compared to total blood, all belonging to the serum 5th quantile, except miR-933, and miR-191-3p (Table S2). Among them, the liver specific miRNA miR-122-5p shows more than 348 fold increase in serum. Several other miRNAs were already associated to cancer, such as miR-455-3p12, -93313, -94014, including HCC13,15 in which miR-455-3p and miR-940 were downregulated in tumor liver tissue, compared to the surrounding disease13 or normal livers15, while mir-933 was upregulated13. To our knowledge, no evidence exists about their presence and clinical significance in serum of HCC patients. Regarding the recently discovered
miRNAs, the lack of experimental evidences linking them to cell pathways or specific diseases make difficult any speculation about their cell origin or clinical meaning.

Seventy-eight miRNAs have a ≥10-fold higher levels in blood than in serum. Among them, miR-150, -155, -223 were already known for their blood origin, being highly expressed in hematopoietic cells and mature blood cells. miR-223, together with miR-197, -574-3p and let-7a represent the previously identified myeloid miRNA signature with substantial differences in the expression between serum and total blood (Table S2). In addition, miR-150 is reported to be highly expressed in human lymphocytes. Twenty-six miRNAs have ≤2 Fold of Change (<10) higher in total blood compared to serum, in this group the red blood cells (RBC) miR-451-3p, -92a-3p, -16-5p, -486-5p are highly expressed in both compartments. In plasma hemolytic samples their expression increase with the extent of the hemolysis suggesting the high impact of RBC in the circulatory miRNA profile. The differences in the average expression level in serum of the RBC-derived and white-cells-derived miRNAs suggest a significant contribution from the erythrocytes even in normal conditions (non-hemolyzed samples). Twenty-seven miRNAs, mostly belonging to the serum 5th quantile, have ≤2 FC <10 higher expression in serum.

Circulating miRNome cluster analysis. The unsupervised hierarchical clustering using Euclidean distances correlation measures of the intensities values for the set of blood and serum shared miRNAs showed the expected clustering of the cases indicating presence of blood and serum-specific miRNA expression profiles (Fig. 3).

Six distinguishable clusters were identified among the expressed miRNA. Cluster 1 (red) includes miRNAs with high expression level in blood, and the 50% of the serum samples have miRNA expressions comparable to blood. These RNAs include miR-486-5p, let-7b-5p, miR-92a-3p, miR-103a-3p, miR-150, -155, -223, -16-5p, -486-5p, -451a, -324a-3p, -17-5p, -191-3p, miR-25-3p, miR-425-5p, miR-93-5p. These miRNAs are widely and highly expressed among different human tissues (Fig. S2) and regulate key cellular pathways, such as cell
proliferation, cell death, kinase activities, aging, metabolic pathways, as evidenced by a validated target enrichment analysis performed with GOrilla (Fig. S3 and Supporting File 1). Those miRNAs have been considered as circulating biomarkers for multiple diseases including HCC. However, they are highly expressed in a wide range of tissues, including blood cells. Therefore blood must be considered as a source of these miRNAs in case of improper sample collection, causing a misinterpretation of the results in biomarker discovery studies.

Figure 3. Heatmap with the pseudocolor scale underneath, of the differentially expressed miRNAs among the 40 samples. Unsupervised hierarchical clustering is used to order samples and miRNAs, the log2-transformed microarray signal was considered. The sample tree with optimized leaf-ordering is drawn using Euclidean distances and average linkages for cluster-to-cluster distance. The six major clusters are identified by different colors: Cluster 1 (red), cluster 2 (blue), cluster 3 (green), cluster 4 (yellow), cluster 5 (orange), cluster 6 (light blue).
Cluster 2 (blue) include miRNAs with high expression levels in blood and in some cases comparable expression in serum. This cluster includes a heterogenic profile of circulating miRNAs, involved in a wide variety of cellular pathways, some of them referring to blood-cells-related pathways: response to oxygen levels, regulation of kinase activity, regulation of immune system, hemopoiesis and regulation of apoptotic process (Fig. S4 and Supporting File 2).

Cluster 3 (green) includes the low expressed miRNAs in both serum and blood. Only four miRNAs of this cluster were previously studied: MiR-27a-3p, -29a-3p, -30a-5p, -143-3p. In HCC, miR-29a-3p, 30a-5p and 143-3p work as oncosuppressors21–23, while contrasting information exists about the oncogenic role of miR-27a, probably due to a different role of the two mature isoforms coming from the hairpin precursors24,25. Unfortunately, we were not able to identify the mature form studied in previous works in HCC.

Cluster 4,5 and 6 include miRNAs with higher expression in serum. The expression of miRNAs in Cluster 4 is highly variable, going from 5.89 FC of miR-2277-5p to 97 FC of miR-1228-3p. Very limited information regarding the cellular role of these miRNAs exist. MOAP1 (Modulator Of Apoptosis 1) and CSNK2A2 (Casein Kinase 2 Alpha 2)26,27 are the only validated targets for miR-1228-3p, with opposite effects on cancer behavior, leading to a still questionable role of this miRNA in cancer.

