METHODS MANUSCRIPT

Plasma or serum? A qualitative study on rodents and humans using high-throughput microRNA sequencing for circulating biomarkers

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Abstract

microRNAs are small non-coding RNAs gaining interest for their potential roles as reliable biomarkers for the diagnosis and therapeutics of numerous pathologies, ranging from cancer to neurodegenerative or psychiatric disorders. Indeed, microRNAs are present in various accessible biofluids, including peripheral blood, and specific dysregulation of their expression may be associated with these different pathological conditions. microRNAs can be isolated from plasma or serum for sequencing with commercial kits. However, these two biofluids might exhibit some differences in their microRNA contents, due notably to the coagulation process occurring during serum collection. It remains unclear from previous studies and commercial recommendations which blood fraction is preferable. Because of the small amount of circulating microRNAs in a given blood volume, this question appears crucial for qualitative and quantitative optimization of microRNA profiling, especially in animal models used for investigating the pathophysiological relevancy of this approach. We therefore evaluated the efficiency of RNA isolation and microRNA levels from plasma and sera isolated from rats and humans, with a widely used extraction kit (QIAGEN miRNeasy), and assessed microRNA quality and quantity with high-throughput sequencing. Fewer reads with length corresponding to non-miRNAs sequences were observed in plasma than in serum, both from rats and humans. Moreover, rat plasma produced twice as many aligned reads compared to sera, as well as more aligned reads corresponding to microRNAs (84.6% against 38.7%), differences that were not find in human samples. Our results, therefore, clearly indicate that plasma should be preferred for miRNA investigations, particularly for translational studies.

Keywords: microRNA, plasma, serum, biomarker, high-throughput sequencing

Introduction

MicroRNAs (miRNAs) are small non-coding RNAs of 19–23 nucleotides, playing a crucial role in the control of RNA messenger (mRNA) translation [1–3]. They exert their post-transcriptional silencing properties by binding to targeted RNAs, leading to their degradation or their translational repression. Moreover, a given

miRNA can control several mRNA targets, and a given mRNA can be silenced by several miRNAs. Thus, miRNAs form a complex regulatory network and, unsurprisingly, their dysregulation has been implicated in a large number of human diseases, ranging from cancer to neurological and psychiatric disorders, such as Parkinson's disease, addiction or depression [4–8].

Due to their biological relevance and their presence in readily available biofluids, miRNAs are now widely studied for their potential use as non-invasive biomarkers [9]. Serum and plasma prepared from peripheral blood are the most commonly chosen because they are easily accessible [10–12]: serum is obtained by letting the blood coagulate, while plasma is the liquid portion of blood that has been prevented from clotting [10–12]. Although the choice between serum or plasma samples is rarely explained in publications, these two biofluids frequently appear to be used as similar materials. However, they are likely to exhibit substantial differences in their miRNA content, making comparison of data between studies very difficult [13–16]. For instance, the presence of fibrinogen in plasma samples might be source of contamination, affecting the extraction quality. On the other hand, the coagulation process occurring during serum collection increases sample-to-sample variability, as RNA released during clotting may change the true repertoire of circulating miRNAs. Therefore, differences in the processing of peripheral blood may lead to important discrepancies among studies and complicate the identification of reliable and relevant miRNA markers. It is thus of prime importance to identify the best biofluid fraction to use, to facilitate discovery of miRNA-based biomarkers and to increase consistency among studies. This appears particularly crucial for animal models aimed at investigating the pathophysiological relevancy of miRNA-based biomarkers in which the amount of circulating miRNAs as well as blood volumes collected are relatively low.

Here, both plasma and sera were isolated from rats and humans, before extracting miRNA-enriched fractions with a widely used commercial kit, QIAGEN miRNeasy Mini kit, coupled with the QIAGEN minElute Cleanup kit [17, 18]. Small-RNA-enriched fractions were then processed using high-throughput miRNA sequencing in order to evaluate differences between serum and plasma miRNA profiles, and finally determine the best suitable preparation for miRNA study.

Materials and methods

Animals

Blood was collected from 12-week-old male Sprague-Dawley rats (Janvier, Le Genest-Saint-Isle, France). The experiment was performed to optimize miRNA analysis, before starting a large-scale sequencing project on this rat strain, including behavioural experiments requiring food restriction. Hence, to be consistent with the future experimental conditions, animals were maintained at 90% of their free-feeding weight and housed individually, under standard laboratory conditions (12 h light/dark cycle, with lights on at 7 a.m.), with water available *ad libitum*. Two rats were used for a miRNA sequencing experiment and two other animals were processed for RT-qPCR validation. All experimental protocols complied with the European Union 2010 Animal Welfare Act and the new French directive 2010/63 and were approved by the French national ethics committee no. 004.

