

RESEARCH

Two miRNAs enriched in plasma extracellular vesicles are potential biomarkers for thyroid cancer

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Abstract

Differential diagnosis of thyroid cancer and benign nodules is still one of the most challenging issues in the field of endocrinology. To overcome overdiagnosis of papillary thyroid carcinomas (PTC) and the consecutive overtreatment of multinodular diseases, the search for easily accessible, sensitive and accurate biomarkers is critical. Several micro-RNAs (miRNAs) freely circulating in peripheral blood or enclosed in extracellular vesicles (EVs) have been proposed as potential biomarkers from non-invasive liquid biopsies. However, protocols are rarely comparable and conflicting data exist in the literature. In this work, we aimed to assess the diagnostic value of six micro-RNAs by comparing their expression in thyroid tissue to their abundance in bulk plasma and in plasma-EVs, before and after thyroid surgery. Plasma-EVs were isolated using a sequential density- and size-based fractionation, followed by in-depth characterization, confirming EV purity. Micro-RNA levels were measured by RT-qPCR in thyroid tissue, plasma and plasma-EVs. Among the six candidates, only miR-146b-5p and miR-21a-5p displayed a significant differential abundance in purified plasma-derived EVs from patients with PTC and benign disease. However, no difference could be demonstrated in bulk plasma through our cohort of patients. Overall, our work supports the use of a well-defined protocol of plasma-EV miRNAs purification for biomarker discovery, rather than the use of freely circulating miRNAs in bulk plasma. Our work also demonstrates that standardized pre-analytical and analytical procedures as well as optimized EV-miRNAs detection methods are essential.

Key Words

- ▶ liquid biopsy
- ▶ thyroid cancer
- ▶ biomarker
- ▶ circulating micro-RNAs
- ▶ extracellular vesicles

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Introduction

Thyroid nodules are the most frequently diagnosed pathologies of the endocrine system. Up to 65% of randomly selected individuals display nodular disease, especially women (Durante *et al.* 2018). The intensification

and the sensitivity of neck ultrasonography testing inevitably increase detection of nodules and the discovery of smaller ones (<1 cm³) (Li *et al.* 2020b). Furthermore, differential diagnosis of these nodules by cytopathology

on ultrasound-guided fine-needle aspiration (FNA) remains challenging (Russ *et al.* 2017, Durante *et al.* 2018). Surgical resection of the thyroid gland is still the preferred treatment for nodules with indeterminate cytology (Russ *et al.* 2017). Strikingly, anatomopathological analysis of resected tissues does not show malignant lesion in 60–80% of the cases, meaning unnecessary surgeries, psychological prejudices and social costs (Duranter *et al.* 2018). It is now widely accepted that the dramatic increase in thyroid cancer (TC) incidence observed in most countries since the 70s is mainly due to overdiagnosis of papillary thyroid cancer (PTC), accounting for 85% of TC (Fagin & Wells 2016, Vaccarella *et al.* 2016, Li *et al.* 2020b, Zaridze *et al.* 2021). To reduce overtreatment, the discovery and the development of more accurate diagnostic tools are a necessity (Oblak *et al.* 2021).

Recently, liquid biopsy has emerged as a promising opportunity to develop non-invasive and sensitive biomarkers for various malignancies, including PTC (Khatami & Tavangar 2018, Rappa *et al.* 2019, Toraih *et al.* 2021). Special attention was paid to small non-coding RNAs, micro-RNAs (miRNAs), and small lipid bilayer-enclosed extracellular vesicles (EVs), both produced by the tumor and released in peripheral blood. Several studies reported that miRNA profile in bulk plasma or in plasma-EVs can inform histological features and clinical outcome of thyroid tumors and could be integrated into the routine diagnostic approach for patient with thyroid nodules (Rappa *et al.* 2019, Zembska *et al.* 2019, Lu *et al.* 2020).

However, several factors impede the development and the clinical application of miRNAs and EV-miRNAs detection from blood biopsies. Logistical and technical difficulties due to the low quantity of circulating components, lack of reproducibility and the absence of consensus about the most suitable protocol for EV purification are partly responsible for discrepant results between studies highlighting the need for standardization of pre-analytical and analytical procedures (Zhao *et al.* 2019, Pogliaghi 2021). For example, a study reported that the majority of miRNAs detectable in serum are concentrated in EVs (Gallo *et al.* 2012), while another showed the opposite (Chevillet *et al.* 2014). Some reports concluded that bulk miRNA measurement in plasma is sufficient to measure differences between patient with benign or malignant diseases, while others demonstrated that EVs are a more stable, consistent and specific source of tumor-derived RNA (Cheng *et al.* 2014, Ge *et al.* 2014, Sanz-Rubio *et al.* 2018). In addition, there is still no agreement concerning the more accurate and predictive miRNA candidates to assess.

In this work, we evaluated the diagnostic value of the most cited 'biomarker' miRNAs in plasma-EVs as compared to bulk plasma. We selected six miRNAs (miR-21a-5p, miR-221-3p, miR-222-3p, miR-146b-5p, miR-31-5p and miR-204-5p) that were proposed to discriminate benign nodules from PTC in more than three independent studies on tissue, on plasma-/serum-EVs and on bulk plasma/serum. To get a global and comparable vision, we used biological materials from the same patients, with multinodular goiter (MNG) or PTC, and measured the level of those miRNA candidates in tissue, in highly purified plasma-EVs and in bulk plasma. In addition, circulating levels were assessed before and after thyroid surgery. While the deregulation of the miRNA candidates was confirmed in PTC tissues as compared to benign tissues, no difference in their abundance was observed in bulk plasma. On the contrary, abundance of miR-146b-5p and miR-21a-5p associated with plasma-EVs could discriminate between the two groups of patients. Our data demonstrate that miRNAs associated with plasma-EVs could serve as biomarkers of the cancerous tissue and that optimized isolation protocol and detection method are required.

