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Research Article

Association of the immediate perioperative dynamics of circulating DNA levels and neutrophil extracellular traps formation in cancer patients

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Abstract

Objectives: Elevated circulating DNA (cirDNA) concentrations were found to be associated with trauma or tissue damage which suggests involvement of inflammation or cell death in post-operative cirDNA release. We carried out the first prospective, multicenter study of the dynamics of cirDNA and neutrophil extracellular trap (NETs) markers during the perioperative period from 24 h before surgery up to 72 h after curative surgery in cancer patients.

Methods: We examined the plasma levels of two NETs protein markers [myeloperoxidase (MPO) and neutrophil elastase (NE)], as well as levels of cirDNA of nuclear (cir-nDNA) and mitochondrial (cir-mtDNA) origin in 29 colon, prostate, and breast cancer patients and in 114 healthy individuals (HI).

Results: The synergistic analytical information provided by these markers revealed that: (i) NETs formation contributes to post-surgery conditions; (ii) post-surgery cir-nDNA levels were highly associated with NE and MPO in colon cancer [$r = 0.60$ ($P < 0.001$) and $r = 0.53$ ($P < 0.01$), respectively], but not in prostate and breast cancer; (iii) each tumor type shows a specific pattern of cir-nDNA and NETs marker dynamics, but overall the pre- and post-surgery median values of cir-nDNA, NE, and MPO were significantly higher in cancer patients than in HI.

Conclusion: Taken as a whole, our work reveals the association of NETs formation with the elevated cir-nDNA release during a cancer patient's perioperative period, depending on surgical procedure or cancer type. By contrast, cir-mtDNA is poorly associated with NETs formation in the studied perioperative period, which would appear to indicate a different mechanism of release or suggest mitochondrial dysfunction.

Keywords: circulating DNA; perioperative period; neutrophil extracellular traps; neutrophil elastase; myeloperoxidase; minimal residual disease

Introduction

Circulating DNA (cirDNA) has gained considerable attention in recent decades because of its potential for clinical diagnostics in oncology [1–4]. It is already being routinely implemented in cancer theragnostics, for example in the detection of *EGFR* mutations in lung cancer. It also has significant potential for researchers and clinicians in many other areas of cancer management care, including the detection of minimal residual disease (MRD) [5–7], treatment monitoring [8, 9], cancer recurrence surveillance [6, 7], and even cancer screening [10–13].

Treatment of many types of non-metastatic cancers is accompanied by prior surgical intervention. Major surgery shares a number of characteristics with trauma, including tissue damage, inflammation, and activation of the endothelium and immune cells [14, 15]. Cheng *et al*. have documented the increased risk of surgical site infections (SSIs) associated with prolonged operative duration [16]. With regard to certain effects of surgery and anesthetic in the perioperative period, immunosuppression, notably, might cause an increase in the perioperative period (transfusion, hypothermia, anesthetic drugs etc.), resulting in an increase in cancer-related mortality [17], and could be monitored through the analysis of circulating biomarkers in the blood. Lo *et al*. have shown that cirDNA levels increase after trauma and may be a potentially valuable prognostic marker [18]. It is known that in patients with multiple trauma, cirDNA concentration reflects the severity of injury [19]. Surgical trauma has been found to increase circulating nuclear DNA (cir-nDNA) levels for at least 1 week, with a subsequent sharp decrease to basal levels 4 weeks post-surgery, in various cancers [20]. Post-surgery elevation of total cirDNA during this time-frame may cause dilution of circulating mutant DNA

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(cir-mutDNA, 1) among total cirDNA and may affect cir-mutDNA detection sensitivity, as part of MRD detection [20].

Most existing studies reporting on post-surgery cirDNA analysis were based on tracking mutant cirDNA and were carried out after 3 to 4 weeks, given that this time-frame offers adequate guidance to clinicians as to adjuvant therapy, and because this represents the typical delay before the subsequent visit to the oncologist. For these reasons, cirDNA dynamics in the immediate perioperative period have until now been understudied if not neglected. This study focused on the perioperative level of total nuclear cir-nDNA and on circulating DNA of mitochondrial origin (cir-mtDNA), and aims at evaluating the influence of neutrophil extracellular traps (NETs) degradation recently found to be associated with cirDNA release [21].

