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Extracellular vesicles from plasma have higher tumour RNA fraction than platelets

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ABSTRACT

In addition to Circulating Tumour Cells (CTCs), cell-free DNA (cfDNA) and Extracellular Vesicles (EVs), the notion of "Tumour-Educated Platelets" (TEP) has recently emerged as a potential source of tumour-derived biomarkers accessible through blood liquid biopsies. Here we sought to confirm the suitability of the platelet blood fraction for biomarker detection in comparison to their corresponding EV fraction. As publications have claimed that tumour RNA and other tumour-derived material are transferred from tumour cells to the platelets and that tumour-derived transcripts can be detected in platelets, we chose to focus on RNA carrying a mutation as being of bona fide tumour origin. After informed consent, we collected prospective blood samples from a cohort of 12 melanoma patients with tissue-confirmed BRAF V600E mutation. Each blood specimen was processed immediately post collection applying two published standard protocols in parallel selecting for EVs and platelets, respectively. The RNA of each fraction was analysed by a highly sensitive ARMS RT-qPCR enabling the quantification of the mutant allele fraction (%MAF) of BRAF V600E down to 0.01%. In a direct comparative analysis, the EV fraction contained detectable BRAF V600E in 10 out of 12 patients, whereas none of the patient platelet fractions resulted in a mutant allele signal. The platelet fraction of all 12 patients contained high amounts of wild-type BRAF signal, but no mutation signal above background was detectable in any of the samples. Our observations suggest that the phenomenon of tumour RNA transfer to platelets occurs below detection limit since even a very sensitive qPCR assay did not allow for a reliable detection of BRAF V600E in the platelet fraction. In contrast, EV fractions derived from the same patients allowed for detection of BRAF V600E in 10 of 12 blood specimens.

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Introduction

Minimally invasive technologies such as liquid biopsies capturing tumour markers in body fluids are starting to complement or replace tissue-based techniques for molecular diagnostics, which are often impaired by sample availability, tumour heterogeneity and increased risk to the patient.

Analysis of blood or other biofluids from cancer patients can be used for diagnosis [1], stratification [2,3], prognosis [4] and longitudinal monitoring [5]. Peripheral blood provides a pool of tumour-derived components such as Circulating Tumour Cells (CTCs), cell-free DNA (cfDNA), ribonucleoproteins, Extracellular Vesicles (EVs) as well as non-tumour derived components such as platelets and red and white blood cells [6]. All these blood fractions potentially harbour important biomarker information, either from the tumour itself or from the body's response to it [7].

When developing assays for the detection of biomarkers in blood, the most important consideration is whether the biomarker is present in the biofluid sample in sufficient copies for detection. It has repeatedly been shown that mutated copies of cfDNA in plasma are low especially in early stages of cancer [8] and we have previously demonstrated that the sensitivity of liquid biopsy mutation detection can be increased by combined isolation of both cfDNA and exoRNA from the same plasma sample, especially in stages of the disease where copies are limited [9–11].

Secondly, biomarker detection must be sufficiently specific to distinguish the biomarker from the complex background of a biofluid sample. The specificity of detection is governed by the uniqueness of the biomarker and the ability to develop an assay that detects the biomarker without interference from the background. The decision of which fraction of a biofluid sample to use is determined by where the biomarker signal to background noise is most favourable. When

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a biomarker is very unique, e.g. an RNA fusion transcript or translocation (e.g. EML4-ALK), it is possible to create a very specific assay and it may not be necessary to fractionate the biofluid sample as exemplified by Bettegowda et al. who analysed gene rearrangements in the cell fraction rather than enriching for the circulating tumour cells [8], since any fractionation of the sample may lead to loss of copies of the biomarker. However, in case of expression profiling of RNA of tumour origin or detection of mutations that do not involve large translocations, it is beneficial to enrich the sample. Especially in expression analysis, the tumour- and background-RNAs are *identical* and it is impossible to distinguish the transcripts of tumour and non-tumour origin. Thus, any background copies of non-tumour origin are simply diluting the tumour signal and it is pivotal to use the biofluid compartment with the optimal biomarker-to-background ratio to ensure the least degree of dilution of tumour biomarker signal.

