

## ANESTHESIOLOGY

# Propofol and Sevoflurane Differentially Impact MicroRNAs in Circulating Extracellular Vesicles during Colorectal Cancer Resection

## A Pilot Study

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### EDITOR'S PERSPECTIVE

#### What We Already Know about This Topic

- While total intravenous anesthesia in combination with local-regional anesthesia during cancer resection may result in improved outcomes, potent volatile anesthetics may enhance tumor cell growth and metastasis
- Sera taken from patients receiving propofol, but not from those receiving sevoflurane, induced a reduction in invasiveness, proliferation, and metastatic potential of cancer cells in addition to enhancing their apoptosis
- Extracellular vesicles are nanosized, membrane-encapsulated information carriers secreted by all living cells that play crucial roles in intercellular communication

#### What This Article Tells Us That Is New

- This proof-of-concept study in colorectal cancer patients receiving either propofol (n = 8) or sevoflurane (n = 9) found 64 extracellular vesicle-associated microRNAs to be significantly regulated by total intravenous anesthesia and 33 to be significantly regulated by sevoflurane anesthesia
- All microRNAs downregulated in response to anesthesia were anesthetic agent specific, while most upregulated microRNAs were not
- Total intravenous anesthesia-regulated microRNAs might mediate inhibitory effects on signaling pathways involving cell proliferation, migration, and epithelial-mesenchymal transition of tumor cell line and enhance effects on apoptosis of carcinoma cell lines

### ABSTRACT

**Background:** Extracellular vesicles and their microRNA cargo are crucial facilitators of malignant cell communication and could mediate effects of anesthetics on tumor biology during cancer resection. The authors performed a proof-of-concept study to demonstrate that propofol and sevoflurane have differential effects on vesicle-associated microRNAs that influence signaling pathways involved in tumor progression and metastasis.

**Methods:** Circulating vesicles were investigated in a prospective, matched-case pilot study in two cohorts of colorectal cancer patients receiving either propofol (n = 8) or sevoflurane (n = 9), matched for tumor stage and location. Serum was sampled before anesthesia and after tumor resection. Vesicular microRNA profiles were analyzed by next generation sequencing and confirmed by real-time polymerase chain reaction. Next, we assessed perioperative changes in microRNA expression induced by either anesthetic and compared their biologic effects on tumor-relevant pathways. Additionally, vesicles from pre- and postoperative sera were biologic characterized.

**Results:** Postoperative microRNA profiles were shifted in both groups with overlap in the perioperative response. A total of 64 (48 up, range of log<sub>2</sub> fold change 1.07 to 3.76; 16 down, −1.00 to −1.55) and 33 (32 up, 1.02 to 2.98; 1 down, −1.36) microRNAs were significantly regulated (adjusted P value less than 0.05) by propofol and sevoflurane, respectively. Thirty-six (propofol) and five (sevoflurane) microRNAs were specifically responsive to either anesthetic agent. *In silico* target analyses of microRNA expression patterns indicated an inhibitory effect of propofol on crucial carcinoma-related pathways such as proliferation (z-score, −1.73) and migration (z-score, −1.97), as well as enhanced apoptosis (z-score, 1.19). While size distribution and protein markers of circulating vesicles were not affected by anesthesia, their concentration was reduced after surgery using both anesthetic procedures.

**Conclusions:** This proof-of-concept study provides preliminary evidence that anesthetic agents have specific effects on microRNA profiles in circulating vesicles. These findings could form the basis for larger and mechanistically oriented outcome studies in cancer patients.

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Colorectal cancer is among the three most common and most deadly cancers worldwide.<sup>1</sup> Despite a continuing decline in the incidence of colorectal cancer, recent reports estimate that it will lead to 1.1 million cancer deaths by 2030.<sup>2</sup> Surgical resection remains the best chance of survival for many colorectal cancer patients.<sup>3</sup>

Paradoxically, there is evidence that removal of the primary tumor is associated with tumor progression and metastasis. Surgical manipulation and removal of the tumor or nearby blood vessels seems to enhance the dissemination of tumor cells, implantation of circulating tumor cells and growth of metastases.<sup>4,5</sup>

Large retrospective studies have suggested a beneficial effect of propofol on cancer outcomes of patients after colorectal cancer surgery when compared to volatile anesthetics.<sup>6–8</sup>

Recently, extracellular vesicles have come into focus as functional mediators of carcinogenesis and metastasis.<sup>9</sup> Extracellular vesicles are nanosized, membrane-encapsulated information carriers secreted by all living cells.<sup>10</sup> Consequently, they have been detected in various body fluids including serum, plasma, urine, and cerebrospinal fluid, where they transport macromolecules such as proteins, cytokines, messenger RNA (mRNA), and microRNA. Importantly, as RNAs transported in vesicles are biologically functional in recipient cells, extracellular vesicles play crucial roles in intercellular communication between both distant and nearby tissues.<sup>11</sup>

In physiologic situations, these vesicles are involved in tissue homeostasis, organ repair and immune surveillance. They are a key part of the immune response and are utilized by the innate and adaptive immune system to execute functions ranging from antigen presentation to T-cell activation. As the quantity and composition of secreted vesicles change based on the state of secreting cells, so do their functions. Extracellular vesicles are consequently involved in many diseases such as diabetes and cardiovascular and infectious diseases, as well as cancer.<sup>12</sup> Adipocyte-derived extracellular vesicles from obese subjects were shown to further drive chronic inflammation and insulin resistance, whereas vesicles secreted by neurons are involved in the propagation of Alzheimer disease and Parkinson disease by spreading amyloid- $\beta$  and  $\alpha$ -synuclein, respectively. Importantly, extracellular vesicle secretion is increased in many cancers, and vesicles shed from primary tumors seem to selectively home to specific organs. Various studies demonstrated an important role of secreted vesicles in cancer progression and metastasis, presumably by modulating the host immune system and educating distant tissues to favor subsequent metastasis.<sup>13</sup> These effects could be mediated by the vesicular noncoding RNA content.<sup>14</sup>

Despite significant interest in deciphering the differential effects of various anesthetic agents on tumor proliferation and distal spread, the potential role of circulating vesicles in this process has not yet been elucidated. Even though a previous *in vitro* study indicated that propofol prompts the release of vesicles with anticancer properties, there are no *in vivo* data on how different anesthetic drugs might impact the secretion and molecular composition of vesicles.<sup>15</sup> We performed a proof-of-concept study to demonstrate that propofol has differential effects on vesicular microRNA profiles in comparison to sevoflurane in colorectal cancer surgery patients and to provide preliminary insight into their potential effects on signaling pathways involved in colorectal cancer proliferation and metastasis.

This article is featured in "This Month in Anesthesiology," page 1A. Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site ([www.anesthesiology.org](http://www.anesthesiology.org)). This article has a visual abstract available in the online version. D.B. and F.B. contributed equally to this article.