Neutrophils are the most prevalent innate immune cells, acting as the immune system's first line of defense against pathogens such as bacteria, viruses, parasites, yeast, and fungi [22–24]. Neutrophils kill pathogens by four different processes: phagocytosis, degranulation, cytokine production, and NETosis [25]. The cirDNA produced in the context of cancer-related surgery may have, amongst various possibilities, neutrophils as their source [3, 26]. NETs are an extracellular web-like chromatin decorated with cytosolic and bactericidal granules proteins, such as neutrophil elastase (NE) and myeloperoxidase (MPO), which are released by activated neutrophils during the NETosis phenomenon [27, 28]. Studies show that NETs are capable of sequestering circulating tumor cells and promote tumor cell invasion and metastasis [29–31]. Tohme *et al*. showed a negative correlation between increased NETs formation and disease-free survival in a cohort of patients undergoing curative-intent liver resection for metastatic colorectal cancer [32]. Several studies have demonstrated that various cancer types, such as breast, lung or colorectal cancer, are associated with an increase in circulating neutrophil numbers [33, 34]. Currently, there is an exponential growth in the literature reporting the role of NETs in tumor progression and metastasis [35, 36]. Recent evidence also links NETs to wound healing after trauma or surgery [37]. Furthermore, it is known that general anesthesia (especially when using anesthetic drugs such as opioates, ketamine, volatile agents) accompanied by surgical stress suppresses immunity [38] and could diminish the release of NETs. It has also been shown that anticoagulant drugs inhibit the formation of NETs [39]. We ourselves have shown that cir-nDNA concentrations in newly-diagnosed metastatic colorectal cancer (mCRC) patients are positively associated with NETs protein markers (NE and MPO) [21]. Similarly, we have demonstrated that cirDNA can derive from chromatin or NETs [40].

This prospective multicenter study addresses a number of questions related to perioperative cirDNA analysis in patients with cancer, notably concerning: (i) the effect of anesthesia on cirDNA release; (ii) the impact of trauma/surgery on cirDNA release; (iii) the origin and structure of cirDNA immediately following surgery; (iv) the extent of the association of NETs with cirDNA release; (v) the impact of surgery protocol on systemic inflammation; and (vi) the correlation of NETs and cirDNA markers with recurrence free survival. We used the synergistic analytical information provided by cirDNA and NETs markers in the perioperative period for three cancer types at stages I–III: colon, prostate, and breast cancer.

In the period ranging from the hours immediately after surgery up to 3 days post-operation, we assessed the dynamics (before surgery and after surgery) of the release into the circulation of cir-nDNA, cir-mtDNA, and NETs protein makers such as MPO and NE, as well as the association of cir-nDNA release with NETs formation and cir-nDNA fragmentation by shallow whole genome sequencing (sWGS). The respective levels and correlation of these circulating biological compounds will be compared with a control cohort constituted of healthy individuals (HI) ($n = 114$). This is the first study involving a perioperative follow-up of cirDNA in cancer patients.

cir-mtDNA may cause an inflammatory response by acting as damage-associated molecular patterns, in combination with other circulating NETs by-products (histones, granule proteins like NE or MPO, and structural proteins). Although HI have lower amounts of cir-mtDNA than cir-nDNA, cir-mtDNA could be a marker for inflammatory diseases [41, 42].

Methods

Design of the study

This is an observational, multicenter and prospective study on the dynamics of circulating DNA in plasma for three cohorts of patients with breast, prostate, or colon cancer. This study was approved by the institutional human investigation committee (Comité de Protection des Personnes EST IV, IDRCB 2017- A00950-53, 9 May 2017) and registered prior to enrollment on ClinicalTrial.gov (NCT03284684). It was conducted in two French university centers (Hôpital Carémeau, Nîmes, and Hôpital Lapeyronie, Montpellier, France) and one national French cancer center (Institut Cancer Montpellier, France). The consolidated standard of reporting trials (CONSORT) recommendations were followed for reporting the trial. The analysis was made once patient enrollment had been completed. Before inclusion, the investigator had obtained written informed consent from each subject in accordance with the Declaration of Helsinki.

Patients and cohorts

Specific characteristics of the patient cohorts are described in Table 1. Eligible patients were adults (18–75 years old, >50 kg) with histologically confirmed non-metastatic (>4 weeks prior to surgery) breast (estrogen and progesterone receptor-positive invasive ductal carcinoma), prostate, or colon cancer. The three elective cancer procedures are: quadantectomy, mastectomy, and nodal picking for breast cancer; robotic radical prostatectomy for prostate cancer; and laparoscopic left or right hemicolectomy for colon cancer. Patients had measurable disease as defined by the Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST v1.1), and were not treated by chemotherapy or radiotherapy prior to enrollment. Written, informed consent was obtained from all participants during the anesthesiologist's consultation. According to the French Public Health Code Article L1131-1 and the following articles, no specific ethical approval is required for this type of study.