Patients and methods

Patients and samples

The study followed the Declaration of Helsinki and was approved by the local ethics committee of the University of Louvain (2017/25OCT/495 and 2017/04OCT/466), Brussels, Belgium. Patients with thyroid nodules were diagnosed and treated at the Surgery and Abdominal Transplantation Division of Saint-Luc Hospital, Brussels, between February 2019 and June 2021. Patients scheduled to undergo thyroidectomy, and who gave written informed consent, were enrolled in the study and were anonymized. Post-operative histological diagnosis was performed by a pathologist. Patients with multinodular goiter ($n = 19$) were included in the benign group, whereas patient with differentiated PTC ($n = 16$) were included in the malignant group. Relevant clinical characteristics of patients are presented in Table 1 and detailed information is provided in Supplementary Tables 1 and 2 (see section on supplementary materials given at the end of this article). Additionally, the plasma of healthy volunteers ($n = 10$) was obtained.

Peripheral blood samples were collected two times, on the day of surgery right after anesthesia (pre-operative samples) and 12–14 days after surgery (post-operative

Table 1 Characteristics of patients included in the study.

	Benign	Malignant
	MNG group	PTC group
<i>n</i>	19	16
Gender	F = 15/19 M = 4/19	F = 16/16 M = 0/16
Age (years)	Mean $\pm$ s.d. = 56.6 $\pm$ 19.2 (18–78)	Mean $\pm$ s.d. = 48.9 $\pm$ 14.7 (23–66)
Diagnosis	MNG = 19/19	Classical PTC = 14/16 Follicular variant of PTC = 2/16
Mutational status		BRAF = 8/16 HRAS = 2/16
Extend of surgery	Lobectomy = 9/19 Total thyroidectomy = 10/19	Lobectomy = 2/16 Total thyroidectomy = 14/16

F, female; M, male; MNG, multinodular goiter; PTC, papillary thyroid cancer.

samples), in EDTA/K⁺-coated vacutainers (SARSTEDT, Germany). Within 60 min, plasma was separated by two successive centrifugations (1880 *g*, 10 min and 2500 *g*, 10 min) to obtain platelet-free plasma, then stored at -80°C . Thyroid tissues were dissected, immediately snap-frozen in liquid nitrogen and stored at -80°C .

Isolation of small extracellular vesicles (sEVs)

EVs were isolated from plasma by a combination of an iodixanol density cushion (IDC) and a size exclusion chromatography (SEC), as described (Karimi *et al.* 2018). Briefly, 3–6 mL of samples were layered on top of an 50–30–10% iodixanol gradient (Optiprep™) and centrifuged at 178,000 *g* for 2 h at $+4^{\circ}\text{C}$ (Ultracentrifuge Optima XPN 80, rotor SW41). The 10–30% junction was collected and passed through a home-made 10 mL Sepharose CL-2B column (GE Healthcare). Finally, 500 μL fractions were collected. For RNA extraction, SEC fractions F8–11 (EV-fractions) were pooled and concentrated on ultrafiltration devices (Amicon® Ultra-4, 10 kDa, cellulose regenerate; Merck) from 2 mL to 100 μL . All relevant data of our experiments were submitted to the EV-TRACK knowledgebase (EV-TRACK ID: EV210295) (EV-TRACK Consortium 2017).

Characterization of individual SEC-fractions

Particles in SEC-fractions were counted by nanoparticle tracking analysis (NTA) with the Zetaview (Particle Metrix, GmbH, Germany), as described (Degosserie *et al.* 2018). Measurement parameters were as follow: sensitivity of 85, camera shutter of 100, medium resolution, two cycles at 11 different positions. Protein concentration

of each fraction was measured using bicinchoninic acid (BCA) assay according to the manufacturer's instructions (Merck). Apolipoproteins ApoA1 and ApoB content were visualized by dot blot. 50 μL of fraction were loaded on methanol-activated PVDF-membrane under vacuum aspiration, air dried, washed and blocked with TBS-BSA 0.5%. Then, membranes were incubated overnight with primary antibodies. After incubation with HRP-conjugated secondary antibody, immuno-reactive dots were detected using Super Signal Chemiluminescent Substrate (Thermo Scientific). Images were acquired using Fusion Solo S (Vilber Lourmat) and quantified with ImageJ software (Schneider *et al.* 2012). CD9, CD81 and CD63 content were quantified by immuno-fluorescence analysis using Europium-ELISA as previously described (Vrancx *et al.* 2021). All antibodies used are reported in Supplementary Table 3.

Transmission electron microscopy

Pools of SEC fractions (F7–8, F9–10 and F11–12) were concentrated and desalted by ultrafiltration using Amicon Ultra-0.5 centrifugal filter unit (10 kDa, cellulose regenerate; Merck) according to the manufacturer's instructions. EV concentrate was observed by transmission electron microscopy after negative staining. A 50 μL drop of sample containing 1% of uranyl acetate stain was put on formvar and carbon-coated grid for 5 min and then excess of liquid was removed by filter paper, specimens were dried overnight in a closed box containing dried silica gel and observed in a transmission electron microscope (CM12, Philips) at 80 kV.

RNA extraction

Total RNA was extracted from thyroid tissues, plasma (100 μL) and concentrated EV-fractions using miRNeasy Mini kit (Qiagen) according to the manufacturer's instructions. For EVs, 700 μL of Qiazol Lysis reagent was added directly to the Amicon filter. Cel-miR-54-5p was added to lysis buffer as *spike-in* for plasma- and EVs-RNA isolation (for plasma-EVs: 15 μL of 2 pM/6 mL of plasma; for bulk plasma: 10 μL of 200 pM). Plasma-RNA and EV-RNA were eluted in 30 μL of RNase-free water. Tissue-RNA concentration was measured by nanodrop.

miRNA candidates and RT-qPCR analysis

Six candidate miRNAs that were proposed in the literature to discriminate benign nodules from PTC in more than three independent studies on tissue, on plasma-/