Blood collection

Rats were decapitated under isoflurane anaesthesia. Blood was collected directly from the trunk and processed to provide both
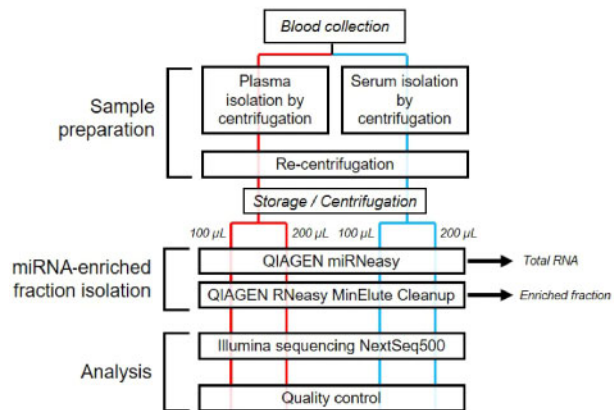
Figure 1: Graphical summary of workflow. Plasma and serum were collected from rats and human subjects and miRNA-enriched fractions were extracted using QIAGEN kits prior to high-throughput miRNA sequencing.

plasma and serum from each animal (Fig. 1). For plasma, 3–4 mL peripheral blood was collected in a K_2EDTA BD Vacutainer tube (Becton Dickinson, Plymouth, UK, no. 367525) and gently agitated at room temperature. After 15 min, plasma was isolated by centrifugation at 1900 g at 4°C for 10 min and the supernatants were transferred to new microtubes (Safe-Lock 1.5 mL, Dutscher, Paris, France, no. 033290), which were centrifuged at 16 000 g at 4°C for 10 min, to remove additional cellular debris and to minimize contamination from damaged blood cells. Aliquots (400 µL) were stored at −80°C, in new microtubes. For serum samples, blood (1.5 mL) was collected and left for 45 min at room temperature in Eppendorf tubes to allow clotting. They were then treated as described above for the plasma samples. For human subjects, all samples were collected from individuals who had given written informed consent for this study (ID-RCB number 2014-A00099-38 and ANSM authorization number B140704-31). Peripheral blood samples were collected using a 21-gauge needle, and processed as for rats. Plasma and serum aliquots (500 µL) were frozen at −80°C and sent to our laboratory for miRNA extraction and miRNA sequencing. The design and the different experimental steps were the same for rats and human samples, and are represented in Fig. 1.

Small-RNA-enriched fraction isolation

Total RNA was extracted from serum and plasma using the commercial miRNeasy Mini kit (Qiagen, Germany, cat. no. 217004) according to the manufacturer's instructions. Fractions were then enriched in miRNAs and other small RNAs (less than 200 nucleotides) by the specific protocol provided by the manufacturer and with the RNeasy minElute Cleanup kit (Qiagen, Germany, cat. no. 74204) (Fig. 1). Prior to extraction, samples were thawed at room temperature and then centrifuged at 16 000 g at 4°C for 5 min, to remove cryoprecipitates. To assess the best amount of starting material, two extractions, using 200 µL or 100 µL of plasma and serum, were performed for each subject and were processed together. For both extraction procedures, small RNAs were washed with RWD buffer (stringent washing buffer containing guanidine salt and ethanol, Qiagen, Germany, cat. no. 1067933) during the enrichment of miRNAs. Finally, miRNA fraction was eluted by adding 14 µL RNAse-free water and stored at −80°C until sequencing or RT-qPCR.

miRNA sequencing

High-throughput miRNA sequencing was performed at the sequencing platform of the 'Institut du Cerveau et de la Moelle épinière' (ICM) in Paris, France. Briefly, libraries were generated from miRNA-enriched fractions using the QIAseq miRNA Library Kit (Qiagen, Germany, cat. no. 331505). Single-end sequencing (75 bp) was performed by Nextseq500 using High Output Kit v2. Prior to sequencing, RNA concentration and the quality of the library were determined using a Tecan Spark 10M (Tecan). RNA concentration and integrity were also analysed using the Agilent Small RNA kit in an Agilent 2200 TapeStation (Agilent Technologies).

miRNA sequencing data analysis

Sequencing data quality was assessed using FastQC. Sequences were trimmed by cutadapt (v0.9.5) before analysis. The proportion of miRNAs in the sequences was evaluated using Bowtie (v0.12.7) [19] for mapping sequences on the rn5 Rat and hg19 human genome assembly with the following command line: '-p 4 -S -q -n 1 -e 80 -l 30 -a -m 5 –best –strata', and using HTSeq [20] and GENCODE v19 to count reads per gene on Ensembl release 75 annotations. miRNA expression was obtained by mapping and quantifying sequences with mirDeep2 [21] on mirbase v21. Based on these read counts, data were normalized using edgeR [22] on R (v.3.2.5). Unsupervised clusterings have been performed using 'dist' and 'hclust' functions in R, using Euclidean distance and Ward agglomeration method.