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## Materials and Methods

### Ethics Approval, Patient Recruitment and Consent to Participate

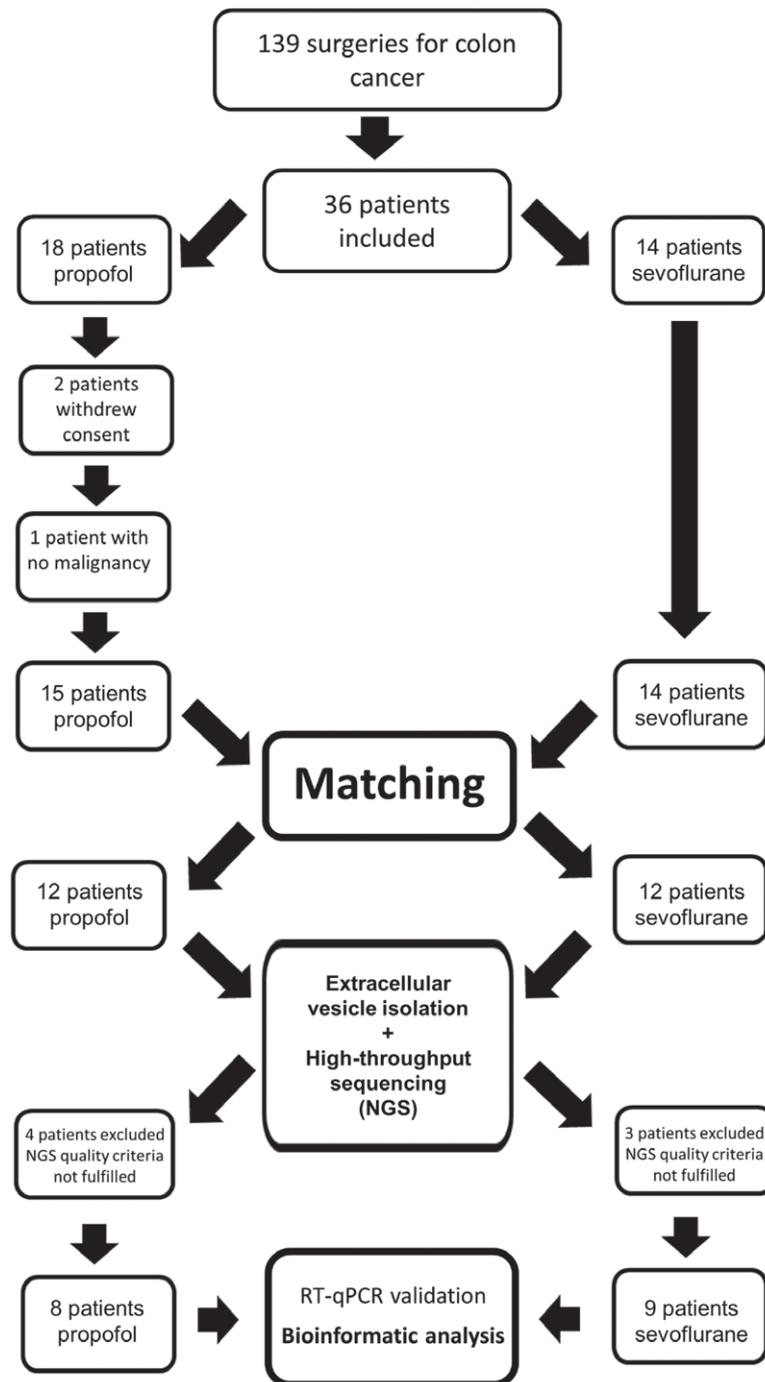
The study was approved by the Ethics Committee of the Medical Faculty of the University of Munich, Munich, Germany (protocol No. 232-16). The study was carried out in accordance with approved guidelines and all study samples were anonymized during analysis. Written informed consent for publication of blinded individual person's data was obtained from each participant.

### Patient Identification and Selection

Patients scheduled to undergo colorectal cancer surgery were identified preoperatively from the operating room schedule of the Department of Surgery at the Ludwig-Maximilians University Hospital of Munich, Germany. Evaluation regarding inclusion and exclusion criteria was performed during a preoperative visit by study personnel (usually on the day before surgery) when informed consent was obtained. The primary inclusion criterion was the presence of a primary colorectal cancer scheduled for operative therapy. Exclusion criteria were (1) nonconsent; (2) age less than 18 yr; (3) pregnancy; (4) colorectal cancer in the presence of another primary cancer; (5) severe organ dysfunction (liver, renal); (6) chronic inflammatory or autoimmune disorders (*e.g.*, rheumatoid arthritis); (7) use of immunosuppressive medication; or (8) contraindications for epidural anesthesia.

Patients were recruited between April 2017 and August 2018. The study was designed as a prospective, matched-case, non-randomized pilot investigation. The patient selection and matching process is illustrated in figure 1. The final study population consisted of eight patients receiving propofol matched to nine patients anesthetized with sevoflurane. Because the study was designed as a proof-of-concept investigation with a limited number of participants, patient matching was performed according to (1) colon carcinoma staging using *Union for International Cancer Control* (Eighth edition, 2017) criteria and (2) colon cancer location. These matching targets were selected because tumor stage and location are the major determinants of patient long-term survival.<sup>16,17</sup> As shown in table 1, patient cohorts were not significantly different concerning the primary matching targets tumor stage ( $P = 0.708$ ) and location ( $P = 0.501$ ). Other variables with possible effects on concentrations of circulating vesicles such as intraoperative fluid administration, duration of surgery, and age and sex of the patients were also not different between study groups (tables 1 and 2).

After removing patients with less than 750,000 mapped microRNA reads at each time point, the final study population consisted of eight patients receiving propofol and nine patients receiving sevoflurane. The exclusion based on stringent quality criteria did not impact the overall matching. Clinical, demographic, and perioperative data of the final patient sample are displayed in table 1 and table 2.



**Fig. 1.** Illustration of the patient selection and matching process. In both groups, extracellular vesicles were isolated from pre- and postoperative sera. Vesicular microRNA profiles were subsequently assessed by next generation sequencing (NGS). After stringent quality filtering, eight (propofol) and nine (sevoflurane) patients were included for *in silico* analyses and validation by real-time quantitative polymerase chain reaction (RT-qPCR).

### Anesthesiologic Procedures and Sample Collection

Standard balanced anesthesia consisting of epidural anesthesia in combination with general anesthesia was planned for all patients. Due to certain preoperative and technical circumstances, epidural anesthesia was not possible in two

patients in the propofol cohort. General anesthesia was induced with propofol in both groups, and subsequently maintained by propofol or sevoflurane. The choice of anesthetic agent was left to the attending anesthesiologist, following standard procedures at our institution.

**Table 1.** Comparison of Demographic and Tumor Staging Data between the Propofol and Sevoflurane Group

Parameter	Propofol	Sevoflurane	P Value
Sex (male/female)	5/3	8/1	0.479
Age (yr)*	65 (60–68)	73 (67–82)	0.074
Body mass index (kg/m <sup>2</sup> )*	25.4 (22.6–27.6)	26.0 (24.8–34.2)	0.180
ASA Physical Status (III/II)	7/1	9/0	0.952
Epidural anesthesia (yes/no)	6/2	9/0	0.399
Tumor location (colorectal/colon [R]/colon [L]/other/sigmoid)	4/2/1/0/1	6/2/0/1/0	0.501
UICC† tumor stage (3/2/1)	4/2/2	3/3/3	0.784
Irradiation (yes/no)	4/4	4/5	0.797
Chemotherapy (yes/no)	4/4	6/3	0.839
Coronary artery disease (yes/no)	8/0	6/3	0.245
Arterial hypertension (yes/no)	4/4	5/4	0.797
Diabetes type II (yes/no)	8/0	6/3	0.245
Renal dysfunction (yes/no)	8/0	8/1	0.952
Atrial fibrillation (yes/no)	8/0	7/2	0.506

\*Data are median (p<sub>25</sub> – p<sub>75</sub>). †Union for International Cancer Control, 8th edition 2017. ASA, American Society of Anesthesiologists; L, left; R, right.

**Table 2.** Comparison of Intraoperative Drug and Volume Use between the Propofol and Sevoflurane Group

Parameter	Propofol	Sevoflurane	P Value
Propofol (mg)*‡	2612 (1665–4009)	285 (88–615)†	< 0.001
Sevoflurane (max. MAC)*‡	N/A	1.3 (1.1–1.5)	N/A
Sufentanil (ug)*‡	95 (60–100)	70 (40–100)	0.296
Ropivacaine (mg)*§	170 (120–190)	170 (160–180)	0.358
Norepinephrine (ug · kg <sup>-1</sup> · min <sup>-1</sup> )*	0.051 (0.042–0.068)	0.081 (0.065–0.098)	0.056
Fluid administration*‡ (l)	3.75 (2.88–5.38)	4.00 (3.50–5.00)	0.423
Duration of surgery* (min)	250 (197–403)	237 (196–306)	0.405

\*Data are median (p<sub>25</sub> – p<sub>75</sub>). †Propofol was administered for induction of anesthesia. ‡Total intraoperative and intravenous dose. §Administered for epidural anesthesia. MAC, minimum alveolar concentration; max., maximum; N/A, not applicable.