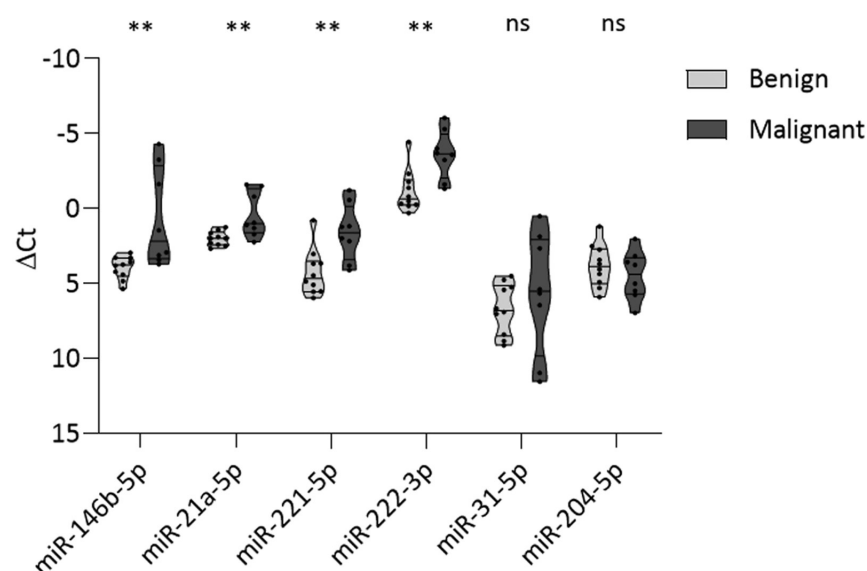
Table 2 Studies proposing a potential diagnostic for six candidate miRNAs in three sources of biological material.

	In tissue	In plasma/serum	In plasma/serum-EVs
miR-146b-5p	(Nikiforova <i>et al.</i> 2008, Rosignolo <i>et al.</i> 2017, Castagna <i>et al.</i> 2019, Li <i>et al.</i> 2019, Pamedylyte <i>et al.</i> 2019, Mahmoudian-Sani <i>et al.</i> 2020)	(Lee <i>et al.</i> 2013, Rosignolo <i>et al.</i> 2017)	(Lee <i>et al.</i> 2015, Jiang <i>et al.</i> 2020, Toraih <i>et al.</i> 2021)
miR-21a-5p	(Sondermann <i>et al.</i> 2015, Pamedylyte <i>et al.</i> 2019)		(Samsonov <i>et al.</i> 2016, Wu <i>et al.</i> 2019, Jiang <i>et al.</i> 2020)
miR-221-3p	(Nikiforova <i>et al.</i> 2008, Rosignolo <i>et al.</i> 2017, Castagna <i>et al.</i> 2019, Pamedylyte <i>et al.</i> 2019, Mahmoudian-Sani <i>et al.</i> 2020)	(Rosignolo <i>et al.</i> 2017)	(Rappa <i>et al.</i> 2019, Jiang <i>et al.</i> 2020)
miR-222-3p	(Nikiforova <i>et al.</i> 2008, Rosignolo <i>et al.</i> 2017, Castagna <i>et al.</i> 2019, Pamedylyte <i>et al.</i> 2019, Zarkesh <i>et al.</i> 2019, Mahmoudian-Sani <i>et al.</i> 2020)	(Yu <i>et al.</i> 2012, Lee <i>et al.</i> 2013, Yoruker <i>et al.</i> 2016, Rosignolo <i>et al.</i> 2017, Rezaei <i>et al.</i> 2019, Zhang <i>et al.</i> 2019)	(Lee <i>et al.</i> 2015, Jiang <i>et al.</i> 2020, Pan <i>et al.</i> 2020)
miR-31-5p	(Ab Mutalib <i>et al.</i> 2016)	(Yoruker <i>et al.</i> 2016)	(Samsonov <i>et al.</i> 2016)
miR-204-3p	(Ab Mutalib <i>et al.</i> 2016, Rosignolo <i>et al.</i> 2017, Du <i>et al.</i> 2021)		(Jiang <i>et al.</i> 2020, Pan <i>et al.</i> 2020)

serum-EVs and on bulk plasma/serum were selected (Table 2). Table 2 also presents the source of biological material used in these studies. Pulsed RT was performed on 6 µL of plasma or EV-RNA or on 250 ng of tissue-RNA using M-MLV Reverse Transcriptase (Invitrogen) and a mix of specific miRNA stem-loop primers (Mestdagh *et al.* 2008). 1 µL of cel-miR-34-5p at 2 pM was added to the RT mix as second *spike-in*. Real-time quantitative PCR was performed as described (Chen *et al.* 2005), in the presence of 300 nM of universal reverse primer, 300 nM of specific forward primer, 250 nM of probe with Takyon Probe Master Mix (Eurogentec, Belgium) on a CFX96 touch real-time PCR Detection System (Bio-Rad). Primers and probes sequences are listed in Supplementary Table 4. Data were analyzed using the ΔCT method, with let-7b-5p or the geometric mean of the two *spikes-in* and the endogenous miR-191-5p as reference miRNAs for tissues samples or EV/plasma samples, respectively.

Statistical analysis

All statistical analyses were performed with Prism software (GraphPad Software). Real-time qPCR values were presented as ΔCt . Graphs were designed with an inverted y axis and each point represents one sample (individual values, mean $\pm$ s.d.). Normality was verified using D'Agostino–Pearson normality tests, and unpaired t-tests were used to compare benign to malignant groups. Paired t-tests were used to compare pre-operative to post-operative samples. When equal s.d. could not be assumed, Welch's correction was applied. For data series with less than ten samples (qPCR analyses on tissues samples), non-parametric Mann–Whitney tests were used. Correlation analyses between miRNAs expression in tissue and its abundance in plasma were performed by calculation of Pearson's coefficient. Differences and correlations were considered statistically significant when $P < 0.05$ (*); $P < 0.01$ (**).

**Figure 1**

miRNA candidates are differentially expressed in PTC tissues. Analysis of miRNAs expression in resected thyroid tissue from patient with benign thyroid diseases (MNG = gray) or malignant thyroid diseases (PTC = black). For each miRNA, data are shown as violin plot with ΔCt (i.e. Ct normalized on let-7b-5p as housekeeping miRNA) presented on an inverted y axis for a more intuitive lecture. Unpaired t-tests with Welch's correction were applied: * $P < 0.05$; ** $P < 0.01$.