RT-qPCR

To validate the sequencing results in plasma and sera, specific miRNA sequences were quantified with the same experimental design for two rats, and using specific stem-loop primers for reverse transcription (RT) and PCR primers/TaqMan probes for each miRNA. The concentration of each miRNA-enriched fraction was determined by spectrophotometric measurement at 260 nm (Nanodrop). RT was performed with the TaqMan MicroRNA Reverse Transcription Kit (Life Technologies, France, cat. no. 4366597) according to the manufacturer's instruction, with the following reaction conditions: 30 min at 16°C, followed by 30 min at 42°C and 5 min at 85°C. Each RT was carried out in a total reaction volume of 15 μL with a fixed volume of 5 μL of diluted miRNA-enriched fraction (corresponding to 1 ng of miRNA per reaction). RT products were stored at −25°C until further use. PCR reaction was achieved using the TaqMan Universal Master Mix II, no UNG (Life Technologies, France, cat. no. 4440048), in addition to the TaqMan microRNA assay (Life Technologies, France, cat. no. 4427975) corresponding to the previously reverse transcribed miRNA (assay ID 000391 for miR-16a-5p, assay ID 002299 for miR-191a-5p, assay ID 000379 for let-7c-5p, assay ID 002619 for let-7b-5p, assay ID 001973 for U6-snRNA). A 20 μL reaction mixture containing 1.33 μL of RT product was run in duplicate on the CFX96 (BioRad), at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

RT-qPCR analysis

RT-qPCR data were analysed using CFX Manager (BioRad) and Excel. Probe efficiency was determined for each miRNA with a standard curve, in order to calculate the relative miRNA quantity in each sample. A No Template Control and a No RT control were systematically added for each experiment. RT-qPCR data for miR-191a-5p, miR-16a-5p and let-7c-5p were normalized by combination of two reference genes, stably expressed among all our samples: U6-snRNA and let-7b-5p or with U6-snRNA only for let-7b-5p validation.

Results

## Serum and plasma samples from rats are dissimilar and clustered by type of biofluid

An unsupervised analysis was performed in order to determine how rat samples cluster without a priori (Fig. 2). Interestingly, samples clustered first by biofluid fraction (plasma vs serum), and then by rat, suggesting that miRNAs differ between plasma and sera, independently of individuals. Furthermore, the amount of starting material (100 μL vs 200 μL) does not seem to make any difference, since it did not influence the clustering of our samples.

Plasma but not serum samples from rats mainly contain miRNA-related sequences

In order to uncover the factors contributing to the differences between plasma and serum samples after extraction and enrichment, we first investigated the sequence length profile of the reads obtained for each sample (Fig. 3A and B). Very short sequences ranging from 4 to 13 base pairs (bp) certainly correspond to fragments of reads. A peak at 20–22 nucleotides is retrieved in both fractions, but with a number of sequences 5–7 times higher for plasma samples compared to sera. In view of their length, this peak is likely to include miRNA sequences. For serum samples, there is an additional, much larger peak of 30–31 nucleotides, corresponding to other small non-coding RNAs (Fig. 3B). This peak is virtually absent from plasma samples. Therefore, plasma and serum differ in terms of the RNA sequence lengths obtained, with plasma samples containing mainly miRNA-related sequences and serum mainly other non-coding RNA populations.
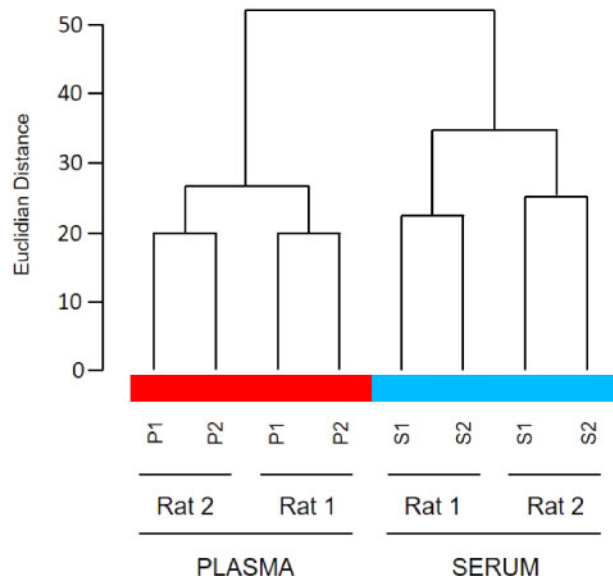