Venous blood was drawn through intravascular catheters at the pre-operative time point and after termination of surgery during wound closure. Nine-milliliter serum tubes (S-Monovette; Sarstedt, Germany) were used for blood collection, samples were immediately centrifuged at 3,400 g for 10 min at room temperature. Serum was aliquoted and stored at –80°C until further processing.

### Extracellular Vesicle Isolation

Extracellular vesicles were isolated from 1 ml of thrombin-digested serum using a commercial method (miRCURY Exosome Serum/Plasma Kit; Qiagen, Germany). Following a preclearing step (10,000 g; 5 min), vesicles were precipitated according to the manufacturer’s instruction. Pellets were resuspended in lysis buffer or phosphate-buffered saline for RNA extraction or biologic characterization, respectively.

### RNA Extraction, Library Preparation, and Small RNA Sequencing

Total RNA was extracted from vesicle pellets using the miRCURY RNA Isolation Kit – Biofluids (Qiagen) per the

manual’s instructions. RNA was eluted in 30 µl of nuclease-free water. Next, size distributions of total vesicular RNA were assessed by capillary electrophoresis using the RNA 6000 Pico Kit and a 2100 Bioanalyzer (Agilent Technologies, Germany).

Sequencing libraries were prepared as reported elsewhere.<sup>18</sup> Briefly, the NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England BioLabs, USA) was used to construct libraries from 6 µl of concentrated vesicle RNA using the manufacturer’s protocols with minor adjustments. Size distribution of final libraries was assessed by capillary electrophoresis (High Sensitivity DNA Assay, 2100 Bioanalyzer; Agilent Technologies) before 50 sequencing cycles on the Illumina HiSeq2500 (Illumina, USA).

### Data Analysis

As no previous investigations on the effect of anesthetics on extracellular vesicles or vesicular microRNA expression patterns were available, no statistical power calculation that would allow sample size estimation could be performed before the study. Furthermore, as the study was designed as a proof-of-principle investigation demonstrating the

feasibility of studying anesthetic effects on vesicular microRNA expression, the sample size was based on our previous experiences with high-throughput sequencing of microRNAs in circulating vesicles.<sup>19,20</sup>

Sequencing data were processed using an in-house pipeline, as described previously.<sup>18</sup> After assessing technical sequencing quality using FastQC, adaptor sequences were clipped using Btrim. Reads without adaptors, as well as reads less than or equal to 15 nucleotides, were removed. Remaining reads were sequentially aligned to sequences from human noncoding RNAs (ribosomal RNA, transfer RNA, small nuclear RNA, small nucleolar RNA) downloaded from RNACentral. Next, reads that did not map to these RNA classes were aligned to miRBase (release 22).<sup>21</sup> All alignment steps were performed using the Bowtie “best” algorithm and allowed one mismatch. Read counts for each RNA class were extracted from Bowtie output and only samples with at least 750,000 microRNA reads were included in subsequent analyses. Normalization, differential expression analysis and false discovery according to Benjamini–Hochberg correction (adjusted *P* value) were carried out for paired pre- and postsurgical samples using DESeq2.<sup>22</sup> False discovery rate correction according to Benjamini–Hochberg is the most commonly performed and widely accepted method to mitigate Type I error accumulation for high-throughput data. Using much stricter correction method for multiple comparisons (e.g., the Bonferroni method) would result in a substantial increase in Type II error risk, limiting the detection of important and genuinely regulated candidates,<sup>23</sup> which would not be adequate for a pilot study like this one.

Only transcripts identified as differentially regulated using stringent filtering criteria (mean expression greater than or equal to 50 reads; log<sub>2</sub> fold change greater than or equal to |1|; adjusted *P* value less than or equal to 0.05) were considered for subsequent analyses of anesthesia-responsive microRNAs. Data were tested for normal distribution using the Shapiro–Wilk test. Nonparametric data and data that are not normally distributed were compared using the nonparametric Mann–Whitney U test and are reported as median and quartiles (Q25, Q75). Normally distributed data were compared using two-tailed, paired Student’s *t* test and are shown as mean ± SD. A *P* value less than 0.05 was regarded as statistically significant. Except for propofol subgroup analyses that excluded the two patients unable to receive epidural anesthesia, all analyses were preplanned. Statistical programs used in this study were Python (version 3.7; Python Software Foundation, USA) and GraphPad Prism (version 7.00; GraphPad Software, USA). Venn diagrams were created using an online tool (<http://bioinformatics.psb.ugent.be/webtools/Venn/>; accessed December 19, 2018).

### Pathway Analysis

Ingenuity Pathway Analysis (Qiagen, USA) was used for the identification of gene targets and causal networks from our high-throughput microRNA expression data. Only microRNAs meeting the abovementioned filtering criteria

were entered into the software, and only experimentally confirmed relationships were considered for the analysis of microRNA targets and gene networks. Target genes of microRNAs significantly regulated by either propofol or sevoflurane were identified using the *microRNA Target Filter*. Filters were set to *cancer*, *colorectal cancer cells*, and *colon cancer metastasis signaling*. For both groups, the involvement of canonical carcinoma-related pathways was subsequently analyzed using the *Comparison Analysis* feature. The *Diseases and Biofunction Analysis* was limited to colorectal and cancer cells, *z*-scores were calculated<sup>24</sup> and represented as heat maps.

### Real-time Quantitative Polymerase Chain Reaction Validation

As patient samples were limited, we used sera from patients previously analyzed by small RNA-sequencing for validation of microRNA expression. Extracellular vesicle isolation and RNA extraction were carried out as described in the Anesthesiologic Procedures and Sample Collection section. RNA extracts were evaporated and resuspended in 4 μl nuclease-free water. Two μl of resuspended RNA were subjected to reverse transcription *via* the miRCURY LNA RT Kit (Qiagen) in a 10-μl reaction volume. Resulting complementary DNA was diluted 1:20 for real-time polymerase chain reaction using the miRCURY LNA SYBR Green PCR Kit (Qiagen) and a Rotor-Gene Q thermal cycler (Qiagen). Reference microRNA candidates were selected from next generation sequencing data using geNorm and NormFinder.<sup>25,26</sup> Subsequent to quantitative polymerase chain reaction, data were normalized with the geometric mean of reference microRNAs miR-21-5p, miR-126-3p and miR-30d-5p (propofol) or miR-21-5p, miR-24-3p and miR-30d-5p (sevoflurane), and relative quantification was performed using the ΔΔCq method.<sup>27</sup> Statistical significance was assessed by paired Student’s *t* test.

### Extracellular Vesicle Characterization

To assess potential anesthesia-induced changes in extracellular vesicle size and concentration, particles were analyzed by Nanoparticle Tracking Analysis using a ZetaView PMX 110 device equipped with a 250-nm laser (Particle Metrix, Germany) in a subset of five patients per group that were previously analyzed by small RNA-sequencing. Vesicle suspensions were appropriately diluted in particle-free phosphate-buffered saline and analyzed in two imaging cycles at 11 positions each. Settings for video capture were adjusted to 70 and 75 for shutter and sensitivity, respectively. Data analysis was performed using the ZetaView software (8.04.02), including only samples with a minimum of 500 completed tracks. Initial particle concentrations in serum were calculated as described by Eitan *et al.*<sup>28</sup>

The expression of common extracellular vesicle marker proteins was assessed by immunoblotting in vesicles from preoperative sera. As precipitation co-isolates large amounts of contaminating protein that mask target proteins,<sup>18</sup> vesicles were washed by ultracentrifugation (Beckman Coulter

Optima LE-80K; SW40 rotor, 100,000g, 2h, k-factor 299.2), as suggested elsewhere.<sup>29</sup> Next, vesicles were lysed in radioimmunoprecipitation assay buffer supplemented with protease inhibitors and separated on reducing or nonreducing 4 to 12% NuPAGE Bis-Tris Gels (Invitrogen, USA). Proteins were subsequently transferred onto 0.45 μm nitrocellulose membranes (GE Healthcare Life Sciences, USA). Membranes were blocked in 1% skim milk powder in Tris-buffered saline with Tween and incubated with primary antibodies overnight. After three washing steps with blocking buffer, secondary antibodies were added for 1 h. Membranes were subsequently washed three times with blocking buffer and developed using the Clarity Western ECL Blotting Substrate Kit (Bio-Rad, Germany). All marker proteins except CD63 and CD81 were analyzed under reducing conditions. Primary antibodies were purchased from Abcam (rabbit anti-Alix, ab92726, 1:1000; rabbit anti-Syntenin, ab133267, 1:5000; mouse anti-CD63, ab59479, 1:500; mouse anti-CD81, ab79559, 1:800) and Biomol (goat anti-Calnexin, WA-AF1179a, 1:2500).