Results

miRNA candidates are differentially expressed in PTC tissues

From the literature, we selected six miRNAs (miR-21a-5p, miR-221-3p, miR-222-3p, miR-146b-5p, miR-31-5p, miR-204-5p) that were proposed to discriminate benign nodules from PTC (see Table 2 for references and the biological material studied). To confirm the differential expression of the selected candidates in our cohort of patients, we measured miRNA expression in resected tissues samples of seven PTC and ten MNG. As expected, expression levels of miR-146b-5p, miR-21a-5p, miR-221-3p and miR-222-3p were significantly higher in PTC tissues compared to MNG tissues (Fig. 1). Even if not significant in this small cohort, a trend was observed for miR-31-5p (higher in PTC tissues) and for miR-204-5p (lower in PTC tissues). Those results indicate that our cohort of patients exhibits deregulation of miRNA expression in PTC tissues similar to published studies.

miRNA levels in bulk plasma do not allow for discrimination of thyroid diseases or ablation

We then measured miRNAs abundance in bulk plasma from 15 patients with PTC and 15 patients with MNG (Fig. 2A). miR-146b-5p, miR-21a-5p, miR-221-3p and miR-222-3p were easily detected, while miR-31-5p and miR-204-5p were below the detection limit in bulk plasma of most patients. Abundance of the four detected miRNAs was not different in bulk plasma from benign group as compared to malignant group (Fig. 2A), despite demonstrated deregulation in the tissues (Fig. 1). Moreover, comparison of their abundance in pre-operative plasma samples with post-operative ones did not reveal a particular trend for these four miRNAs candidates (Fig. 2B). We concluded that malignant thyroid disease and thyroid ablation do not significantly impact on miR-146b-5p, miR-21a-5p, miR-221-3p and miR-222-3p abundance in bulk plasma of patients, as compared to benign diseases.

Highly purified EVs are isolated from plasma by density cushion combined with size exclusion chromatography

Plasma is a complex biofluid, containing lipoproteins and protein aggregates that can be co-isolated with EVs and carry over bound, that is non-encapsulated, contaminant miRNAs. To analyze the abundance of the selected candidates in plasma-derived extracellular vesicles (plasma-EVs) with limited confounding factors, we chose a protocol

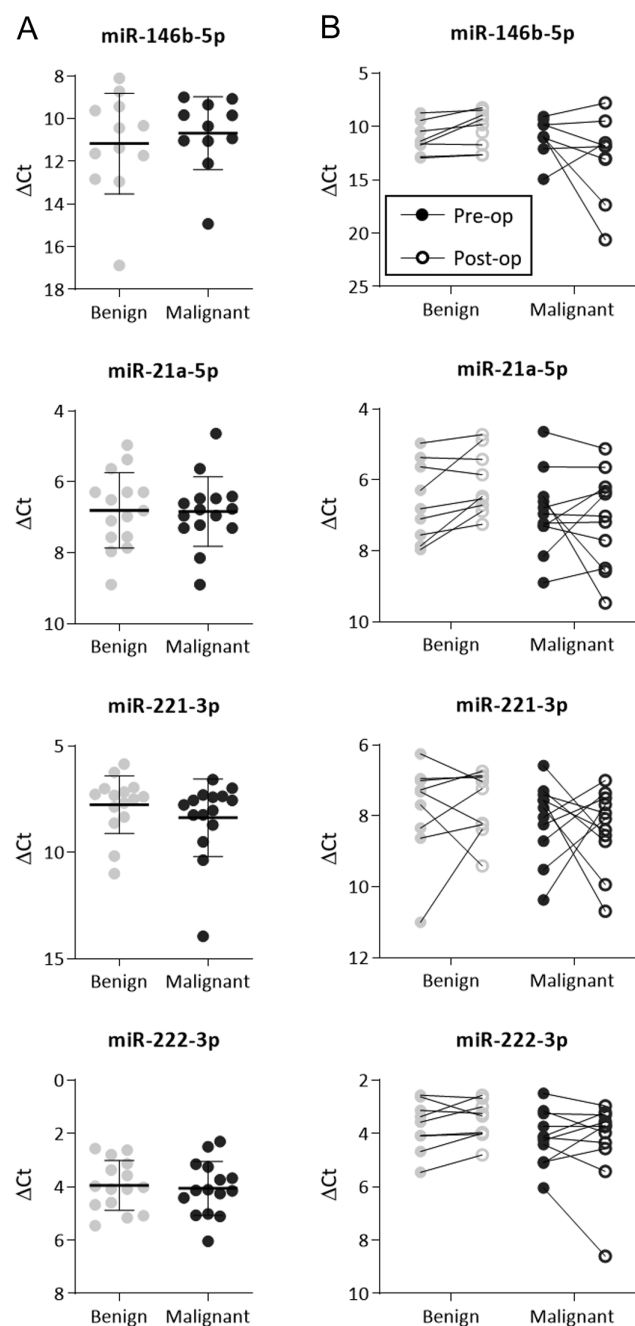
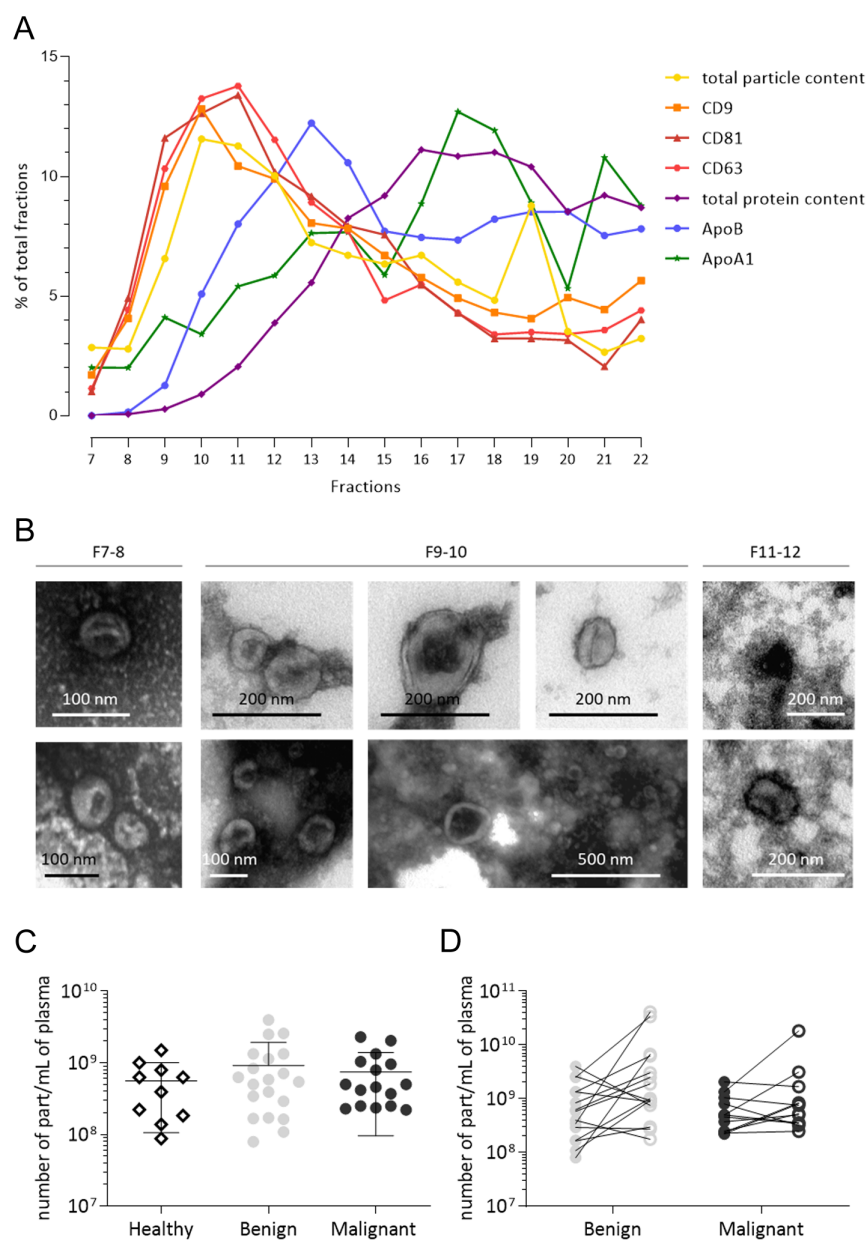