Over the past few years, there have been several reports suggesting that Tumour-Educated Platelets (TEP) could harbour tumour-derived RNA, for example, by taking up tumour exosomes [12–15]. Most recently, a publication in Nature Protocols described the isolation of platelets from blood for RNA analysis [16], so we sought to confirm the suitability of this blood fraction for liquid biopsy applications using this protocol in direct comparison to the EV fraction from the same samples isolated by a commercially available method described previously [17]. If platelets contain significant amounts of tumour RNA and have a favourable tumour-to-non-tumour RNA ratio, this could be a valuable blood fraction for detection of RNA biomarkers of tumour origin. We chose to focus on melanoma patients with tissue confirmed *BRAF* V600E positive tumours and consider RNA carrying the BRAF V600E mutation as being of *bona fide* tumour origin. We compared blood fractions, including platelets and EVs, for the presence of this mutation and the corresponding amount of BRAF wildtype background.

Material and methods

Ethics statement

The study was performed under a clinical study protocol approved by the Ludwig-Maximilians-University (Munich, Germany) ethics review board. All participants provided written informed consent prior to participation in the study.

Sample collection and processing

A group of 12 treatment naïve or progressing high-grade melanoma patients (stage III–IV) was defined for their tumour grading and confirmed by tissue testing to carry the BRAF V600E mutation (Supplemental Table S3). This cohort as well as two healthy controls (NHC) were recruited for single specimen donation per patient.

Fresh blood samples were prospectively collected by venipuncture in 2 × 8.5 mL EDTA plasma tubes (BD Vacutainer® PPT™ Plasma Preparation Tube IVD, Cat# 362799) per patient or healthy control over a 6-month period. To prevent sample degradation and platelet activation, all tubes were kept upright at room temperature (rt) and were transported with minimal agitation to Exosome Diagnostics' ISO 15189 accredited clinical laboratory for immediate processing of each individually collected sample within 2 hours post collection. Each specimen was subjected to two different protocols in parallel on the day of collection for separation of both EVs and platelets. See Figure 1 for experimental design and protocol overview.

EV separation and RNA purification

To prepare plasma for isolation of EVs from whole blood, the EDTA tube was first centrifuged at 1,100 x g for 10 min (Beckman Coulter, Allegra X-15 R, rotor SX4750A), followed by filtration through a 0.8 µm filter (Millex-AA, MerckMillipore) to remove remaining cells, cell debris and platelets. The EV fraction was then prepared from plasma according to the manufacturer's instructions using Qiagen exoRNeasy (#77164), a dedicated product for spin column-based isolation of extracellular vesicles, followed by extraction of the RNA content [17].

In brief, 2 mL plasma per specimen were mixed with 1 volume of binding buffer and added to the exoEasy membrane affinity column to bind the EVs to the membrane. After centrifugation (500 x g, 1 min, rt, Beckman Coulter, Allegra X-15 R, rotor SX4750A), the flow-through was discarded and wash buffer was added to the column to wash off non-specifically retained material. After another centrifugation (5,000 x g, 5 min, rt), and discarding of the flow-through, the vesicles were lysed by adding 700 µl QIAzol to the spin column, and the lysate was collected by centrifugation (5,000 x g, 5 min, rt). The *miRNeasy Plasma Spike-In Control* (QIAGEN, #219600) was added. Following addition of 90 µL chloroform, thorough mixing and centrifugation (12,000 x g, 5 min, 4°C, Eppendorf,