Secondary antibodies were purchased from Abcam (goat anti-Rabbit HRP, ab97080, 1:10000; rabbit anti-Goat HRP, ab97105, 1:10000; goat anti-Mouse HRP, ab97040, 1:10000).

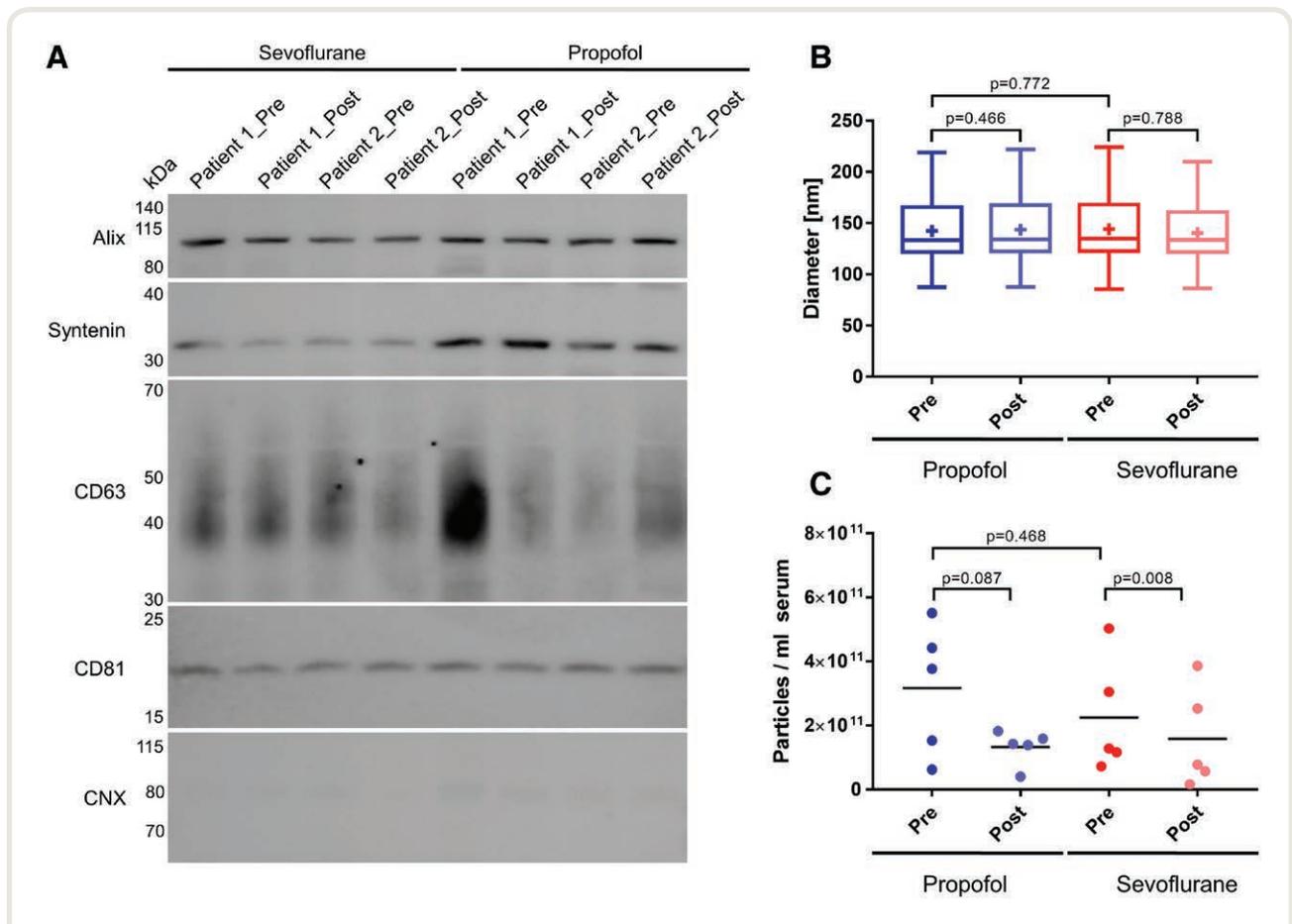
**Results**

**Composition and Drug Exposure of the Study Groups**

Matching of the two study groups resulted in comparable demographic data, tumor localization, and tumor stage (table 1). Likewise, both groups were comparable regarding the use of opioids, perioperative vasopressor or intravenous fluid requirements, and the duration of surgery (table 2).

**Anesthesia Does Not Alter Extracellular Vesicle Characteristics**

Extracellular vesicle-enriched and nonenriched proteins were analyzed by immunoblotting (fig. 2A). Vesicle markers alix, CD63, and CD81 were detected in samples from both the propofol and sevoflurane group without systematic



**Fig. 2.** Characterization of extracellular vesicles. (A) Pre- and postoperative vesicles in the sevoflurane (left) and propofol (right) group were positive for the protein markers alix, syntenin, CD63, and CD81. Calnexin (CNX) was not detected, indicating the absence of contamination with cellular debris. (B) Nanoparticle tracking analysis revealed homogenous size distributions of vesicles from pre- and postoperative sera in both groups. (C) Postoperative particle concentrations were reduced in both groups, whereas preoperative baseline concentrations were not significantly different. Whiskers indicate 10th and 90th percentiles; line indicates modal diameter; and + indicates mean diameter.

differences in signal intensities for vesicles sampled before surgery and after tumor resection. Similarly, the extracellular vesicle marker syntenin was detected in patient vesicles from both groups, albeit with a slight enrichment in patients anesthetized using propofol. All vesicle lysates were negative for the ribosomal marker calnexin, indicating the absence of contamination with cellular material.

Analysis of vesicle size and concentration by nanoparticle tracking analysis detected particles with exosome-like diameters in all samples (fig. 2B). Average modal diameters were  $135.0 \pm 10.8$  nm (propofol),  $133.6 \pm 9.5$  nm (postpropofol),  $133.4 \pm 13.3$  nm (presevoflurane) and  $134.1 \pm 15.2$  nm (postsevoflurane). Particle concentrations per ml serum varied significantly between individual patients (see figure, Supplemental Digital Content 1, <http://links.lww.com/ALN/C59>, displaying individual particle size distributions for each patient), but presurgical baseline concentrations (propofol:  $3.2E11 \pm 2.0E11$ ; sevoflurane:  $2.2E11 \pm 1.8E11$ ) were similar between groups ( $P = 0.468$ ). Compared to sera sampled before surgery, postanesthetic particle concentrations were lower in patients anesthetized with either propofol ( $1.3E11 \pm 5.4E10$ ) or sevoflurane ( $1.6E11 \pm 1.6E11$ ), reaching statistical significance ( $P = 0.008$ ) for the latter (fig. 2C). Despite lower particle concentrations, size distributions for individual patients were highly similar in pre- and postsurgical sera (Supplemental Digital Content 1, <http://links.lww.com/ALN/C59>).