Exclusion criteria were as follows: refusal to participate, age > 75 years, age < 18 years, weight < 50 kg, emergency or bilateral surgery (breast), open surgery (prostate, colon), cognitive disorders (delirium, dementia), pregnancy (beta human chorionic gonadotropin (hCG) test performed systematically for women < 45 years), patients with history of alcohol or drug abuse (questionnaire), patients unlikely to be fully cooperative during the study, and those who had participated in another study within the previous 30 days. Patients were also excluded when presenting: renal failure (creatinine clearance < 50 $ml.min^{-1}$, Cockroft formula), liver failure (transaminases and/or alkaline phosphatases > 3 times the normal upper value, and/or prothrombin time < 70% of control), acute or chronic

Table 1. Patients and perioperative characteristics. Details of the perioperative characteristics data.

	Breast	Prostate	Colon
	$n = 9$	$n = 10$	$n = 10$
Age, median years (IQR)	52.0 (39.5–64.5)	63.1 (60.5–68.2)	63.1 (55–67.5)
Gender			
Male	0	10 (100%)	6 (60%)
Female	9 (100%)	0	4 (40%)
BMI ($kg.cm^{-2}$)	26.0 (21–28.7)	27.5 (24.7–28.2)	28.0 (22.3–38.0)
ASA physical status, 1/2/3	4(44%)/5(66%)/0	3(30%)/7(70%)/0	4(40%)/5(50%)/1(10%)
Preoperative biological data			
– Creatinine clearance ($ml.min^{-1}$)	78.5 (56.0–84.5)	76.0 (65–84.7)	82.1 (73.0–96.2)
– Hemoglobin ($g.dl^{-1}$)	13.9 (12.5–14.5)	14.6 (12.2–15.4)	13.6 (10.6–14.4)
Primary site of disease and surgical procedure			
Colon left	–	–	10 (100%)
Colon right	–	–	0
Prostate Gleason score			
7	–	4 (40%)	–
6	–	6 (60%)	–
Type of surgery			
Open	9 (100%)	0	0
Closed (robotic)	0	10 (100%)	10 (100%)
Axillary nodal dissection	9 (100%)	–	–
Previous chemotherapy treatment	0	0	0
Synchronous metastasis (yes)	0	0	0
Intra-operative period			
Duration of surgery (min), median (IQR)	75.0 (57.2–97.7)	130.0 (117.5-0.182.5)	240.1 (187.5–262.5)
Blood loss (>250 ml)	0	4 (40%)	0
Blood glycemia (>1.8 g.dl)	0	0	1 (10%)
Anesthetic and analgesia drugs			
Propofol	9 (100%)	10 (100%)	10 (100%)
Ketamine	9 (100%)	10 (100%)	10 (100%)
Neuromuscular agent	0	10 (100%)	10 (100%)
Dexamethasone	9 (100%)	8 (80%)	7 (70%)
Volatile agent	9 (100%)	10 (100%)	10 (100%)
Ropivacaine infiltration	9 (100%)	0	0
Opioid			
Sufentanil	9 (100%)	7 (70%)	8(80%)
Remifentanil	0	3 (30%)	2(20%)
Analgesia intravenous			
Paracetamol	9 (100%)	10 (100%)	10 (100%)
NAIDs	9 (100%)	10 (100%)	7 (70%)
Morphine in PACU	0	1 (20%)	2 (20%)
Hypothermia < 35.5°	1 (11%)	2 (20%)	3 (30%)
Perioperative transfusion	0	1 (10%)	0
Early postoperative outcome (day: 0–5)			
Opioid rescue	0	1 (10%)	0
Surgical adverse event	0	1 (10%)	0
Medical adverse event	0	1 (10%)	0
Length of stay (days), median (IQR)	1 (0–1)	4 (3–5)	4 (3–6)
Tumor			
TNM staging system			
T1N0M0	0	1 (10%)	4 (40%)
T1 N1M0	1 (11%)	0	0
T2N0 M0	2 (22%)	4 (40%)	0
T2N1-3 M0	4 (45%)	0	2 (20%)
T3N0 M0	1 (11%)	3 (30%)	3 (30%)
T3N1-3 M0	1 (11%)	2 (20%)	1 (10%)
T4	0	0	0
Gleason score (prostate)			
<5		0	
5–6	–	4 (40%)	–
7	–	6 (60%)	–
7–8	–	0	–