Figure 2: Dendrogram of rat sample clustering. Dissimilarity was calculated using the Ward method D2 criteria; P1: 100 μL starting material for plasma; P2: 200 μL starting material for plasma; S1: 100 μL starting material for serum; S2: 200 μL starting material for serum.
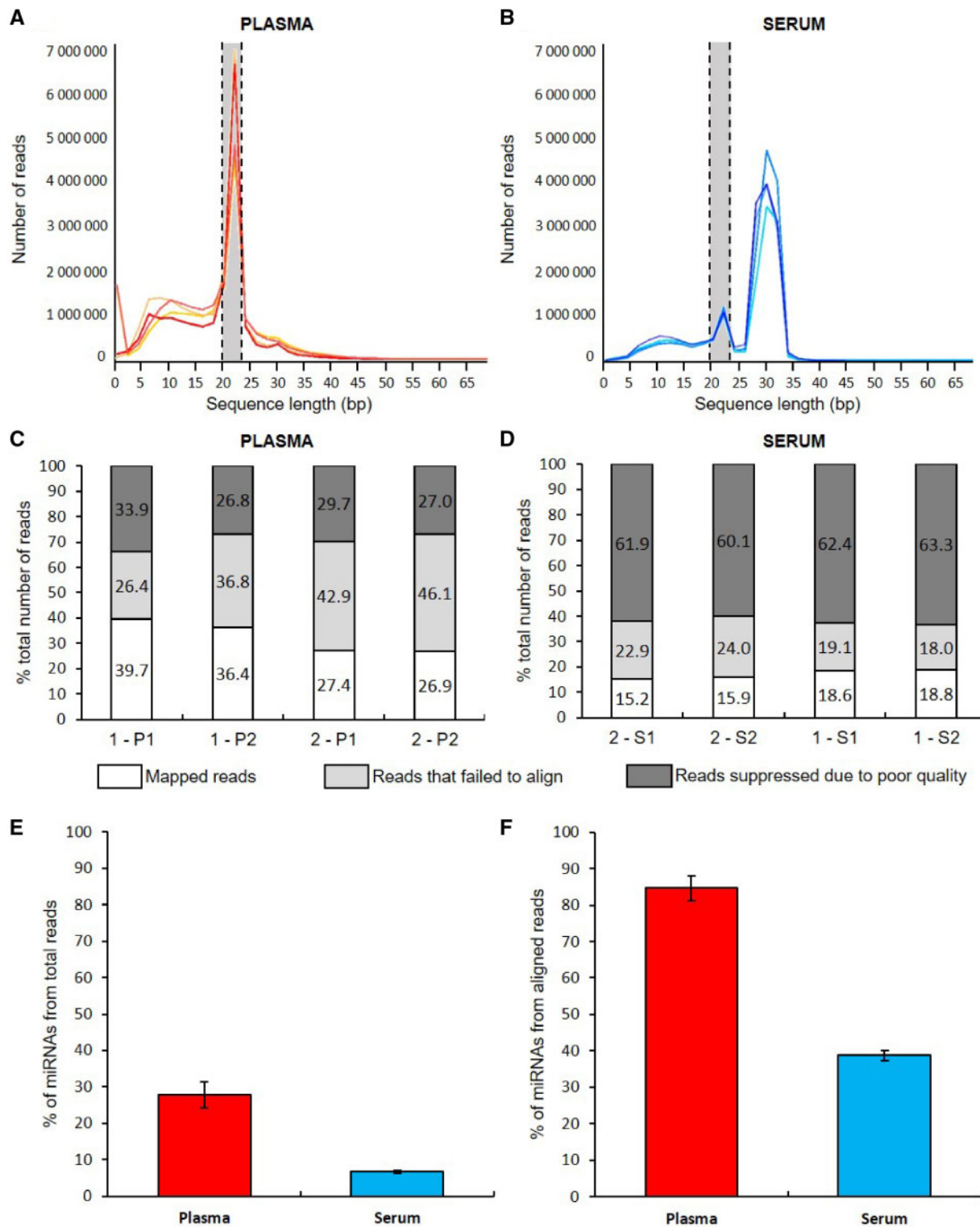
Figure 3: Evaluation of rat miRNA content from plasma and serum samples. (**A–B**) Number of reads, according to their length (in base pairs), for rat plasma (A) and serum (B). Each coloured line corresponds to the distribution of reads from one sample and the grey zone between two dotted lines corresponds to the 20–23 nucleotides-length sequences. (**C–D**) Alignment rates for plasma (C) and serum (D). 1 and 2 correspond to rats 1 and 2; S1: 100 µL starting material for serum; S2: 200 µL starting material for serum; P1: 100 µL starting material for plasma; P2: 200 µL starting material for plasma. (**E–F**) Percentage of miRNA content over total (E) and aligned reads (F) (mean ± S.E.M, *n* = 4).
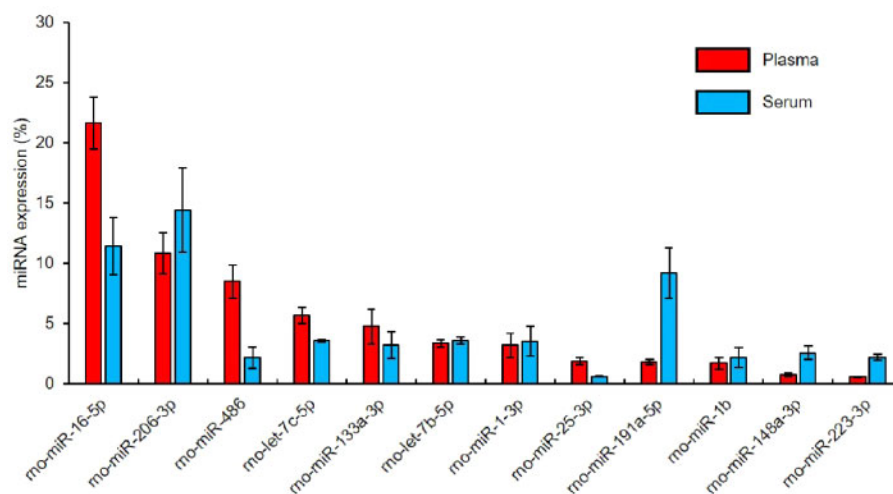
Figure 4: Comparison of the most expressed miRNAs between rat plasma and sera. The 10 most expressed miRNAs from plasma and serum samples were selected for this analysis (mean $\pm$ S.E.M, $n = 4$).