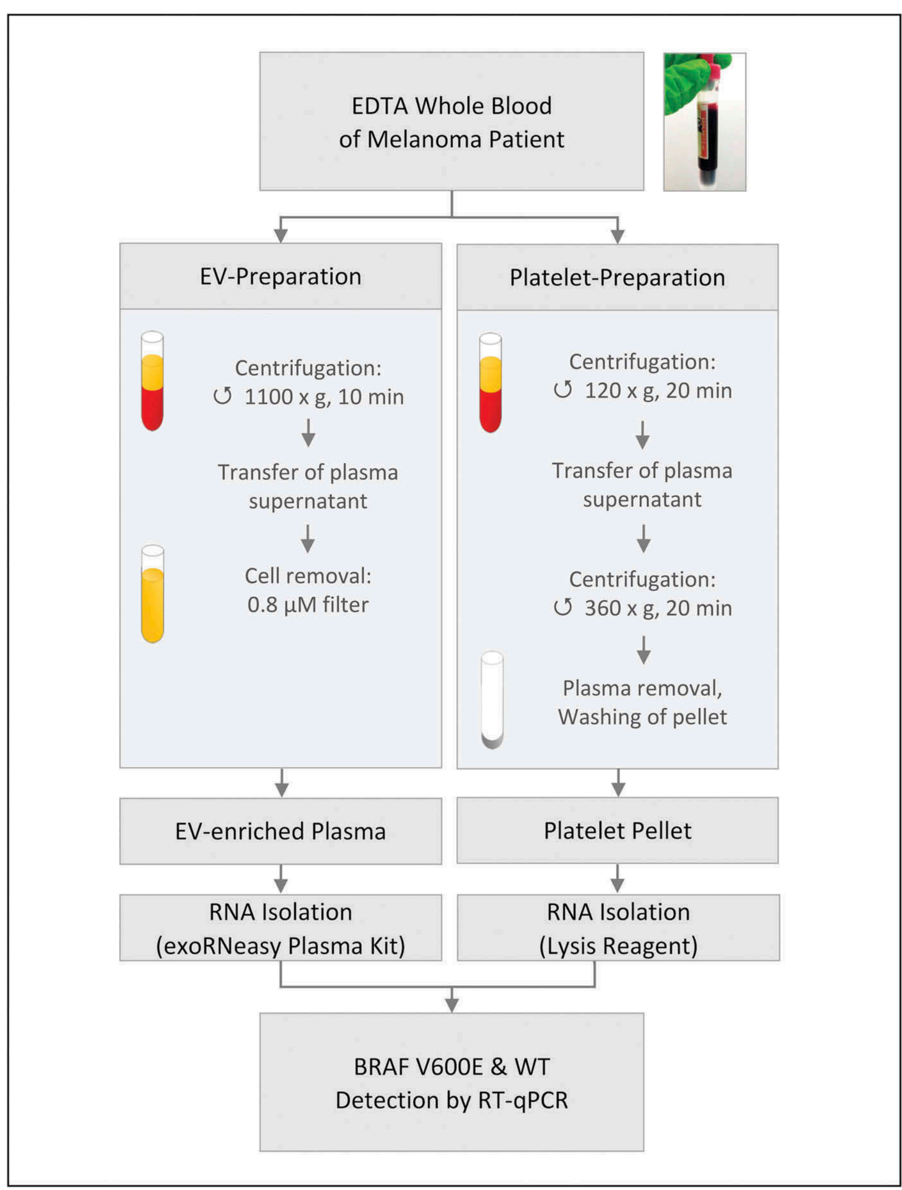

Figure 1. Experimental outline of blood fractionation and molecular analysis. Collection of platelet pellet was done by low spin of whole blood (120 xg, 20 min) followed by low spin of supernatant plasma (360 xg, 20 min). Collection of EV-enriched plasma was done by high spin of supernatant (1,100 xg, 10 min) followed by filtration (0.8 µm) of the supernatant. (EV: Extracellular vesicles).

5424 R, rotor 5424 R) to separate organic and aqueous phases, the aqueous phase was recovered and mixed with 2 volumes of 100% ethanol. The sample-ethanol mixture was added to a RNeasy MinElute spin column and centrifuged (8,500 x g, 15 sec, rt). The column was washed once with buffer RWT (8,500 x g, 15 sec, rt), and then twice with buffer RPE (8,500 x g, 15 sec and 2 min, rt) followed by membrane drying (17,000 x g, 5 min, rt) and elution of RNA into nuclease-free water (17,000 x g, 1 min, rt). This procedure allowed concentrating the extracellular RNA from 2 mL plasma into a final volume of 14 µL of water (see Figure 1). The total RNA yield of plasma-derived EV was determined by Bioanalyzer RNA 6000 Pico assay (Agilent) according to the manufacturer's protocol (see Supplemental Figure S1) and resulted in high purity of RNA (see Supplemental Figure S2).