Data are numbers (percentage), American Society of Anesthesiology (ASA) physical status, inter-quartile (IQR), body mass index (BMI), nonsteroidal anti-inflammatory drugs (NAIDS), TNM Classification of Malignant Tumors (TNM), post anaesthesia care unit (PACU)

respiratory insufficiency (Spo2 < 94% in ambient air), acute (in the 3 months prior to surgery) inflammatory disease or chronic inflammation pathology. Lastly, all patients already taking steroids, narcotics, opioids, oral direct thrombin inhibitor, platelet antiaggregant, or low molecular weight heparin were not included. Patients who had received radiotherapy or chemotherapy preoperatively for the current cancer, or who currently or in the past had had a cancerous lesion or who had received neo-adjuvant therapy (immunotherapy, radiotherapy, chemotherapy) were also excluded.

A total of 30 patients (10 patients x 3 types of cancer) from three clinical centers [CHU of Nîmes, CHU of Montpellier, and Montpellier Cancer Institute (ICM)] were initially included in this study in the period between 22 January 2018 and 11 October 2018. One patient was excluded from the study because he reached the age of 75 years (Fig. 1A). Routine tumor tissue determination and blood collection were conducted in each clinical center. The study incurred no change to the usual patient management, apart from the addition of blood collections during patient care in the hospital [blood collection volume per patient: 45 ml (5 ml x 9 samples) in total in 4 days]. A total of nine blood samples were taken for each patient from 1 day before surgery to the third day after surgery (Fig. 1B).

Surgery, anesthesia, and perioperative care

For surgery, procedures were similar in the three centers and standardized, and were performed by senior surgeon (> 5 years experience). For all patients, surgical skin preparation used chlorhexidine.

For prostate and colon surgery, all procedures were performed under a robotic approach (da vinci X, Intuitive, USA), in a proclive position, with no drain at the end of the procedure.

For breast surgery, there was no particularity, as the procedure was open, using a drain in the nodal axillary compartment at the end.

For the three groups (breast, colon, prostate), the patients were managed with a similar standardized surgical and anesthetic perioperative protocol, which ran from the day before surgery up to hospital discharge. In the preoperative period, all patients were admitted the day before surgery for a blood sample. No premedication was used and preoperative fasting consisted of no food restrictions until 6 h and no drink restrictions until 2 h prior to anesthesia.

In the intraoperative period, patients were admitted to the operating room and intravenous access was secured. Intravenous fluid (Ringer lactate) was used for all patients, with administration restricted to < 2 $ml.kg^{-1}h^{-1}$. Central body temperature was maintained at > 36°C using hot air blankets and fluid warmers in the three groups.

For prostate and colon surgeries, a urinary catheter and a gastric tube were placed after induction of anesthesia and removed at the end of surgery for the gastric tube and at day 2 for the urinary catheter. Induction of anesthesia was performed with intravenous propofol (2–3 $mg.kg^{-1}$), cisatracurium (0.6–1 $mg.kg^{-1}$), and sufentanil (0.25 $\mu g.kg^{-1}$), and maintained with sevoflurane 1%–2% and additional intravenous (IV) sufentanil (5 μg every hour or when variations in mean arterial pressure or heart rate were > 20% from baseline). Intubation and protective mechanical ventilation (low tidal volume: 6–8 $ml.kg^{-1}$, PEEP (positive end-expiratory pressure) level: >5 cmH_2O, <30% FiO_2 (fraction of inspired oxygen) < 50% for SpO_2 (peripheral oxygen saturation) > 94% and 25 < $EtCO_2$ (end-tidal carbon dioxide) < 33 mmHg, plateau pressure: <25 cmH_2O) were used intraoperatively. At 30 min before the end of the surgery, for postoperative pain relief all patients received 1 g of IV paracetamol for 15 min, 100 mg of ketoprofene, 20 mg of nefopam, and ketamine (0.25 $mg.kg^{-1}$). The first two were continued for 48 h (at 6 h intervals for paracetamol and 12 h for ketoprofene). For prevention of postoperative nausea and vomiting, intraoperative injection of IV dexamethasone (8 mg) was performed. Reversal of anesthesia was performed at the end of surgery (when body temperature was > 35.5°), patients were then placed in the postoperative care unit (PACU) for 2 h, and subsequently transferred to the hospital ward.