Mapping is better in rat plasma samples than in rat serum

In order to assess the overall quality of the sequences obtained depending on the biofluid fraction used, we looked at the mapping rate in plasma and sera. After alignment of the data to the reference genome, the mapping rate was higher for plasma samples, with a mean of $32.6 \pm 3.2\%$ of mapped reads (Fig. 3C; Supplementary Table S1A), while only $17.1 \pm 0.9\%$ of the sequences mapped on the rat genome for serum samples (Fig. 3D; Supplementary Table S1A). Therefore, the serum samples present a weaker alignment rate, compared to plasma samples. It is noteworthy that most of the suppressed reads were discarded due to failure to align (adaptators, or unspecific sequences that mapped more than once) for plasma samples (Fig. 3C, Supplementary Table S1A), while the quality of serum samples was clearly too poor (Fig. 3D, Supplementary Table S1A).

Reads corresponding to miRNAs are more enriched in rat plasma than in rat serum samples

Because of differences in sequence content and mapping efficacy, miRNAs represent $27.9 \pm 3.6\%$ of total reads for rat plasma and only $6.6 \pm 0.4\%$ for serum (Fig. 3E). Even when restricted to aligned reads, miRNA enrichment appears superior for plasma than for serum. Indeed, $84.6 \pm 3.4\%$ of aligned reads correspond to miRNAs in plasma samples according to Gencode annotations (Fig. 3F). Moreover, the number of miRNAs reads ($6\,268\,614 \pm 798\,928$) indicates that nearly all the sequences ranging from 20–22 nucleotides seen in Fig. 3A ($7\,596\,287 \pm 671\,084$) were correctly mapped and were indeed miRNAs. In contrast, only $38.7 \pm 1.5\%$ of aligned reads in serum samples corresponded to miRNAs (Fig. 3F).

More miRNAs are detected in rat plasma than in rat serum

Next, we determined whether the differences in alignment and enrichment influence the detection of miRNAs. With a minimum depth of five reads per transcript, we found that 490.5 ± 14.6 known miRNAs were detected (i.e. miRNA with at least five reads) for plasma samples against 393.8 ± 8.7 for serum samples. Using another normalization method (sequencing values normalized to the total number of reads for each sample) leads to the same conclusion, with an increase in detection of known miRNAs in plasma compared to serum (380.8 ± 10.5 and 306.0 ± 12.1, respectively). Although the main miRNAs are globally the same in plasma and serum (Fig. 4), such as miR-206-3p, miR-133a-3p, let-7b-5p or miR-1-3p, significant differences in the overall distribution between plasma and serum samples were observed. For instance, some miRNAs, such as miR-16-5p, let-7c-5p and miR-486, were under-represented in serum samples, while others were over-represented, such as miR-191a-5p. As a result, miR-16a-5p appears as the most expressed for plasma, let-7c-5p as the fourth one and miR-191a-5p the ninth, while they, respectively, correspond to the second, fifth and third most expressed miRNA for sera (Fig. 4). In order to validate these results with another quantification method, expression levels of miR-16a-5p, let-7c-5p, miR-191a-5p and let-7b-5p in plasma and serum samples were further evaluated using RT-qPCR. The expression profiles obtained were basically the same as by sequencing (Supplementary Fig. S1A-D). Therefore, miRNA content and profiles are clearly different in rat plasma and serum.