Figure 2

miRNA levels in bulk plasma do not allow for discrimination of thyroid diseases or thyroid ablation. (A) Comparison of miRNAs abundance in 100 μ L of bulk plasma from patient with benign thyroid diseases (MNG = gray; $n = 15$) and malignant thyroid diseases (PTC = black; $n = 15$). (B) Paired comparison of miRNAs abundance in 100 μ L of bulk plasma from patient with benign thyroid diseases ($n = 9$) or malignant thyroid diseases ($n = 11$) before (pre-operative samples = solid circles) and after surgery (post-operative samples = open circles). Data are shown as dot plot with Δ Ct (i.e. Ct normalized on the geometric mean of Cts obtained for hsa-miR-191-5p, Cel-miR-54-5p and Cel-miR-39-5p) presented on an inverted y axis.

**Figure 3**

Highly purified EVs are isolated from plasma by density cushion combined with size exclusion chromatography. (A) Illustration of particle content (NTA, yellow), biochemical abundance of EV markers CD9 (orange), CD81 (dark red) and CD63 (red), of ApoB (blue) and ApoA1 (green) and protein content (BCA assay, purple) of SEC fractions 7–22. CD9, CD81 and CD63 abundance was measured by ELISA-Europium, ApoB and ApoA1 by dot blot ($n = 3$). (B) Transmission electron microscopy of concentrated and desalted pools of SEC fractions 7–8, 9–10 and 11–12. (C) Nanoparticle tracking analysis (NTA) of pooled fractions 8–11 isolated from plasma (6 mL) of healthy volunteers ($n = 10$) and of patients with benign ($n = 20$) and malignant ($n = 16$) thyroid diseases. (D) Paired comparison of the number of particles per mL of plasma from patient with benign ($n = 15$) or malignant ($n = 11$) thyroid diseases before (pre-operative samples = solid circles) and after surgery (post-operative samples = open circles).

that isolate highly purified EVs by combining an iodixanol density cushion (IDC) with size exclusion chromatography (SEC) (Karimi *et al.* 2018). NTA counting revealed that particles were mainly detected in SEC fractions 9 to 13, reported by several groups as EV-fractions, and in fraction 19 (Fig. 3A, yellow). Five *bona fide* EV markers, CD9, CD81, CD63, TSG101 and HSP70 were quantified in fractions 7 to 22 by Europium-ELISA or dot blot and showed clear enrichment in fractions F9 to F12, exactly matching the first peak of NTA-detected particles (Fig. 3A and Supplementary Fig. 1). To evaluate the presence of plasmatic contaminants, we then measured protein content by BCA assay, along with ApoB and ApoA1 contents, respectively markers of low-

density lipoproteins (LDL, VLDL, chylomicrons) and high-density lipoproteins (HDL), by dot blot assay. Starting from extremely low levels in the first fractions, protein content increased in F12 and peaked in F16–F19. ApoB peaked in F11–15, while ApoA1 peaked in F16–19, in accordance with their expected sizes (Fig. 3A). Endoplasmic reticulum markers calnexin and protein disulfide-isomerase were also measured to assess intracellular contamination and were completely absent from the first fractions until F12 (Supplementary Fig. 1). Electron microscopy images of fractions F7–8, F9–10 and F11–12 pools confirmed these results (Fig. 3B). Small extracellular vesicles recognizable by their size, their delineating membrane and their biconcave

shape were present in fractions F7–8 (scarce) and F9–10 (more abundant). In fractions F11–12, EV structures were rarer, and more lipoproteins and protein aggregates were clearly visible. Altogether, this in-depth characterization revealed that the IDC/SEC protocol allowed to separate biologically different particles. For subsequent EV analyses, we only considered fractions F8–11 as EV-enriched fractions (high in particles and EV markers, low in proteins, ApoA1 and ApoB).

Abundance of purified EV from plasma is not different between MNG and PTC patient

The concept that tumor cells produce more EVs than their normal counterparts and, consequently that EVs are more abundant in plasma of patients with cancer, is largely spread in the literature (Konig *et al.* 2017, Osti *et al.* 2019). To test this idea in patients with TC, we quantified the number of particles present in F8–11 obtained from plasma of healthy volunteers and of patients with benign nodular disease or PTC. We observed no differences in the number of isolated particles per milliliter of plasma from the three groups (Fig. 3C). Furthermore, thyroid ablation in patients with MNG or PTC did not cause a drop in particle counts (Fig. 3D). Altogether, we conclude that thyroid diseases, and more particularly TC, do not impact on the abundance of EVs purified from circulating peripheral blood.

miR-146b-5p and miR-21a-5p levels in purified plasma-EVs are higher in malignant thyroid diseases

Although EV abundance could not segregate patients with benign or malignant thyroid disease, we tested whether miRNA content within purified EVs from MNG and PTC patient was different. We analyzed miRNA levels on RNA extracted from concentrated EV-fractions (F8–11). As in bulk plasma samples, miR-31-5p and miR-204-5p levels were below the detection limit in most of the patients and are not presented. Interestingly, miR-146b-5p and miR-21a-5p were readily detected and significantly different between the two groups, with a higher level in PTC as compared to the MNG group (Fig. 4A). Although not significant, a similar trend was observed for miR-221-3p and miR-222-3p. Surprisingly, comparison of miRNAs abundance in purified EVs from pre-operative vs post-operative plasma samples did not reveal any difference, even for miR-146b-5p and miR-21a-5p (Fig. 4B). In both the MNG group as well as the PTC group, the abundance of these miRNA candidates in purified EVs remained stable, increased or decreased after surgical resection, depending on the patient.