For breast surgery, the preoperative period and induction were performed as previously described for other groups, but neuromuscular agents and fluid were not used. At the end of surgery of the breast, local anesthetic (ropivacaine) was injected over the skin incision by the surgeon to prevent opioid rescue (Table 1). Pain relief and prevention of postoperative nausea and vomiting and care in PACU were similar to other groups (see above).

For the postoperative period, early oral intake on the day of surgery, no fluid administration, and sitting up and walking on day 1 were observed for all patients. Intra- or post-operative transfusion was performed only if the hemoglobin level was < 7 $g.dl^{-1}$. From surgery to day 2, the analgesia protocol was standardized: paracetamol (1 g, at 6-h intervals), ketoprofene [100 mg at 12-h intervals and, if necessary, in the event of nausea or vomiting, ondansetron (IV, 8 mg) or droperidol (IV, 1.25 mg)]. From surgery to day 2: all patients received a similar morphine rescue analgesia protocol, as previously described by the authors [43].

For thromboprophylaxia, patients in the colon and prostate groups received enoxaparin (subcutaneous, 40 units) for up to 12 h after the end of surgery, and then each day at 8 pm, whereas the breast group did not receive enoxaparin.

Surgery was performed under coelioscopy for hemicolectomy using low intra-abdominal pressure (<15 mmHg) and lateral skin incision <5 cm at the end of the procedure for tumor extraction (under plastic bag protection); similarly for robotic procedures (prostate surgery), with tumor extraction through umbilical skin incision.

Clinical assessments

The characteristics of the patients, anesthesia (duration, drugs, fluid, and blood transfusion), perioperative treatment (analgesia, enoxaparin), and surgery (type and duration) were recorded. All intraoperative parameters (heart rate, blood pressure, oxygen saturation, and central temperature) and intraoperative adverse outcomes (blood transfusion, conversion to open surgery) were recorded. All medical or surgical complications (including readmission) were recorded throughout the study period. Cancer type was confirmed with a preoperative biopsy, and cancer staging was confirmed after surgery (TNM staging system). After 3 months and 1 year, patients were contacted to record recurrence.

Healthy individuals

We also analyzed the blood samples collected in ethylenediaminetetraacetic acid (EDTA) tubes (V = 6 ml) from 114 HI (59 men and 55 women) from the Etablissement Français du Sang (EFS), which is Montpellier's blood transfusion center (Convention EFS-