Dissimilarities in plasma and serum samples are specific to rats

Human samples were processed and analysed in order to assess the specificity of the findings described for rodents. First, unsupervised analysis showed that plasma and sera samples also clustered by biofluid fraction, with however two exceptions (Supplementary Fig. S2). Interestingly, the differential sequence length profile found in plasma and serum samples from rats was also identified within human samples, with the presence of the additional peak (30–31 nucleotides) in serum samples (Fig. 5A and B). However, and in contrast to what was observed in rats, the number of sequences of 20–23 nucleotides appeared equivalent between the two biofluids. Moreover, the overall quality of the sequences, looked similar, with an alignment of $23.0 \pm 2.2\%$ and $25.6 \pm 2.0\%$ of reads for plasma and sera samples (Supplementary Table S1B and Supplementary Fig. S3A-B), and plasma and serum miRNAs represented, respectively, $9.3 \pm 1.8\%$ and $11.4 \pm 2.1\%$ of total reads, and $38.9 \pm 4.6\%$ and $44.1 \pm 4.1\%$ of
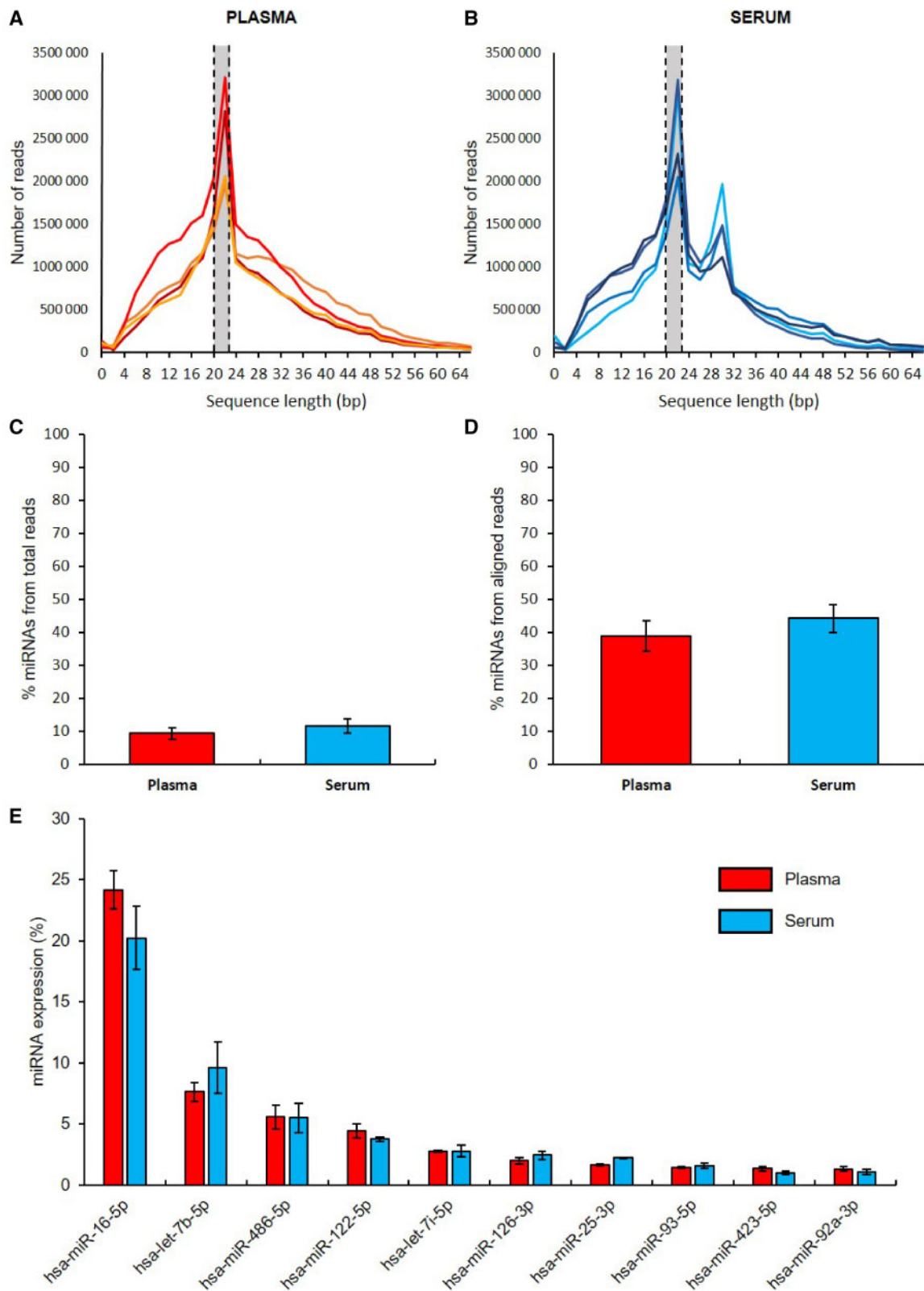
Figure 5: Comparison of human plasma and serum sequences, alignment and miRNA expression. (**A–B**) Numbers of reads, according to their length (in base pairs), for human plasma (A) and serum (B). Each coloured line corresponds to the distribution of reads of one sample and the grey zone between two dotted lines corresponds to the 20–23 nucleotides-length sequences. (**C–D**) Percentage of miRNA content over total (C) and aligned reads (D). (**E**) Expression comparison of the 10 most expressed miRNAs from plasma and serum samples (mean ± S.E.M, $n = 4$). It should be noted here that some miRNAs, such as miR-16-5p, let-7c-5p or miR-486-5p, were also identified as highly expressed in rat samples.
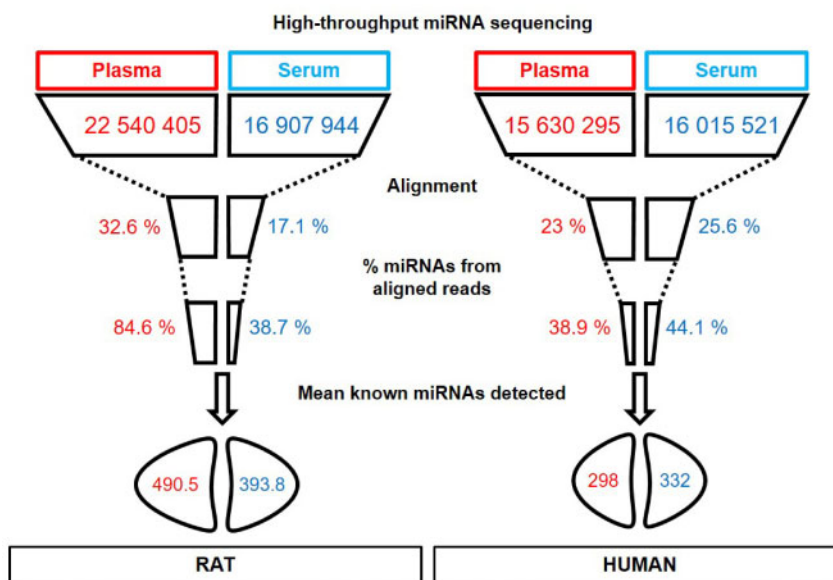
Figure 6: Graphical summary of step-related differences between rat and human plasma and serum.