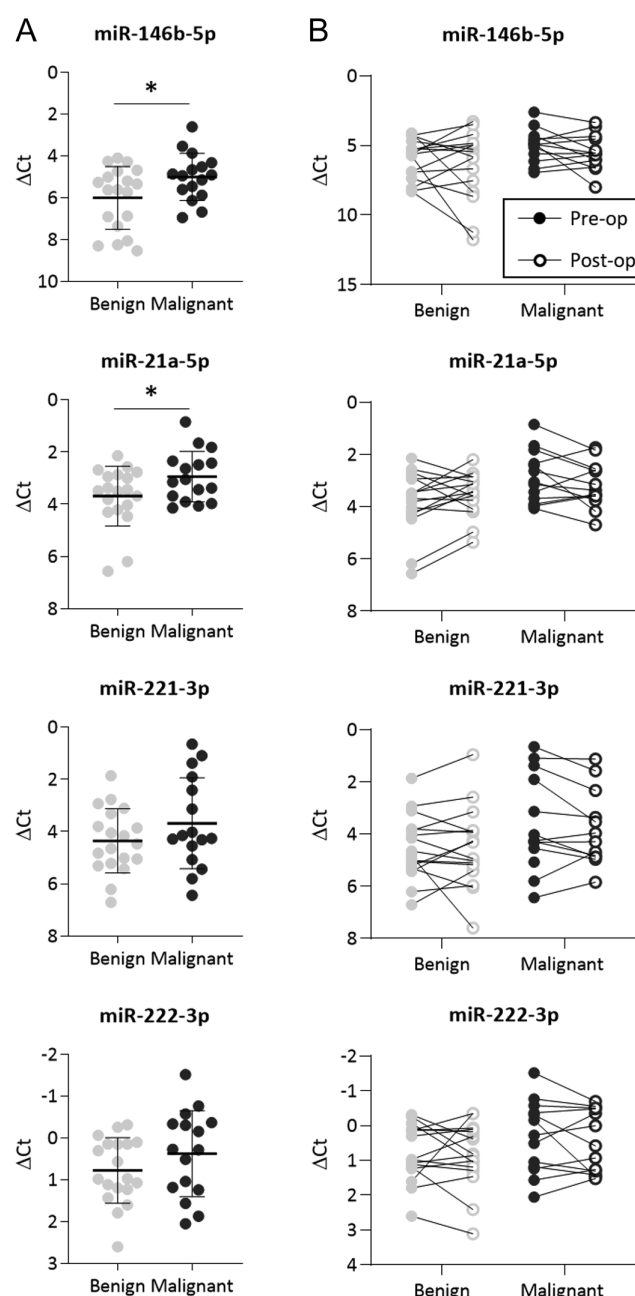


Figure 4

miR-146b-5p and miR-21a-5p levels in purified plasma-EVs are higher in malignant thyroid diseases. (A) Comparison of miRNAs abundance in EVs (SEC fractions 8–11) purified from 3–6 mL of plasma from patient with benign thyroid diseases (MNG = gray; $n = 19$) and malignant thyroid diseases (PTC = black; $n = 16$). (B) Paired comparison of miRNAs abundance in EVs (SEC fractions 8–11) purified from 3–6 mL of plasma from patient with benign thyroid diseases ($n = 16$) or malignant thyroid diseases ($n = 13$) before (pre-operative samples = solid circles) and after surgery (post-operative samples = open circles). Data are shown as dot plot with Δ Ct (i.e. Ct normalized on the geometric mean of Ct obtained for hsa-miR-191-5p, Cel-miR-54-5p and Cel-miR-39-5p) and presented on an inverted y axis. Unpaired *t*-tests with Welch's correction were applied: * $P < 0.05$.

Finally, we evaluated the relationship between the expression of candidate miRNAs in tissue and their respective abundance in plasma using Pearson's coefficient. Pearson's coefficient (r) is a measure of linear correlation between two data sets, essentially a normalized measurement of covariance, thus giving a value comprised between -1 (strong inverse correlation) and 1 (strong direct correlation). We compared tissue miRNA expression with miRNA abundance in bulk plasma or in purified EVs for each patient. Pearson's coefficients were comprised between -0.15 and 0.46 with an associated P -value >0.05 , showing

no significant linear relationship (Fig. 5). However, with our limited number of samples, the best positive associations were observed for miR-146b-5p (plasma-EVs and bulk plasma) and for miR-21a-5p (plasma-EVs), above 0.3 .

Discussion

Increase in TC incidence is almost exclusively due to the overdiagnosis of (small) PTC. Since clinical guidelines preconize active surveillance of small thyroid nodules