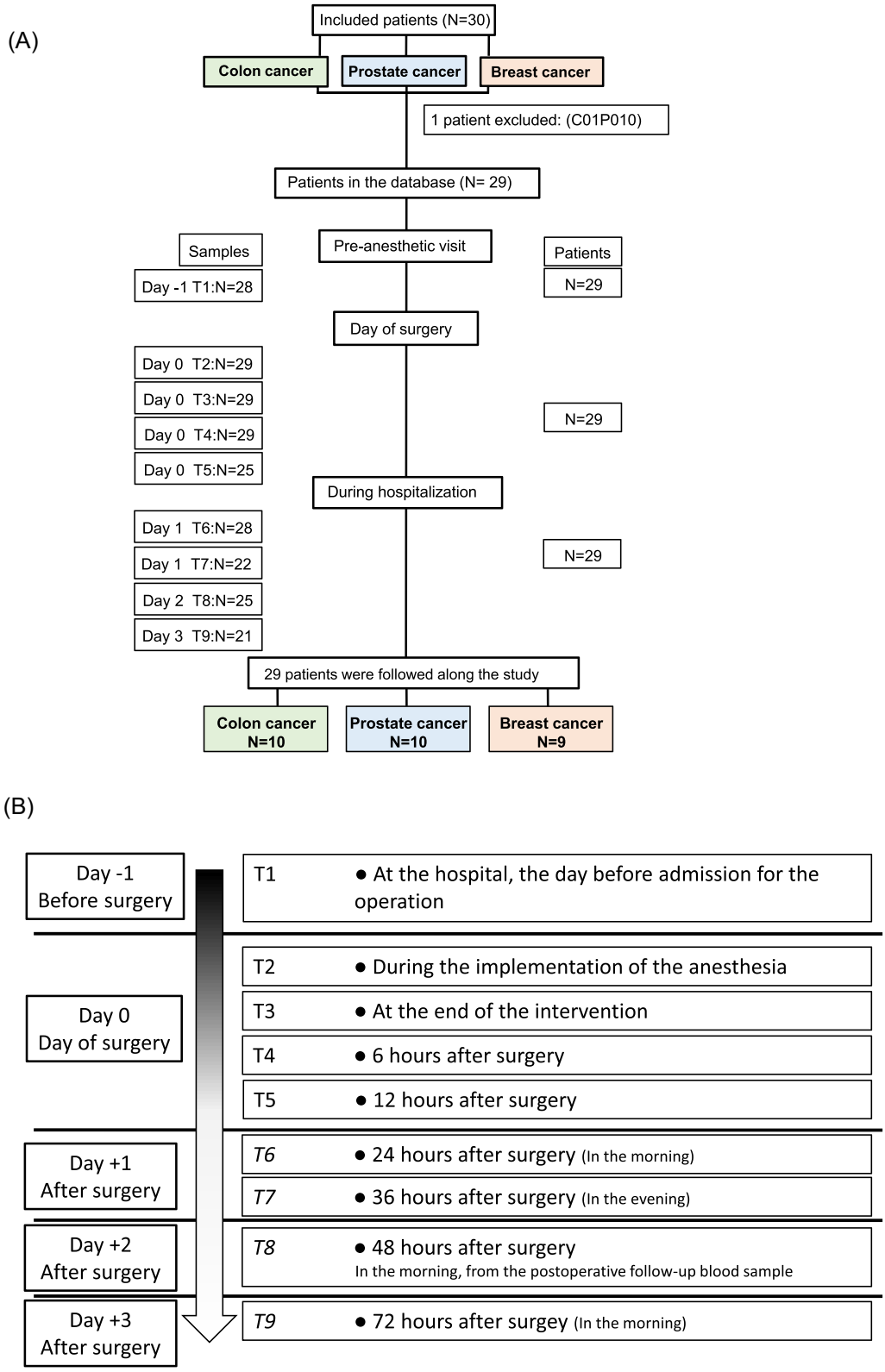

Figure 1. Flowchart of the study. (**A**) Thirty patients were included, with 29 patients followed post-surgery, including patients with colon cancer ($n = 10$), prostate cancer ($n = 10$), and breast cancer ($n = 9$). (**B**) Time points of the perioperative blood collections for cirDNA and NETs marker analysis during patient's management care. Blood samples from patients were collected in 5 ml EDTA tubes 24 h before surgery (Day −1, T1), on the day of surgery (Day 0, T2 to T5), on the day following surgery (Day +1, T6 and T7), two days after surgery (Day +2, T8), and finally 3 days after surgery (Day 3, T9).

PM N° 21PLER2015-0013). These samples were initially screened (virology, serology, immunology, blood numeration) and ruled out whenever any abnormality was detected.

Plasma isolation and cirDNA extraction

Blood samples from patients were collected in 5 ml EDTA tubes and centrifuged at 1600g at 4C° for 10 min directly at the hospital laboratory after collection. Then plasma samples for cirDNA analysis were stored at −80°C and transferred between institutions on dry ice. The plasma isolation protocol as well as the handling and storage conditions have been described previously [44]. Plasma samples were centrifuged at 16 000g at 4°C for 10 min. Supernatant was immediately used for cirDNA extraction and stored at −20°C.

cirDNA (cir-nDNA and cir-mtDNA) was extracted in an elution volume of 80 μl from 0.2 ml of isolated plasma using the Maxwell RSC ccfDNA plasma kit (Promega Corporation, Madison, WI, USA), in accordance with the pre-analytical guidelines we have described previously [44]. The Maxwell RSC ccfDNA plasma kit has the advantage of using a fully automated, magnetic beads-based protocol, which avoids human manipulation errors [45]. DNA extracts were kept at −20°C until use, or were used immediately. In total, we analyzed 229 serial plasma samples from 29 cancer patients.