To cluster 5 (orange) belong high expressed miRNAs in both biofluids, although there is a clear difference between the two compartments. Among the 21 members of the cluster only 6 miRNA have experimentally validated target genes on miRTarBase (http://mirtarbase.mbc.nctu.edu.tw), (miR-638; -663a; -762; -1908-5p; -4516; 4674). The GO target enriched analysis evidenced the involvement of these miRNAs in key cell pathways such as cell cycle, cell migration, cell death regulation and drug response, often dysregulated in cancer (Fig. S5 and Supporting File 3). Since their high expression in both compartments, we hypothesize these miRNAs could represent a portion of miRNA widely released from body cells, although still missing information exists about tissue profiling on these miRNA (Fig. S6).

In cluster 6 (light blue), 10 miRNAs show high differences in the serum compared to blood samples, going from 68.61 FC, of miR-3613-3p, to 633.01 FC of miR-4484. To this cluster belong rather unknown miRNAs both in terms of function and clinical significance. The only validated targets listed in miRTarBase are DUSP1 (Dual Specificity Phosphatase 1) and CD274 for -miR-940 and MUC1 (Mucin 1) for miR-455-3p. Interestingly DUSP1 and CD274, also known as B7H1 (B7 Homolog 1) or PD-L1 (Programmed Cell Death 1 Ligand 1), are involved in cancer progression and vascular invasion28, and cancer immune surveillance29. The importance of these two targets was reported in two recent publications in which a relevant role of these two targets was discovered in HCC30,31. DUSP1 was linked to HCC development and progression34, while the expression of PD-L1 in neoplastic or intra-tumoral inflammatory cells was related to tumor aggressiveness and response to treatments. Further evidence links the expression of miR-940 to a suppression of tumor invasion and migration35 and to apoptosis induction of cancer cells36. For the remaining miRNAs, only a weak experimental association to their putative targets exist.

**Mirna families.** MiRNA families are classified based on the mature miRNA sequence similarities and/or structure of the miRNA precursor34. There have been evidences that miRNAs within the same family have similar expression patterns and coordinate regulate cell biological processes37, ensuring robustness to gene regulatory networks38. In the present study, we further performed a global analysis of miRNA families in the two biofluids of HCC patients, in order to identify potential functional correlations among miRNAs. Fifty-eight miRNA families are present in blood with at least two members. The top expressed families are the let-7/98/4458/4500, miR-378/422a/378bdefhi, miR-17/17-5p/20ab/20b-5p/93/106ab/427/518a-3p/519d,
miR-15abc/16/16abc/195/322/424/497/1907, miR-181abcd/4262, miR-25/32/92abc/363/363-3p/367 and the miR-320abcd/4429 family. Twenty-four families are entirely represented in blood (Table S3) including the top ranked miR-320abcd/4429 family.

In serum 214 miRNA families are present. The let-7/98/4458/4500, miR-320abcd/4429, miR-378/422a/378bc-defhi and miR-17/17-5p/20ab/20b-5p/93/106ab/427/518a-3p/519d families are the most represented families. Six families are fully represented in serum (Table S3) including the top ranked miR-320abcd/4429 family, as for blood.

MiRNA families are differently expressed in the two biofluids. The let-7 family and the miR-378 family are mostly expressed in blood, in which their expression is higher compared to serum (Fig. 4). Furthermore, there are some overlapping families between the two biofluids such as the miR-320 and the miR-548abakhjiwy families (Fig. 4).

In humans the let-7 family consists of 14 miRNA members, let-7a,b,c,d,e,f,g,i, miR-98, miR-4458 and miR-4500 normally involved in hematopoietic stem progenitor cell homeostasis37. In cancers the expression is commonly down-regulated38. In HCC evidences of reduced let-7 family members was observed in tissues 39,40. On the contrary, in serum those miRNA have been shown to be increased41. In our blood samples the let-7 family members are highly represented and have a high average expression (avg log2 expr. 10.35; min–max 8.87–15.27 for the let miRNAs, except for miR-4458 (avg log2 1.67; min–max 0.77–2.52) Table S3). The family members correlate in the expression in blood except miR-4458 that has the lowest correlation coefficient (Fig. 5a). The high correlation is also explained by the overlapping among validated mRNA targets (Fig. S7). This observation agrees with the important role of the family in the hematopoietic stem cells and with the previous finding that reported those miRNA highly expressed in blood42. Interestingly, when comparing blood and serum, we observed no correlation in the expression among the blood and serum miRNAs (Fig. 5b), suggesting a possible extra-blood origin of the serum miRNAs. To notice that the overall correlation among the serum miRNA is generally lower than in blood (Fig. 5b).