Platelet separation and RNA purification

Platelets were fractionated from whole blood strictly following a two-step centrifugation protocol as previously described in papers reporting on TEPs [12,13,15,16,18]. Briefly, a second EDTA plasma tube was centrifuged at 120 x g for 20 min (Beckman Coulter, Allegra X-15 R, rotor SX4750A, no brake) to sediment cells and generate platelet-rich plasma. Two

milliliters of platelet-rich plasma were transferred to a separate tube staying clear of the cell/plasma interface, taking care not to transfer any cells and centrifuged again, this time at 360 x g for 20 min (Beckman Coulter, Allegra X-15 R, rotor SX4750A, no brake) to generate a platelet pellet (Platelets) and a supernatant. After discarding the supernatant, the pellet was carefully washed twice with 1 mL PBS each to remove any remaining plasma and lysed by directly adding 700 µL QIAzol. Subsequently, platelet RNA was purified on a RNeasy MinElute spin column (Qiagen, #77164) following the manufacturer`s instructions as described for EV-derived RNA earlier (see also Figure 1). The total RNA yield of plasma-derived platelets was determined by Bioanalyzer RNA 6000 Pico assay (Agilent) according to the manufacturer's protocol (see Supplemental Figure S1). As Best et al. [12] "advise users of this platelet-isolation protocol to regularly assess the presence of leukocytes in fresh platelet preparations", we evaluated two platelet preparations for potential leukocyte contamination using both microscopy cell counting and qPCR for CD45 [19] (leukocyte marker; Thermo Fisher Hs04189704_m1) and PF4 (platelet marker; assay details in Podmore et al. [20]). None of our investigations, which are summarized in Supplemental Figure S3, indicated any contamination with leukocytes.

Tumour RNA detection by RT-qPCR

The RNA of each blood fraction (EVs and Platelets) was analysed by a highly sensitive and specific RT-qPCR for detection of the *BRAF* V600E mutation. This assay is based on an enhanced Amplification Refractory Mutation System (ARMS) assay containing a wild-type blocker to accurately detect single-digit copies of the V600E mutation down to a mutant allele fraction (% MAF) of *BRAF* V600E of 0.01%, limited only by the error rate of the reverse transcriptase enzyme (1:20,000) [21,22]. To achieve maximum assay sensitivity, the entire amount of isolated RNA per sample fraction was applied as input into a single reverse transcription followed by triplicate qPCR mutant quantification (SD ≤0.4 CT). Inter-sample and inter-assay consistency were monitored by in-sample controls. Development and validation of this *BRAF* V600E assay were performed in Exosome Diagnostics' ISO 15189 certified laboratory and has been published previously [23]. Further, in a cohort of 62 tumour-V600E-negative plasma samples, the clinical specificity was 100% using the 0.01% MAF cut-off (Supplemental Table 1).

Additional EV characterization

Plasma was collected and separated and EVs isolated by exoRNeasy (Qiagen) exactly as previously described [17] and followed by comprehensive qualitative and quantitative characterization of the EV fraction [17,24]. Briefly, in Enderle et al., EV morphology was characterized by scanning electron microscopy of intact vesicles separated by the exoRNeasy affinity membrane and compared to separation via ultracentrifugation; nanoparticle tracking analysis was performed on vesicles eluted from exoRNeasy affinity column and the corresponding flow-through; the yield and size distribution of total RNA from plasma isolated by membrane affinity was measured by Bioanalyzer RNA Pico assay in a cohort of 57 plasma samples to a median value of 7 ng/mL plasma. In the trace profile of the total RNA preparation, the majority of transcripts appeared around an approximate size of 125 bp, although many transcripts of larger sizes were clearly visible and qPCR analysis indicated that plasma mRNAs (e.g. GAPDH, KRAS) were easily detectable. Western blot analysis showed vesicle-enriched proteins such as TSG101 in amounts comparable to ultracentrifugation. Separately, the exoRNeasy platform has also been extensively characterized and compared among 10 different isolation platforms, including the gold standard ultracentrifugation across 5 different types of biofluids in a large multicenter cross-platform evaluation performed by The Extracellular RNA Communication Consortium and published in Cell earlier this year [25]. The platforms were compared for inter-lab reproducibility, yield, EV specificity and diversity of RNA targets evaluated by qPCR and RNAseq. The exoRNeasy kit ranked the highest integrated quality score (IQS) values across all platforms for both plasma and serum due to its high reproducibility and it was concluded that exoRNeasy has the best quality metrics for EV specific RNA profiling [25].