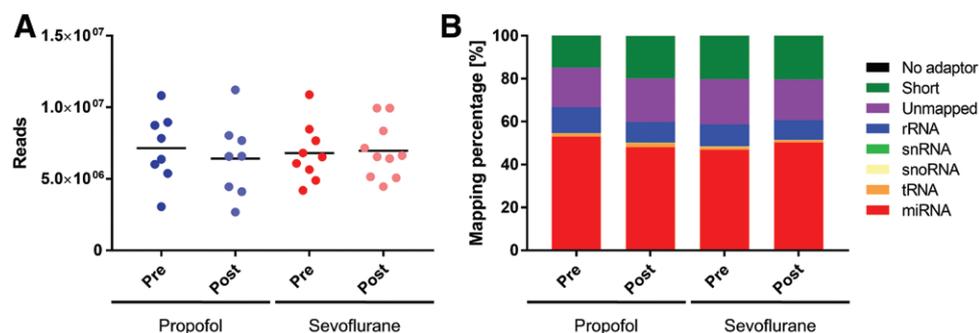
In summary, extracellular vesicle marker-positive particles with exosome-like sizes were isolated in all groups. Regardless of the respective anesthetic agent, particle concentrations in serum were reduced after tumor resection at the termination of surgery.

### Characterization of Extracellular Vesicle RNA

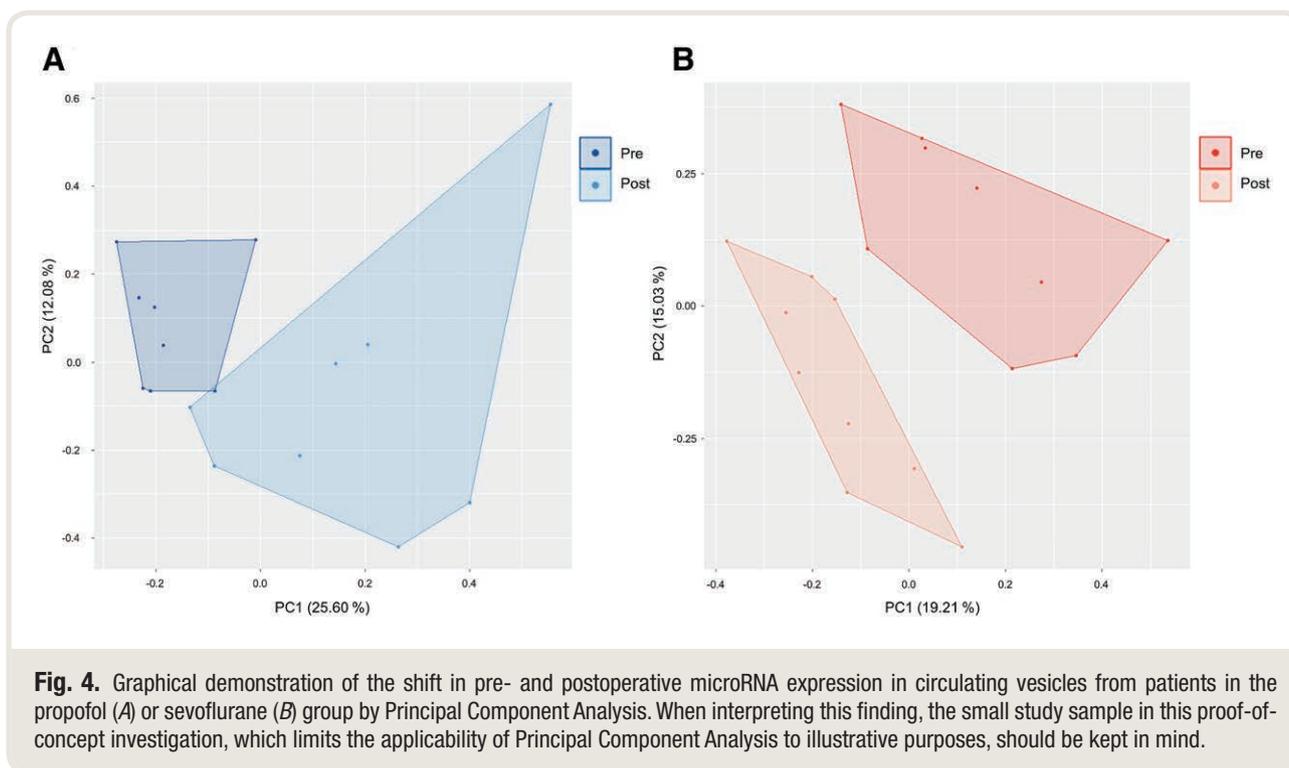
Concentration and size distribution of RNA in circulating vesicles were analyzed by capillary electrophoresis. The

median RNA yield in vesicles from 1 ml of serum was 4 (3 to 9) ng (propofol), 3 (2 to 7) ng (postpropofol), 5 (4 to 8) ng (presevoflurane) and 6 (4 to 16) ng (postsevoflurane). Baseline differences in RNA concentration between the groups were not statistically significant ( $P = 0.984$ ), neither were intergroup differences after surgery ( $P = 0.209$ ) or perioperative differences within groups (propofol:  $P = 0.279$ ; sevoflurane:  $P = 0.748$ ).

Sequencing libraries for patients in both groups were reasonably homogenous in size and composition (fig. 3, A and B). Mean library sizes were  $7.15E6 \pm 2.43E6$  reads (propofol),  $6.41E6 \pm 2.68E6$  reads (postpropofol),  $6.80E6 \pm 2.02E6$  (presevoflurane) and  $6.64E6 \pm 1.72E6$  reads (postsevoflurane). Analysis of relative mapping frequencies revealed an enrichment of microRNA reads in all groups (fig. 3B). On average,  $53 \pm 11\%$  (propofol),  $45 \pm 11\%$  (postpropofol),  $45 \pm 14\%$  (presevoflurane) and  $49 \pm 8\%$  (postsevoflurane) of all reads mapped to microRNAs. Four (propofol) and two (sevoflurane) patients were excluded from the original cohort due to insufficient (less than 750,000) microRNA reads. Additionally, one patient from the sevoflurane group was identified as an outlier by hierarchical clustering (see figure, Supplemental Digital Content 2, <http://links.lww.com/ALN/C60>, displaying hierarchical clustering of microRNA expression in all patients). This patient had received several units of a fresh frozen plasma preparation due to the development of an intraoperative bleeding tendency. As transfused plasma products very likely contain extracellular vesicles and microRNAs, this could have resulted in the observed shift in microRNA expression values. This patient was therefore excluded from further analyses. There were no significant differences in preanesthetic baseline for library sizes ( $P = 0.751$ ) and number of microRNA reads ( $P = 0.452$ ) between the propofol and sevoflurane



**Fig. 3.** Characterization of sequencing libraries. (A) Individual library sizes for extracellular vesicles isolated from pre- and postoperative sera. No significant differences were detected between all time points and groups. (B) Mean relative mapping distributions for various classes of small RNA revealed a strong enrichment of microRNAs in circulating vesicles from all groups. Less than 0.2% of reads carried no adaptor or mapped to snRNA or snoRNA. rRNA, ribosomal RNA; Short, read less than or equal to 15 nt; snRNA, small nuclear RNA; snoRNA, small nucleolar RNA; tRNA, transfer RNA.



**Fig. 4.** Graphical demonstration of the shift in pre- and postoperative microRNA expression in circulating vesicles from patients in the propofol (A) or sevoflurane (B) group by Principal Component Analysis. When interpreting this finding, the small study sample in this proof-of-concept investigation, which limits the applicability of Principal Component Analysis to illustrative purposes, should be kept in mind.

group. To assess microRNA detection in each group, we filtered sequencing data for microRNA species with a mean expression of at least 10 reads per group. A total of 494 (prepropofol), 458 (postpropofol), 473 (presevoflurane), and 483 (postsevoflurane) distinct microRNAs were detected above this threshold.

### Anesthesia-induced Changes in MicroRNA Profiles

Potential changes in microRNA expression profiles were assessed by principal component analysis of normalized, regularized, log-transformed read counts obtained from DESeq2 (fig. 4). Anesthesia by both propofol and sevoflurane induced a clear shift in microRNA expression as evidenced by separate clustering of pre- and postsurgical samples. While there was minimal overlap of groups in propofol-induced anesthesia (fig. 4A), samples in the sevoflurane group were separated more clearly (fig. 4B).