aligned reads (Fig. 5C and D). Similar results between the two biofluids were still observed when identifying the most expressed miRNAs, as the 10 most expressed miRNAs were the same, with equivalent miRNA percentage expression and conserved ranking (Fig. 5E). Finally, the number of miRNAs detected was virtually the same in plasma (298.0 ± 17.2) and serum (332.0 ± 29.4). Therefore, it appears that miRNA quality, content and profile in human plasma and serum differ less than in rodents.

Discussion

Biofluids are now widely used as an easily accessible provider of biological material for the development of new therapeutical and diagnostic tools. As miRNAs can be extracted from plasma and serum, these two blood fractions have rapidly become references for the investigation of these potential biomarkers in various pathological conditions or preclinical models [5, 6, 8, 23, 24]. However, they are usually considered to be equivalent, although their miRNA content may significantly differ. For instance, some miRNAs, such as miR-191-5p or miR-34c-5p, were found dysregulated in both plasma and sera of Alzheimer's disease patients, but each fraction also exhibited several non-overlapping dysregulated miRNAs (see for review [25]), leading to different miRNA-related signatures. The same review did not list even one miRNA common to plasma and serum from Parkinson's disease patients [25]. These distinct profiles may be explained by different inclusion criteria (age, sex, evolution of the disease, treatments ...) or the technique used for the miRNA detection (RT-qPCR, sequencing, nanostring ...). Nonetheless, when plasma and sera were collected from the same patients and compared with the same technical approach, substantial differences in miRNA expression profiles remained [13, 26], clearly indicating that the biofluid itself has a significant impact on the miRNAs identified. In the present study, using high-throughput sequencing, we showed striking differences in the detection of miRNAs isolated and enriched from plasma and serum of rats. First, the vast majority of sequences obtained from plasma samples were sequences of around 20 nucleotides long corresponding to miRNAs, whereas most of

the sequences obtained from rat sera were 30 nucleotides long corresponding to other non-coding RNAs. Interestingly, this 30 nucleotide-long sequence peak was also observed in human sera. Regarding the sequences obtained in rodents, plasma samples led to 25% more reads at the sequencer output compared to serum samples, with a read alignment twice as high as in serum (Fig. 6). In addition, from these aligned reads, plasma samples were twice as concentrated in miRNAs compared to serum (Fig. 6). Therefore, at each step of the process, plasma appears much better than serum, finally resulting in the detection of far more miRNAs in plasma. Indeed, 20% of miRNAs reliably identified in plasma (about 100 miRNAs) were not detected in serum at all (Fig. 6). The study of human biofluids, however, show that expression profile of the most expressed miRNAs, alignment rates and average miRNA detection were similar in both plasma and serum (Fig. 6). Consistently, the two biofluids were less clearly separated by unsupervised analysis (Supplementary Fig. S2).

The lower number of reads for miRNAs in rat serum samples is not due to a defect in the miRNA data sequencing analysis, as the number of miRNA-related sequences before alignment concords with the number of mapped reads corresponding to miRNAs after mapping, as for plasma samples (Fig. 3B and F). As a result, this indicates that there is less miRNA in serum than plasma even before analysis for this species. A defect during extraction or sequencing is unlikely to account for this decrease in miRNA content. Both biofluid fractions were collected at the same time from the same rats, and both fractions were processed together during the extraction procedure (Fig. 1). All extractions were equivalent, as the internal spike-in control expression (cel-miR-39) added to our samples before the procedure was equivalent across all samples, as evaluated by RT-qPCR (data not shown). Moreover, a library pre-sequencing quality control was done using an Agilent 2100 TapeStation and the same amount of each library was loaded onto the sequencer and multiplexed together. As all parameters were counterbalanced and controlled, any technical issue would have affected both biofluid fractions similarly.