Real-time quantitative PCR analysis

Analysis of cirDNA (both nuclear and mitochondrial DNA) was done by IntPlex®, an allele-specific blocker quantitative PCR (ASB Q-PCR), which we have described previously [9, 46–48]. Concentrations of cir-nDNA were analyzed using a 67 bp long wild-type sequence of the *KRAS* gene. The cir-mtDNA was analyzed using a 67 bp short fragment of the mitochondrial cytochrome oxidase III gene (MT-CO3). *KRAS* primers have previously been used in the IntPlex® diagnostic system [46]. Primers for the cir-mtDNA have also shown their effectiveness previously [44, 49]. The Q-PCR assays were performed according the MIQE guidelines [50]. A coefficient of variation of 24% for Q-PCR quantification of the cir-nDNA was determined when considering variation due to the extraction procedure and analysis in the same plate [46]. Q-PCR amplifications were carried out in at least triplicate, in a 25 μl volume, on a CFX96 instrument using the CFX manager software (Bio-Rad, Hercules, CA, USA). Each Q-PCR reaction was composed of 12.5 μl of Sso advanced mix Sybr Green (Bio-Rad), 2.5 μl of free water (Qiagen, Hilden, Germany), 2.5 μl of forward and reverse primers (0.3 pmol/ml), and 5 μl of template. Thermal cycling comprised of three repeated steps: a hot-start activation step at 95°C for 3 min, followed by 40 cycles of denaturation–amplification first at 95°C for 10 s, then at 60°C for 30 s. Melting curves were investigated by increasing the temperature from 60 to 90°C with a plate reading every 0.2°C. Standard curves were performed for each run with a genomic extract of the DiFi cell line at 1.8 ng/μl of DNA. Validation of Q-PCR amplification was made by melt-curve differentiation. Note that we quantify cirDNA from different origins, and to ease manuscript reading we use the "cir-nDNA" abbreviation for circulating cell-free nuclear DNA and the "cir-mtDNA" abbreviation for circulating mitochondrial DNA, which showed very different physical characteristics and biological stability [1, 51].

Library preparation and cir-nDNA analysis by sWGS

In this study, we analyzed DNA size profiles using sWGS, which allows the accurate estimation of the number of fragments of a certain length with a resolution of 1 bp. Double strand DNA prepared (DSP) libraries were prepared with the NEBNext® Ultra™ II kit. For library preparation, a minimum of 1 ng of cirDNA was engaged without fragmentation, and the kit providers' recommendations were followed. Briefly, for DSP with the NEB kit, Illumina paired-end adaptor oligonucleotides were ligated on repaired A-tailed fragments, then Solid Phase Reversible Immobilization (SPRI) purified and enriched by 11 PCR cycles with unique dual indexes (UDI) primers indexing, and then SPRI purified again. The SPRI purification was adjusted to keep the small fragments around 70bp of insert. Finally, the libraries to be sequenced were precisely quantified by Q-PCR, to ensure that the appropriate quantity was loaded on to the Illumina sequencer, in order to obtain a minimum of 1.5 million clusters.

All libraries were sequenced on NovaSeq (Illumina) as paired-end 100 reads. Image analysis and base calling was performed using Illumina Real Time Analysis with default parameters. The individual barcoded paired-end reads were trimmed with Cutadapt v1.10 to remove the adapters and discard trimmed reads shorter than 20 bp. Trimmed fastq files were aligned to the human reference genome (GRCH38) using the Modal EM (MEM) algorithm in the Burrows–Wheeler Aligner v0.7.15. The insert sizes were then extracted from the aligned bam files with the total length (TLEN) column for all pairs of reads having an insert size between 0–1000 bp.

Human MPO and NE assays

MPO and NE levels were measured using enzyme-linked immunosorbent assays according to the manufacturer's standard protocol (Duoset R&D Systems, DY008, DY3174, and DY9167-05). Briefly, capture antibodies were diluted at the working concentrations in the reagent diluent (RD) provided in the ancillary reagent kits (DY008), and coated overnight at room temperature (RT) on 96-well microplates at 100 µl per well. Then, capture antibodies were removed from the microplate and wells were washed three times with 300 µl of wash buffer (WB). Microplates were incubated at RT for 2 h by adding 300 µl of RD to each well. RD was removed from the microplates and wells were washed three times with 300 µl of WB. Then, 100 µl of negative controls, standards, and plasma samples (diluted 1/10) were added to the appropriate wells for 1 h at RT. Samples, controls, and standards were removed from the microplates and wells were washed three times with 300 µl of WB. Detection antibodies were diluted at the working concentrations in the RD and added at 100 µl per well for 1 h at RT. Detection antibodies were removed from the microplates and wells were washed three times with 300 µl of WB. Then, 100 µl of streptavidin-horseradish peroxidase was added to each well and the microplates were incubated at RT for 30 min. The wash was repeated three times. Finally, 100 µl per well of substrate solution was added and incubated for 15 min, and the optical density of each well was read immediately at 450 nm with the PHERAstar FS instrument using the PHERAstar control software.