### Detection of Anesthesia-responsive MicroRNAs

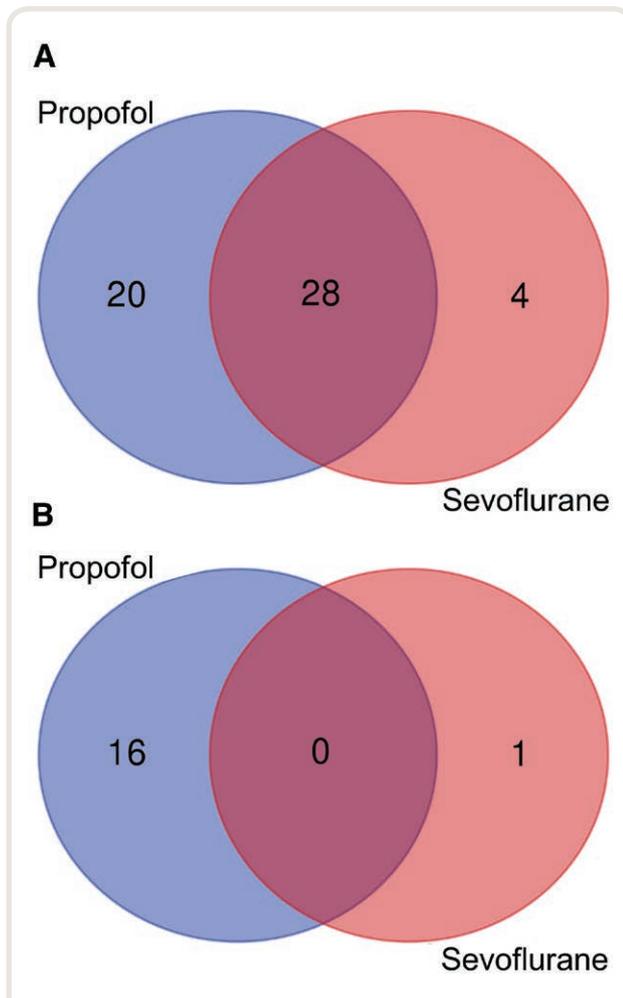
To assess which microRNA species displayed altered expression in response to anesthesia by propofol or sevoflurane, we performed differential gene expression analysis of paired samples in either group (propofol:  $n = 8$ ; sevoflurane:  $n = 9$ ). Using stringent filtering criteria for microRNA expression and magnitude of expression changes, a total of 64 microRNAs was found to be significantly regulated by propofol-induced anesthesia (48 up, range of  $\log_2$  fold change = 1.07 to 3.76; 16 down,  $\log_2$  fold change range = -1.00 to -1.55). Expression levels of 33 microRNAs were

significantly changed by sevoflurane-induced anesthesia (32 up,  $\log_2$  fold change range = 1.02 to 2.98; 1 down,  $\log_2$  fold change = -1.36). All regulated microRNAs, respective expression levels, fold changes, and adjusted  $P$  values are listed in Supplemental Digital Content 3 (<http://links.lww.com/ALN/C61>). Next, we assessed potentially overlapping changes in microRNA expression for both groups. As demonstrated in figure 5, all microRNAs downregulated in response to anesthesia were specific to the respective anesthetic agent, while the majority of upregulated microRNAs was shared in both groups. Of the 48 microRNAs significantly upregulated by propofol, 20 (42%) were not significantly regulated by sevoflurane anesthesia. Of the 32 microRNAs upregulated in response to sevoflurane (13%), 4 were specific to this anesthetic agent. All microRNAs exclusively regulated by either anesthesia are listed in table 3 along with adjusted  $P$  value and  $\log_2$  fold change values.

Regulation of selected microRNAs specific to either anesthetic procedure or part of the shared response was validated by real-time quantitative polymerase chain reaction (table 4). All microRNAs except let-7c-5p, miR-103a-3p, miR-106a-5p, miR-23a-3p, miR-328-3p, and miR-483-5p (propofol), as well as miR-1246 (sevoflurane), were successfully validated.

### Potential Biological Effects of Anesthesia-induced Shifts in MicroRNA Expression

MicroRNAs significantly regulated during either form of anesthesia were separately uploaded into Ingenuity Pathway



**Fig. 5.** Differential regulation of microRNAs by propofol and sevoflurane. (A) The majority of microRNAs significantly upregulated after tumor resection was shared in both anesthetic groups. Twenty and four microRNAs were exclusively upregulated by propofol and sevoflurane, respectively. (B) Postoperatively down-regulated microRNAs displayed no overlap between groups. Sixteen (propofol) and one (sevoflurane) microRNAs had significantly lower expression levels after tumor resection.

Analysis and analyzed identically with mRNA target filtering set to *cancer, colon cancer cells, and colon cancer metastasis signaling*. This identified 13 microRNAs in the propofol group (7 upregulated) with 19 potential mRNA targets from the aforementioned canonical and signaling pathways (table 5). For the sevoflurane group, *in silico* analysis using identical target filtering identified six significant microRNAs (all upregulated) and nine potential target genes (table 6). In order to characterize different biofunctions of vesicle-associated microRNAs from both groups, the *Comparison Analysis* function was used. The analysis indicated that, in contrast to sevoflurane anesthesia, propofol-regulated microRNAs might mediate inhibitory effects on signaling pathways involving cell proliferation (z-score,  $-1.73$ ), migration (z-score,  $-1.97$ ),

**Table 3.** MicroRNAs Exclusively Regulated by Propofol or Sevoflurane

Regulation	MicroRNA	baseMean	log2FC	Adjusted P Value	
Propofol down	miR-106a-5p	67.91	-1.55	8.98E-03	
	miR-96-5p	51.96	-1.51	4.18E-04	
	miR-15a-5p*	121.77	-1.45	1.95E-05	
	miR-144-5p	395.48	-1.34	1.59E-04	
	miR-652-3p	253.18	-1.21	1.47E-03	
	miR-19b-3p*	412.83	-1.21	1.65E-03	
	miR-15b-3p	202.01	-1.18	9.68E-06	
	miR-103a-3p	2129.20	-1.14	9.23E-04	
	miR-16-5p*	3861.74	-1.10	1.36E-04	
	miR-501-3p	580.79	-1.08	1.09E-04	
	miR-7-5p*	2610.65	-1.06	1.57E-06	
	miR-17-5p*	491.99	-1.04	3.48E-03	
	miR-185-5p*	21576.49	-1.03	2.95E-03	
	miR-363-3p*	4662.80	-1.02	1.49E-04	
	miR-598-3p	54.91	-1.00	8.98E-03	
	miR-23a-3p	2314.70	-1.00	1.75E-02	
	Propofol up	miR-206	2312.18	3.57	1.27E-20
miR-205-5p*		51.95	2.80	3.36E-04	
miR-204-3p		58.93	2.13	1.42E-03	
miR-3168		1268.45	2.06	1.22E-02	
miR-1228-5p		241.73	1.80	2.80E-03	
miR-574-5p		68.30	1.73	3.53E-03	
miR-204-5p		57.70	1.55	1.47E-03	
miR-429		77.00	1.43	4.65E-03	
miR-193b-5p		122.32	1.41	2.06E-04	
let-7c-5p		1600.31	1.39	1.99E-04	
miR-4433b-3p		521.45	1.37	4.82E-03	
miR-125b-2-3p		58.73	1.32	1.20E-03	
miR-483-5p		101.04	1.30	3.55E-03	
miR-485-5p		152.11	1.26	9.26E-04	
miR-7706		261.90	1.17	2.44E-02	
miR-27b-3p		16969.53	1.15	7.77E-05	
miR-30a-3p		625.75	1.11	1.68E-05	
let-7b-3p	238.90	1.08	8.98E-03		
miR-499a-5p	347.89	1.08	1.03E-03		
miR-582-3p	826.73	1.07	3.43E-03		
Sevoflurane down	miR-1246	410.41	-1.36	3.73E-08	
	Sevoflurane up	miR-335-3p	196.16	1.31	2.04E-04
		miR-10a-3p	69.29	1.17	2.22E-04
		miR-23b-5p	56.85	1.14	6.74E-03
miR-335-5p*	242.73	1.02	9.82E-07		

\*MicroRNA (miR) validated by real-time quantitative polymerase chain reaction. baseMean, mean microRNA expression; log2FC, log2 fold change.

epithelial-mesenchymal transition of tumor cell line (z-score,  $-0.79$ ), and enhance effects on apoptosis of carcinoma cell lines (z-score,  $-1.19$ ) (fig. 6).