Another highly correlated family in blood and serum is the miR-17 family. The family members miR-106a-5p, miR-106b-5p, miR-17-5p, miR-20b-5p, miR-93-5p are expressed in the two biofluids. However, while the expression in blood is homogeneous, more variability was observed in serum (Table S3 and Fig. 1, 2). Comparing the two biofluids, we found a negative correlation among serum miR-17-5p, -106b-5p, miR-93-5p and the miRNA family members in blood (Fig. 5c), suggesting two distinctive patterns for the two compartments. MiR-17-5p, -106b-5p, -93-5p have been extensively studied both in HCC tissues43 and serum44,45, in which they are considered as oncomirs. Thus, their expression in serum could be influenced by the disease rather than blood cells. The high and relatively stable expression of these miRNA suggests a physiological role in blood cell. A high and stable expression of the miR-320 family was observed in blood. Moreover, a high intra-blood and intra-serum exist among the miR-320a, miR-320b and miR-320c members, probably due to the high conservation among

Figure 5. (a) Correlation chart of the let7 family members in whole blood. Correlation chart of the let7 family (b), miR-17 family (c), and miR-320 family members (d) in both whole blood and serum. The upper-right side shows the correlation coefficient and p-value for each pairwise correlation. The lower-left side show the distribution. b, c and d show the correlation for the miRNA that are expressed in both biofluids. Intra-blood correlations (black quadrants) and intra-serum correlations (grey quadrants) are reported. Outside the quadrants the pairwise inter-biofluids correlations.
the sequences (Fig. 5d). Although the very similar pattern, there is no significant correlation between the two biofluids for the three miRNAs. Only a slightly positive association was observed among serum miR-4429 and the blood miR-320a,b,c.

To the miR-378 family belong miR-378a,b,c,d,e,f,h,i and miR-422 all expressed in blood, while in serum only miR-378a,c,h,i are detectable (Table S3). MiR-378a has been reported to be involved in cell survival, tumor growth and angiogenesis. In liver it inhibits the hepatic stellate cell activation and works as an onco-suppressor by targeting VEGF, PDGFR3 and c-Raf. Very few information exist about the other family members, despite their high expression in blood (miR-378c,d,f,i) and serum (miR-378h). MiR-378h has a 47.02 fold higher expression in serum compared to blood (Table S2), however, to our knowledge no information about this miRNA is available in literature. Interestingly miR-378w was previously detected only in liver and testis (Fig. S8). The weakly associated target genes, downloaded from MirTarBase, link this miRNA to FAS signaling pathway, VEGF signaling pathway, Insulin/IGF pathway-protein kinase B signaling cascade, p53 pathway feedback loops 2, Oxidative stress response, Gonadotropin-releasing hormone receptor pathway, Angiogenesis, although no statistically enrichment was obtained (Table S4). To further clarify the function of this miRNA in HCC more extensively studies are needed.

**MiRNA differently expressed in HCC.** To further verify the consistency of our data with previously identified miRNA HCC, we searched in StarBase v.3.0 database for differently expressed miRNAs in 370 HCC compared to 50 normal livers. Among the 187 miRNAs expressed in the serum of our patients, we identified 139 being already identified in HCC tissues, however only 95 with a log2(RPM + 0.01) > 0.1 (Table S5). Twenty-five miRNAs were significantly up-regulated in HCC tissues compared to normal liver, while 40 were significantly downregulated (Table S5). With this preliminary study we are not able to define a clinical significance for these findings that should be further validated in independent translational studies.

**Conclusions**

In recent years, circulating biomarkers have been considered as a powerful, non-invasive tool for disease detection or cancers follow-up. MiRNAs are eligible biomarker candidates for their stability, easy assessment and role in gene regulation. Although detectable in all biofluids, serum is the most studied source as readily accessible as fresh or frozen material. A portion of cell-free circulating miRNAs released in serum could originate from cancerous cells or tumour progression being promising candidates as cancer biomarkers.

Recently, an increasing interest is paid in the study of the whole blood miRNome originating from monocytes, platelets, mature red blood cells, cells involved in cancer immune-surveillance and circulating cancer cells. Giving the systemic nature of cancer, the whole blood miRNome could represent an important source of information for biomarker discovery studies. In this work we compared the circulating miRNome from two distinct compartments: Whole blood and serum. Despite the multitude of circulatory miRNAs investigations being present in literature, still fragmented and scanty information exists on HCC. We identified different expression patterns for the two biofluids. Some miRNAs, previously identified as serum biomarkers in HCC are also highly expressed in blood, such as miR-107, miR-92a-3p, miR-17-5p, miR-223. On the other hand, the role and clinical significance of some highly expressed miRNAs in serum are still unexplored in HCC, such as miR-4484, miR-904, miR-933, miR-378h, providing hints for new researches in this field. Despite the major limitation of our study consisting in the relatively low number of patients, we believe that this study might represent a step in the way for future studies enrolling larger cohorts for an independent validation. Our contribution in the characterization of the circulating miRNome in blood and serum from HCC patients is still exploratory and clinical and functional studies are desired to further promote the use of miRNAs as biomarkers in a clinical setting.

**Data Availability**

The datasets used during the current study are available from the corresponding author on reasonable request.

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Author Contributions

D.P., R.C. and L.C. designed the study. R.P. and L.C. enrolled the patients, collected the samples and elaborated the clinical database. D.P. extracted the small RNAs. H.K. performed the miRNA gene array. D.P. performed the statistical analysis. D.P., R.C., L.C., J.T.-C. and C.T. analyzed the data and participate in the discussion of the results. D.P., R.C. and C.T. wrote the manuscript. All authors contributed to the manuscript revision.

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Additional Information

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