Results

Platelet- and EV-derived RNA samples were tested by Bioanalyzer and found to have RNA profiles similar to those previously published (see Supplemental Figure S1) [16,17]. Platelet RNA yield was average 38 ng RNA/mL blood, S.D. = 33 ng/mL (compared to 24 ng/mL, S.D. = 22 ng/mL, published by Best et al. [16]) and EV RNA yield was average 6 ng RNA/mL plasma, S.D. = 5 ng/mL (compared to 7 ng/mL, range: 1.6–18,2 ng/mL, published by Enderle et al. [17]). When comparing the two fractions isolated in parallel

from a single blood specimen, all 12 platelet samples predominantly contained wild-type *BRAF* signal and the absolute copy number of *BRAF* wild-type was up to 250-fold higher in platelets (median 679,440 copies/ 2 mL blood) than in EVs (median 2,670 copies/2 mL blood) (Figure 2(a) and Supplemental Table S2). However, none of the 12 platelet samples resulted in a tumour mutant signal of *BRAF* V600E above the detection limit of the RT-qPCR assay (Figure 2(b) and Supplemental Table S2). In contrast, analysis of the EV fraction from the same blood draw, which was prepared from plasma that was depleted of platelets by filtration and centrifugation, resulted in detectable tumour mutant *BRAF* V600E RNA in 10 of 12 patients displaying mutant allele fractions up to 6%.

Discussion

These observations suggest that although platelets of cancer patients contain substantially higher amounts of RNA than EVs, the ratio of tumour RNA to non-tumour RNA is drastically lower in platelets than in EVs and thus platelets may not be the optimal blood fraction for detecting RNA of tumour origin. The ratios of tumour RNA in platelets were permanently below the detection limit in all 12 samples tested, even when