## Discussion

This study was triggered by findings from several retrospective investigations, which indicated that the use of propofol in combination with local-regional anesthesia during cancer resection may result in improved outcomes in a variety of tumors including colorectal cancers.<sup>6,7,30</sup> In a recent retrospective study on colorectal cancer surgery patients receiving propofol or sevoflurane (n = 579 each), propofol-based anesthesia resulted

**Table 4.** Validation of MicroRNAs Significantly Regulated in Patients Anesthetized Using Propofol or Sevoflurane by Real Time Quantitative Polymerase Chain Reaction

Anesthesia	microRNA	Cq pre	Cq post	P Value	
Propofol	let-7c-5p	3.8 ± 1.2	5.1 ± 1.7	0.135	
	miR-103a-3p	2.8 ± 1.8	4.0 ± 1.8	0.228	
	miR-106a-5p	1.0 ± 1.7	2.2 ± 1.9	0.260	
	miR-1-3p	9.2 ± 1.6	4.9 ± 1.0	2.95E-04	
	miR-15a-5p	-0.5 ± 0.4	0.0 ± 0.5	0.037	
	miR-16-5p	-4.1 ± 0.5	-3.6 ± 0.3	0.015	
	miR-17-5p	0.3 ± 0.4	0.8 ± 0.5	0.026	
	miR-185-5p	0.5 ± 0.5	1.2 ± 0.6	0.005	
	miR-19b-3p	-2.0 ± 0.3	-1.4 ± 0.3	0.006	
	miR-205-5p	5.9 ± 0.9	3.3 ± 1.0	0.002	
	miR-23a-3p	-0.7 ± 0.2	-0.8 ± 0.5	0.304	
	miR-328-3p	5.4 ± 0.5	5.0 ± 0.5	0.104	
	miR-363-3p	2.7 ± 0.6	3.5 ± 0.5	0.004	
	miR-483-5p	7.6 ± 4.2	8.7 ± 2.2	0.617	
	miR-7-5p	5.3 ± 0.7	6.1 ± 0.7	0.001	
	Sevoflurane	miR-1246	3.5 ± 2.3	2.0 ± 2.3	0.163
		miR-1-3p	7.8 ± 1.9	5.2 ± 1.7	0.023
miR-335-5p		5.6 ± 1.3	4.6 ± 0.8	0.038	

Lower normalized cycle quantification (Cq) values indicate higher expression levels. Post, postoperative; pre, preoperative.

in significantly longer recurrence-free survival, higher overall survival, and reduced metastasis.<sup>6</sup> Various studies have reported propofol to have anticancer properties *in vitro* and *in vivo*. Song *et al.*,<sup>31</sup> Tsuchiya *et al.*,<sup>32</sup> and Zhang *et al.*<sup>33</sup> demonstrated apoptosis-inducing effects of propofol in several cancers including leukemia, hepatocellular carcinoma, and non-small cell lung cancer.<sup>31-33</sup> In a cellular model, propofol reduced the migration of colorectal cancer cells by inhibiting epithelial-mesenchymal transition,<sup>7</sup> which is in line with findings from our study. In contrast, there is evidence that the use of volatile gases during cancer surgery may enhance tumor cell growth and metastasis. In a recent study comparing intravenous and inhaled anesthesia in 80 breast cancer surgery patients, sevoflurane was shown to significantly increase circulating levels of vascular endothelial growth factor, thereby establishing a proangiogenic environment and potentially fostering tumor progression.<sup>30</sup> These epidemiologic observations and experimental findings represented the primary trigger for our pilot study.

Despite these experimental and preclinical studies demonstrating differential effects of anesthetics on tumor growth and metastasis risk, a common soluble mediator that regulates the interaction of the primary tumor, migratory tumor cells, and premetastatic niches, and that might be influenced by the type of anesthesia used has not been identified.

A recent *in vitro* study compared the effect of sera from patients undergoing colorectal cancer surgery receiving thoracic epidural and propofol *versus* sevoflurane anesthesia on cancer cell biology. Colon cancer cells were cultured with sera obtained from these patients at 24h postoperatively. Interestingly, sera taken from patients receiving propofol, in

**Table 5.** Extracellular Vesicle-associated MicroRNAs Regulated by Propofol Anesthesia during Colorectal Cancer Resection and Their Possible Gene Targets

microRNA	log2FC	Adjusted P Value	mRNA Target
miR-1-3p	3.77	2.66E-20	EGFR
miR-1-3p	3.77	2.66E-20	LRP1
miR-133a-3p	3.37	4.01E-06	CASP9
miR-133a-3p	3.37	4.01E-06	RHOA
miR-143-3p	1.87	6.04E-12	KRAS
miR-143-3p	1.87	6.04E-12	MAPK12
miR-185-5p	-1.03	2.95E-03	AKT1
miR-185-5p	-1.03	2.95E-03	RHOA
miR-19b-3p	-1.21	1.65E-03	CCND1
miR-204-5p	1.54	1.47E-03	MMP3
miR-204-5p	1.54	1.47E-03	MMP9
miR-205-5p	2.80	3.36E-04	VEGFA
miR-218-5p	2.60	1.89E-18	EGFR
miR-218-5p	2.60	1.89E-18	PIK3C2A
miR-23a-3p	-1.00	1.75E-02	SMAD3
miR-23a-3p	-1.00	1.75E-02	SMAD4
miR-30a-3p	1.11	1.68E-05	PIK3C2A
miR-511-5p	1.18	2.95E-06	TLR4
miR-7-5p	-1.06	1.57E-06	EGFR
miR-7-5p	-1.06	1.57E-06	FOS
miR-7-5p	-1.06	1.57E-06	IRS1
miR-7-5p	-1.06	1.57E-06	IRS2
miR-96-5p	-1.51	4.18E-04	ADCY6
miR-96-5p	-1.51	4.18E-04	IRS1

Positive fold changes indicate upregulation of the corresponding microRNA during anesthesia, negative fold changes show downregulation of the corresponding microRNA.

ADCY6, adenyl cyclase type 6; AKT1, RAC-alpha serine/threonine-protein kinase; CASP9, apoptosis-related cysteine peptidase; CCND1, Cyclin D1; EGFR, epidermal growth factor receptor; FOS, cFOS pro-oncogene; IRS1, insulin receptor substrate 1; IRS2, insulin receptor substrate 2; KRAS, GTPase Kras; log2FC, log2 fold change; LRP1, low density lipoprotein receptor-related protein 1; MAPK12, mitogen-activated protein kinase 12; MMP3, matrix metalloproteinase-3; MMP9, matrix metalloproteinase-9; PIK3C2A, phosphatidylinositol-4-phosphate 3-kinase C2 domain-containing alpha polypeptide; PTPN11, tyrosine-protein phosphatase non-receptor type 11; RHOA, ras homolog family member A; SMAD3, mothers against decapentaplegic homolog 3; SMAD4, MAD-homolog 4; TLR4, toll-like receptor 4; VEGFA, vascular endothelial growth factor A.

comparison to those from sevoflurane patients, induced a reduction in invasiveness, proliferation, and metastatic potential, as well as enhanced apoptosis of the cancer cells.<sup>34</sup> This could indicate the presence of one or multiple factors in serum that influence recurrence risk by modulating the inflammatory microenvironment and that are downregulated by propofol. We assumed that circulating extracellular vesicles in patients undergoing tumor resection potentially represent such a factor and that these particles might mediate the long-lasting impact of propofol on colorectal cancer cells. Circulating vesicles are known to play an important role in organ-specific tropism and the preparation of premetastatic niches in colorectal cancer, melanoma, breast cancer, pancreatic cancer, prostate cancer, and brain metastasis, among others.<sup>35-40</sup> This line of reasoning represented a second trigger for our study.

The primary aim of this pilot study was to demonstrate that propofol and sevoflurane do indeed have differential effects on circulating vesicles and their RNA cargo.