Therefore, the difference in miRNA expression is more likely to be due to low miRNA content in serum samples before

extraction. However, this quantitative differences in miRNA content between plasma and serum samples are unlikely to be due to incorrect processing of collected blood. Indeed, for plasma isolation, we used a centrifugation protocol that greatly limits RNA contamination from blood cells [16], in order to avoid any artificial increase in miRNA content in this biofluid fraction. For serum samples, our results indicate that the coagulation process was also well controlled and not affected by red blood cell lysis, as miRNAs known to be released during haemolysis (miR-16, miR-486, miR-451 …) were not overexpressed in serum samples compared to plasma [15, 16, 26, 27]. The correct processing of our plasma and serum samples was confirmed by a similar low level of absorbance at 414 nm, or virtually no absorbance at 541 and 576 nm (Nanodrop measurement, data not shown), indicating a low or moderate level of haemolysis, whatever the blood fraction [27–29]. One factor that can explain the lower level of miRNAs is the coagulation process itself, occurring during serum preparation [30, 31]. As the activation of platelets and other blood cells release RNAses [32–34], degradation of circulating miRNAs could occur in serum samples. Additionally, a portion of RNAs, including miRNAs, could be adsorbed onto the blood clot during coagulation [26]. Coagulation could also modify the RNA content of the sample, with the release of transfer RNA-derived RNA fragments under cellular and physiological stressful conditions [35] such as the clotting process. Interestingly, the large peak of 30-nucleotide length sequences, seen in serum but not in plasma samples (Figs. 3B and 5B), may correspond to these fragments [15, 35, 36]. A combination of these different phenomena might account for the overall reduced miRNA content and quality found in sera. However, no differences were found between plasma and sera when focusing on human samples, while extraction, processing of the sample and sequencing analyses were identical to those used in rats. Moreover, whatever the human blood fraction investigated, the number of sequences obtained after the library preparation and the miRNA sequencing was half the number obtained with rats. As the same amount of biofluid was used for extraction (100 μL or 200 μL), these differences between rat and human biofluids may reflect a weaker concentration of circulating miRNAs in humans, which might be related to a much larger volume of blood compared to smaller species. Another possibility is a putative differential coagulation process in rodents and humans [37, 38].

Among all the miRNAs detected, especially in plasma, some have been already associated with several disorders such as miR-16-5p for breast and gastric cancer proliferation [39, 40], miR-206-3p for amyotrophic lateral sclerosis [41], members of let-7 family for coronary artery disease and depressive disorders [42, 43], as well as miR-34b and miR-34c in different brain regions in Parkinson's disease patients [23, 24]. The presence, in rodents, of targets already identified in the clinic validates the utility of such preclinical approaches, in order to better understand the role of these miRNAs in the development of the corresponding disease, and confirm their use as relevant and predictive biomarkers.

We have chosen a well-validated next generation sequencing approach, commonly used for miRNA screening [14, 36, 44], which appears relevant for the present question. However, it should be noted that the field is rapidly moving forward and the increasing interest in these small non-coding RNAs has led to the recent development of new and more accurate detection/investigation methods. Preparation techniques, such as the HTG EdgeSeq miRNA sequencing system, now simplify sample preparation as RNA extraction or manual library construction is no longer required [45, 46]. Moreover, this technique allows detection of very low expressing miRNAs with very low-input

material, and should be taken into consideration when studying less abundant transcripts. Similarly, for RT-qPCR, the TaqMan system used is highly specific and sensitive, and thereby particularly adapted for the detection and quantification of miRNAs. Nevertheless, Droplet Digital PCR technology, based on massive sample partitioning, allows absolute quantification of the miRNAs of interest, and thus could lead to even more accurate results [47, 48].

Beyond these methodological considerations, our study clearly suggests that plasma, compared to serum, leads to better detection and analysis of miRNAs in rats in particular, favouring the use of the former for preclinical studies. An additional argument for plasma use, rather than serum, is the specific isolation of circulating microparticles (MP). These MP are released by various cell-types upon cell-activation, are rich in stabilized miRNAs, and deliver their content to target cells. These MP, known to play a role in cancer and cardiovascular diseases, bind to platelets and can therefore be isolated when working with plasma samples, broadening the potential application of this biofluid in biomarker studies [49, 50]. When searching for biomarkers in blood, peripheral blood mononuclear cells (PBMCs) also appear as a potentially useful component to use [51]. These white blood cells share more than 80% of the transcriptome with other tissues and may therefore provide a reliable reflection of miRNA dysregulations occurring in organs difficult to access in routine [44, 52–54]. However, working with this material is highly challenging. Indeed, with rodents, it is difficult to obtain enough whole blood to properly isolate PBMCs (T.D. and S.C., unpublished data) and collect enough material for satisfactory miRNA extraction and sequencing. The use of PBMCs as a source of biomarkers therefore appears as an interesting approach in humans, but remains difficult to apply in small animals.

To conclude, miRNAs were isolated and detected using miRNA sequencing both in plasma and serum samples. For each step of the process, we show that it is preferable to use plasma for miRNAs in rodents, since this fraction exhibits a total number of reads, an alignment rate and a miRNA coverage greater than sera. Our study also reveals substantial species dissimilarities, as these important differences between serum and plasma were not observed in human samples. Taken together, this study provides useful information that should be taken into particular consideration for translational studies, with the preferential use of plasma to allow greater reproducibility and validation of results among studies and species.

Supplementary data

Supplementary data is available at *Biology Methods and Protocols* online

Acknowledgements

Author contributions

T.D., D.M., C.C., S.B., E.R., J.L.H. and S.B. performed research; T.D. and S.C. designed research and T.D., N.R., P.G., S.C.

analysed data; T.D. and S.C. wrote the manuscript with the help of N.R., P.G. and the other authors.

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