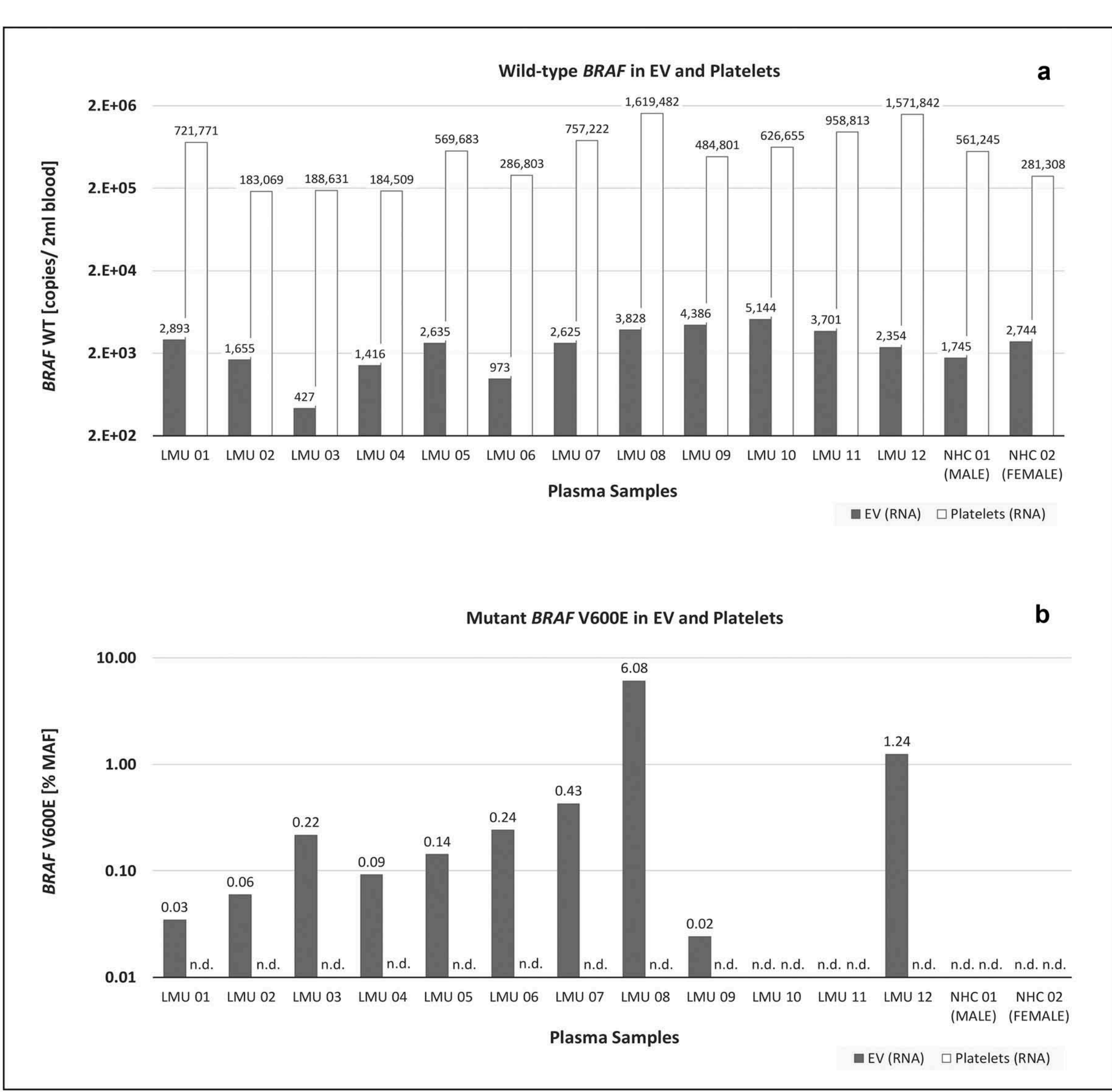

Figure 2. Total BRAF RNA molecules and fraction of V600E mutations in fractionated blood specimens. (a) Copy number of BRAF wild-type RNA per patient sample in platelet and EV fractions from 2 mL plasma. (b) Percent mutant allele fraction of BRAF V600E in the platelet and EVfractions. (Platelets: Platelet pellet; EV: Extracellular vesicle; %MAF: Mutant allele fraction; n.d.: Signal not detectable; LMU: Sample identifier; NHC: Normal healthy control).

applying a highly sensitive RT-qPCR assay only limited by the intrinsic error rate of the reverse transcriptase, and did not allow for the detection of *BRAF* V600E in blood. Directly comparing the parallel EV and platelet fractions, the EVs enabled detection of *BRAF* V600E in 10 of 12 BRAF V600E-positive cancer patients.

We found that platelets contain a very high amount of wild-type RNA (up to 1.6 million copies of *BRAF* per sample/680,000 copies *BRAF* per sample on average), which creates a high non-tumour background even if some tumour-derived exosomes or other tumour-derived RNA were to adhere to or be taken up by the platelets. Even with the highly sensitive and specific assay applied here, we could not detect any tumour-derived RNA above background in the platelet fraction, despite the assay being able to discriminate allelic frequencies down to 0.01%. Of note, most clinical mutation assays are only specific down to mutant allelic frequencies of >0.1% [26–28]. We reviewed the literature for the most appropriate protocol for platelet isolation and applied it across a range of samples in a routine clinical laboratory (ISO 15189). The platelet RNA yields were consistent with previous reports of platelet RNA isolation [12,13,15,16,18].

It is plausible that the RNA content of platelets can be altered by pathological conditions such as infections, inflammation, compromised immune status or activated immune system due to the presence of a tumour or by therapeutic factors such as steroids, chemotherapy or radiation [12,14]. However, our results suggest that no clinically or analytically relevant transfer of tumour-derived *BRAF* V600E RNA to platelets is occurring, and the high amount on non-tumour RNA in platelets renders accurate detection of tumour contributions impractical, if not impossible. In addition, platelets are very sensitive to activation (collection, transport, storage processing) which could affect the total RNA profile as evaluated by NGS/RNA-seq methods.

Previous reports have suggested that platelets can sequester RNA of tumour origin, e.g. through the uptake of exosomes and microvesicles. However, even in the sample that had very high levels (6%) of mutated BRAF RNA transcripts in the EV fraction, we saw no evidence of mutations in the corresponding platelet fraction. With a 0.01% detection limit of the qPCR assay, this indicates that the EV fraction contains at least 600 times more tumour-derived RNA than the platelets. Although our conclusion is based on a single RNA marker of tumour origin, we assume that this finding does not differ for other molecular alterations/RNA species as they are likely subject to the same cellular processes.

As a consequence, it is recommended to remove platelets from a blood specimen for optimal detection of tumour-derived RNA in extracellular vesicles.

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Disclosure of Interest Statement

Kay Brinkman, Lisa Meyer, Anne Bickel, Daniel Enderle, Johan Skog and Mikkel Noerholm are employees and shareholders of Exosome Diagnostics.

Carola Berking declares no conflict of interest and no competing financial interest.

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