**Table 6.** Extracellular Vesicle-associated MicroRNAs Regulated by Sevoflurane Anesthesia during Colorectal Cancer Resection and Their Potential mRNA Targets

MicroRNA	log2FC	Adjusted P Value	mRNA Target
miR-1-3p	2.98	5.86E-05	EGFR
miR-1-3p	2.98	5.86E-05	LRP1
miR-133a-3p	2.58	5.96E-07	CASP9
miR-133a-3p	2.58	5.96E-07	RHOA
miR-143-3p	1.24	1.40E-06	KRAS
miR-143-3p	1.24	1.40E-06	MAPK12
miR-218-5p	2.49	5.22E-21	EGFR
miR-218-5p	2.49	5.22E-21	PIK3C2A
miR-335-5p	1.02	9.82E-07	PTPN11
miR-511-5p	1.08	6.88E-06	TLR4

Positive fold changes indicate upregulation of the corresponding microRNA during surgery.

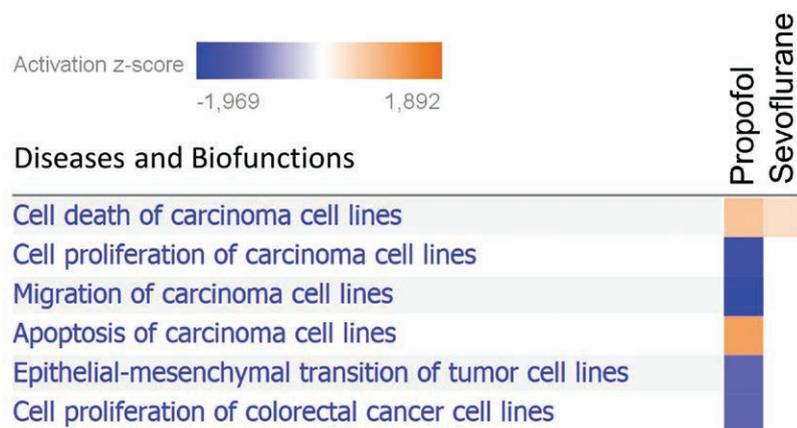
CASP9, apoptosis-related cysteine peptidase; EGFR, epidermal growth factor receptor; KRAS, GTPase Kras; log2FC, log2 fold change; LRP1, low density lipoprotein receptor-related protein 1; MAPK12, mitogen-activated protein kinase 12; PIK3C2A, phosphatidylinositol-4-phosphate 3-kinase C2 domain-containing alpha polypeptide; PTPN11, tyrosine-protein phosphatase nonreceptor type 11; RHOA, ras homolog family member A; TLR4, toll-like receptor 4.

We did not find significant anesthesia-associated differences in vesicle size and quantity, but identified a number of vesicular microRNAs that were specifically responsive to propofol but not to sevoflurane. *In silico* analysis then demonstrated several important signaling pathways regulated by these microRNAs from the propofol group, which included apoptosis, migration, epithelial-mesenchymal transition, and cell proliferation of colorectal cancer cell lines. Interestingly, these propofol effects have previously been

described in *in vitro* studies,<sup>31,32</sup> and also in the aforementioned investigation in which colon carcinoma cells were exposed to sera from patients after propofol anesthesia.<sup>34</sup> None of these earlier studies has addressed the possibility that signaling through circulating vesicles may be involved as a mediator of these anesthetic effects.

Our pilot study was purely correlational, however, and could only demonstrate the feasibility of studying and characterizing anesthetic effects on circulating vesicles and associated cell-free microRNAs. Further confirmatory studies need to both include larger patient cohorts and use a true randomized approach carefully balanced for tumor stage and localization. Since, as shown in Supplemental Digital Content 4 (<http://links.lww.com/ALN/C62>), epidural anesthesia does probably not impact tumor-related biofunctions mediated by circulating microRNAs, additional regional anesthesia could be used in consecutive studies. Epidural anesthesia, however, reduces peri- and postoperative opioid requirements which may have independent effects on tumor biology.<sup>41</sup>

There is no clear evidence that circulating vesicles are actually derived from colorectal cancer cells. This issue could potentially be resolved by comparing RNA signatures and protein markers from tumor tissues to those isolated from extracellular vesicles. When considering this approach, one should keep in mind, however, that circulating vesicles are a mixture of vesicles secreted by various tissues, as well as blood cells, to which tumor vesicles may only contribute to a minor extent. It is therefore very likely that the microRNA response assessed in our—and other—studies does not reflect altered vesicle secretion by tumor cells, but rather a global host response of the



**Fig. 6.** Differential impact of propofol and sevoflurane on tumor-related biologic functions. Lower z-scores (blue) indicate a predicted downregulation of the respective pathway, while positive z-scores (orange) indicate a predicted upregulation. Pathway regulation is based on perioperatively altered microRNAs and microRNAs with the same seed sequence. Four sevoflurane-responsive microRNAs (including miR-143-3p and -214-3p) targeted cell death of carcinoma cell lines, whereas all other biofunctions were not affected in the sevoflurane group. In the propofol group, the cell death/apoptosis cluster was predicted to be regulated by nine microRNAs (including miR-143-3p and -214-3p) while the proliferation of carcinoma/colorectal cancer cell lines cluster and the migration/epithelial-mesenchymal transition cluster were targeted by 12 microRNAs (including miR-205-5p and -19b-3p) and 18 microRNAs (including miR-10a-5p and -96-5p), respectively.

individual which could in turn have effects on tumor outcome. Even though we analyzed the potential impact of altered microRNA profiles on carcinoma cells in our *in silico* analysis, they might also differentially impact host immune cells or premetastatic sites in distant tissues, and this possibility should be addressed in further studies. This issue should also be kept in mind when studying the impact of anesthesia on circulating vesicles in cancers other than colorectal cancer or in control groups of patients undergoing noncancer surgery to delineate general effects of anesthesia on circulating vesicles, which are unrelated to the presence of cancer.

Functional studies performed to test the hypothesis that anesthetic agents have differential effects on circulating extracellular vesicles that, in turn, influence signaling pathways involved in tumor progression and metastasis could initially use the aforementioned approach outlined by Xu *et al.*<sup>34</sup> As described in this work for total serum, colon cancer cell lines could be exposed *in vitro* to circulating vesicles isolated from patients undergoing cancer resection using different anesthetic techniques. Assays to quantify cancer cell proliferation and metastatic properties are readily available and well validated.

In summary, our proof-of-concept study describes a validated work package for perioperative isolation of extracellular vesicles and high-throughput sequencing of associated noncoding RNA and provides preliminary evidence that different anesthetic techniques have specific effects on the expression levels of these RNAs. Our *in silico* finding that propofol-based anesthesia may have inhibitory effects on vesicle-mediated signaling pathways involved in tumor cell apoptosis and metastatic properties could form the basis for larger and mechanistically orientated studies.

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### Competing Interests

The authors declare no competing interests.

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## ANESTHESIOLOGY REFLECTIONS FROM THE WOOD LIBRARY-MUSEUM

# A Clumsy Chloroforming in Connecticut: Look What the Cat Dragged In...



Discovered independently in America, France, and Germany in 1831, chloroform was first used as an anesthetic in 1847 in Edinburgh, Scotland. Six years later, in London, chloroform provided obstetric anesthesia for the birth of Queen Victoria's eighth child. Although initially the preferred anesthetic of Europeans because of its potency, portability, and pleasant odor, chloroform fell out of favor in the United States due to its anesthetic mortality. By 1881, however, the *Danbury News* reported a *nonlethal* chloroforming that took place 95 miles east, in Norwich, Connecticut. (Note that clergyman Henry Ward Beecher had hailed the latter city as "the Rose of New England.") The *News* cited how one Norwich couple had administered chloroform to their "extremely fitty" cat to "put it out of its misery." Then, like proper Norwichians, they had planted not any bush, but "a rosebush over its remains." The next morning, their darling kitty "appeared at the door to be let in," all adorned with bits of roses. (Copyright © the American Society of Anesthesiologists' Wood Library-Museum of Anesthesiology.)

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