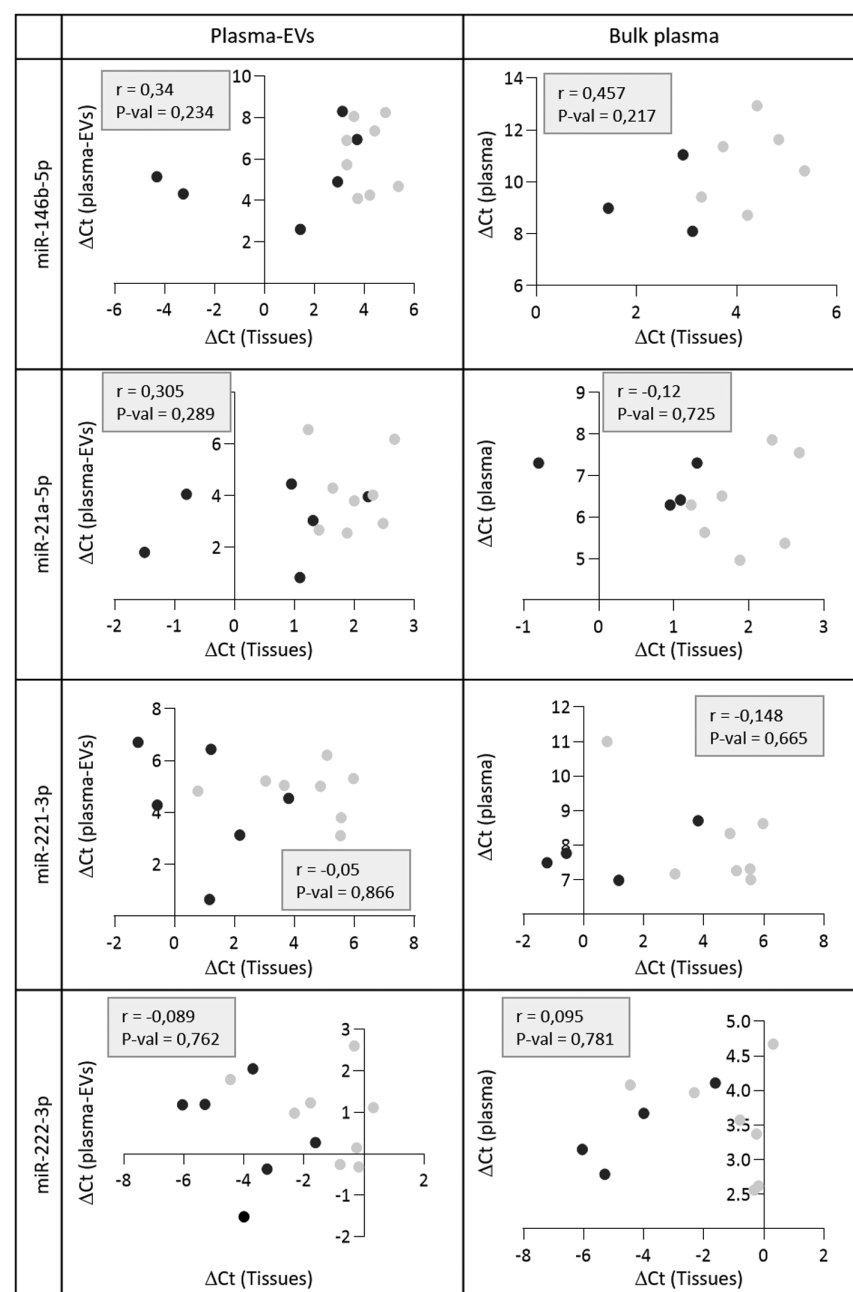


Figure 5

Absence of correlation between miRNA levels in circulation and in thyroid tissue. miRNA levels (expressed as ΔCt) were measured in thyroid tissue, in bulk plasma and in plasma-EVs of the same patient to assess correlation using Pearson's coefficient (r). Black circles represent patients with malignant disease; gray circles represent patients with benign disease.

and microcarcinomas, and limitation of invasive interventions to high-risk patients, efforts must be made in the identification of biomarkers from liquid biopsies (Vaccarella *et al.* 2016, Russ *et al.* 2017, Li *et al.* 2020b). Several studies already proposed miRNA or EV-miRNA candidates that could segregate patients with PTC or benign diseases (Khatami & Tavangar 2018, Rezaei *et al.* 2019, Jiang *et al.* 2020, Pan *et al.* 2020). However, variability in starting material, number of patients, pathologies studied and plasma- or serum-EV isolation protocols generated hardly comparable results (Pogliaghi 2021). In the present study, we aimed to assess the diagnostic potential of circulating miRNAs by measuring miRNA expression in tissue and abundance in bulk plasma and in purified extracellular vesicles (EVs). Data obtained from the same patient allowed us to compare miRNA expression in thyroid tissue and in peripheral blood circulation.

The tested miRNA candidates were selected by literature screening based on differential expression in diseased tissue and on potential diagnostic utility in bulk plasma, serum and/or in circulating EVs (see Table 2 for details on the studies). Among the six selected candidates, miR-21a-5p, miR-221-3p, miR-222-3p, miR-146b-5p (and miR-31-5p) expression was higher in PTC as compared to MNG tissues. miR-204-5p expression, albeit lower, was not significantly different probably due to the high variability in the PTC group. Overall, our results confirmed previous published data on miRNA levels in tissue, FNA or FFPE samples (Castagna *et al.* 2019, Li *et al.* 2019, Mahmoudian-Sani *et al.* 2020), in which they were shown to correlate with different features of tumor malignancy (size, stage, mutational status, local invasion) (Li *et al.* 2019, Pamedytyte *et al.* 2019). It was thus interesting to evaluate if the differential miRNA tissue expression could be consistently measured in peripheral blood.

It is believed that the encapsulation of miRNAs inside extracellular vesicles increases their stability in circulation making them a more reliable and more specific source of biomarkers (Gallo *et al.* 2012, Cheng *et al.* 2014, Chevillet *et al.* 2014, Sanz-Rubio *et al.* 2018, Wang *et al.* 2018). To eliminate part of background noise caused by other circulating miRNAs, we selected a sequential density- and size-based fractionation protocol that has been developed and proven to efficiently isolate high-purity EVs with reliable reproducibility (Karimi *et al.* 2018, Vergauwen *et al.* 2021). Even if this multi-step approach is time-consuming and generates lower yields, we and others think that cleaner samples are necessary for the discovery stages to avoid misinterpretation of the results due to contaminants (Théry *et al.* 2018). Characterization confirmed the purity of the EV

samples analyzed in this study and validated the use of this protocol to analyze the abundance of miRNA by RT-qPCR.

Among the six candidates, miR-31-5p and miR-204-5p were detected in less than 50% of not only plasma-EVs but also bulk plasma samples. Hence, they were not further considered in our analysis and discussion. In pre-operative bulk plasma samples, the four remaining miRNA candidates did not reveal differential abundance in patient with PTC as compared to benign disease. The latest study about circulating miRNA signature in thyroid cancer was also unable to demonstrate any consistent differences in serum profiles of known miRNAs, supporting the powerlessness of miRNA quantification in bulk plasma/serum (Jankovic *et al.* 2022). Remarkably, in our study, two miRNAs, miR-146b-5p and miR-21a-5p, exhibited a significant difference ($P=0.035$ and $P=0.046$, respectively) in purified EVs samples, with changes that were consistent with the differential expression measured in tissues (PTC plasma-EVs > MNG plasma-EVs). miR-146b-5p in EVs has already been proposed to be predictive of lymph node metastasis, while miR-21-5p has been recommended to discriminate between PTC and FTC patients (Samsonov *et al.* 2016, Jiang *et al.* 2020). We thus confirmed the utility of EV-associated miR-146b-5p and miR-21a-5p as potential biomarkers to discriminate patients with PTC from those with multinodular diseases.

Altogether, our report fully supports the use of purified plasma-EV miRNAs for biomarker discovery, rather than bulk plasma miRNAs, and is in accordance with other studies comparing the biomarker potential of freely circulating miRNAs as compared to EV-associated miRNAs in preeclampsia (Biro *et al.* 2019), in breast cancer (Min *et al.* 2019) and in lung cancer (Tian *et al.* 2017). In addition, we found no relationship between the abundance of most free and encapsulated miRNA in plasma of patient with thyroid diseases, as recently reported (Capriglione *et al.* 2021). The parameters governing the abundance of freely circulating and EV-encapsulated miRNAs levels in peripheral blood circulation are still largely unknown but probably influenced by the producing organ, differential miRNAs stability and/or specific sorting mechanisms in EVs. Very recently, cell-specific sorting motifs have been identified in miRNA sequences and demonstrated to control their secretion in EVs, defining the EV-miRNA profile of that cell type (Garcia-Martin *et al.* 2022). Those motifs could help to link EV-miRNAs to tissues of origin.

EV abundance in plasma was not reduced after thyroid ablation, regardless of the type of surgery (lobectomy or total thyroidectomy). Furthermore, plasma from PTC patients did not contain more EVs than plasma from

patients with benign thyroid disease. It is conceivable that thyroid-derived vesicles represent a negligible fraction of the total population of plasma-EVs and that small cancers such as differentiated thyroid cancer, especially microcarcinomas, do not produce sufficient EV quantities to impact on total EV circulating in the blood. Even though we demonstrated that EV-associated miR-146b-5p and miR-21a-5p levels are indicators of PTC, we were surprised to observe that their abundance did not change in plasma-EVs isolated 12–14 days after thyroid surgery, regardless of the extent of surgery. One possibility is that EVs are stable and long-lived particles in blood circulation. Even if the half-life of endogenous EVs in circulation is still unknown (Verweij *et al.* 2019), some studies on the administration of exogenous EV evaluated their half-life from 7 min to up to a few hours (Wiklander *et al.* 2015, Banks *et al.* 2020, Dang *et al.* 2020). A recent study in *Macaca* calculated a half-life of 40 min but a possible detection in plasma up to 24 h (Driedonks *et al.* 2021). We thus believed that tumor-derived EVs should have been eliminated 12–14 days after surgery. A more realistic hypothesis is that the source of EV, and thus miRNAs, in circulation is heterogeneous. In a mouse thyroid cancer model, the analysis of EVs produced *in vivo* indicated that tumor cell-derived EVs were not the main EV population within thyroid tumor (Delcorte *et al.* 2022). Moreover, miR-146b-5p and miR-21a-5p are not thyroid-specific (Keller *et al.* 2021) and other cell types have been shown to produce EVs containing those miRNAs (Chen *et al.* 2016, Liu *et al.* 2019). This could explain the weak correlation between miRNA expression in thyroid tissue and miRNA abundance in plasma. Although the application of EV-associated miR-146b-5p and miR-21a-5p quantification in clinics is promising, it would definitely need further investigation and validation in a larger cohort, and longer time points after surgery, to obtain sufficient predictive value and great specificity.

Our work also underlines the importance of standardization of the pre-analytical and analytical procedures and of improvement of sensitive and reproducible EV-miRNAs detection. To overcome the challenge of the low abundance of circulating components in the search for EV-based biomarker, the development of an approach to specifically enrich EVs released by thyroid cells could be an important breakthrough. Recently, Zabegina *et al.* isolated sufficient amount of thyroid-derived EVs from plasma using thyroid peroxidase as a surface marker, in order to perform miRNA analysis (Zabegina *et al.* 2020). Along the same line, proteomic analysis of serum EVs from PTC patient could also help identifying interesting new surface markers (Luo *et al.*

2018). Meanwhile, current progress in isolation platforms such as chips and microfluidics could potentiate the use of these new markers (Liang *et al.* 2017, Li *et al.* 2020a, Mizenko *et al.* 2021). Finally, high-throughput screenings, using NGS, provide a more integrated view of miRNAs profile in EVs (Dai *et al.* 2020, Pan *et al.* 2020). From those data, a panel of informative miRNAs could be set up to discriminate not only the malignancy of the nodules but also the histological subtype, the stage, the risk of recurrence or of lymph node invasion.

Conclusion

In this study, we identified two miRNAs, miR-146b-5p and miR-21a-5p, that were significantly increased in circulating extracellular vesicles of patients with PTC compared to patient with benign multinodular diseases. Importantly, these miRNA candidates were not differentially abundant when analyzing the entire plasmatic circulating miRNA pool, i.e., without EV purification. Our work demonstrates that EVs isolation before miRNA analysis is thus a critical step to enrich tumor-derived miRNAs and to highlight differences between patient groups. These data support the hypothesis that circulating EVs are a more consistent source of tumor-derived miRNAs and an interesting alternative for biomarker discovery in TC, provided that pre-analytical and analytical procedures are standardized.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-21-0343>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

O D conceived and performed all the experiments, prepared the figures and wrote the manuscript. J C collected all tissue samples. S M and C S helped for plasma sample collection and handling. P V D S managed E M experiments. E M and C D wrote the documents for the ethical committee and revised the manuscript. M M was in charge of patient recruitment,

surgery and collection of blood samples. C E P and O D conceived and designed the project. C E P supervised the project and wrote the documents for the ethical committee and the manuscript. All authors have approved the final version of the manuscript.

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