Statistical analysis

The Mann–Whitney U test was used for non-parametric data. Correlation analysis was performed using the Spearman test (Graph Pad Prism 8.3.1 software). A probability < 0.05 was considered to be statistically significant; $^{*}P < 0.05$, $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

Results

Dynamics of cir-nDNA, NE, and MPO concentrations during the perioperative period

The dynamics of total cir-nDNA concentration in plasma of patients with breast ($n = 9$), prostate ($n = 10$), and colon ($n = 10$) cancer are presented in Fig. 2. Data on the cir-nDNA dynamics for each type of cancer similarly revealed two distinct phases: (i) a stable level of cir-nDNA during the 24 h before surgery (T1) to the implementation of anesthesia (T2), followed by (ii) the most significant increase in cir-nDNA, peaking at the end of surgery (T3). From 6 h (T4) to 72 h (T9) after surgery, most samples showed cir-nDNA increases to various extents in colon cancer patients. In prostate cancer patients, cir-nDNA levels were observed in smaller amounts and with higher heterogeneity, whereas in breast cancer patients cir-nDNA concentrations showed a slight increase or remained stable (Fig. 2A–D).

Median values of cir-nDNA after surgery are significantly higher than before surgery for colon and prostate cancer patients, while their slight increase after surgery observed in breast cancer patients was not significant (Fig. 2E and H). For each cancer type, the pre-surgery cir-nDNA values were rather homogenous, with mean levels of 12.21, 9.81, and 12.56 ng/ml, for colon, prostate, and breast cancer, respectively) (Fig. 2H). We observed that most increases in cir-nDNA concentration by the end of the surgery (T3) were followed by decreases after 6 h (T4) (supplememtary Fig. S1, see online supplementary material). Interestingly, the peak observed at the end of surgery (T3) is less prominent for patients operated on for breast cancer than for patients operated on for colon or prostate cancer (Fig. 2H).

Then, we compared the median values of cir-nDNA, NE, and MPO in cancer patients ($n = 29$) with those in healthy individuals ($n = 114$) (Fig. 3). Data revealed that cir-nDNA, NE, and MPO levels in cancer patients are higher both pre- and post-surgery, as compared to healthy individuals (Fig. 3A–C, and supplementary Fig. S2, see online supplementary material). For both colon and prostate cancer patients, the dynamics of the three markers are similar, showing an increase with time, post-surgery (Fig. 3A and B). For breast cancer patients, in contrast, the dynamics of the three markers are clearly different, showing no increase with time (Fig. 3C).

Association of cir-nDNA levels with NETs protein markers during the perioperative period

We used a Spearman correlation test to assess the association between cir-nDNA concentrations and NETs proteic markers (Fig. 4). Data revealed that only NE and MPO levels are associated in HI, $r = 0.37$ ($P \leq 0.0001$). Moreover, cir-nDNA levels are not associated with NET protein maker levels in HI (Fig. 4A). In cancer patients, there is a significant positive association between NE and MPO before surgery (T1, $n = 25$), at the end of surgery (T3, $n = 28$), and 72 h after surgery (T9, $n = 22$), $r = 0.77$ ($P \leq 0.0001$), $r = 0.62$ ($P \leq 0.0001$), and $r = 0.79$ ($P \leq 0.0001$), respectively (Fig. 4B–D). Data revealed significant statistical positive associations between MPO and cir-nDNA levels at the end of surgery and 72 h after surgery, with $r = 0.40$ ($P \leq 0.01$) and $r = 0.44$ ($P \leq 0.01$), respectively. Data showed a similar association in both cohorts between NE and cir-nDNA levels at the end of surgery and 72 h after surgery, with $r = 0.49$ ($P \leq 0.001$) and $r = 0.46$ ($P \leq 0.01$), respectively (Fig. 4C and D). Moreover, data revealed that the positive associations between NE and MPO are stronger in cancer patients than in HI, whether before or after surgery (Fig. 4A–D).

The positive associations between NE and MPO levels in cancer patients remain high at all-time points before, during, and after surgery (Fig. 4E). Before surgery (T1), data showed positive associations between cir-nDNA and NETs protein marker levels in colon and breast cancer, but cir-nDNA and MPO were not associated in prostate cancer patients. In the period from the implementation of the anesthesia (T2) to 6 h after the surgery (T4), data revealed positive associations between cir-nDNA levels and NE and MPO levels in the three types of cancer. From 36 h after surgery (T7) to 72 h after surgery (T9), the cir-nDNA levels were associated with NE and MPO levels in colon cancer patients, with $r = 0.60$ ($P < 0.001$) and $r = 0.53$ ($P < 0.01$), respectively. In the same period, the cir-nDNA levels were no longer associated with NE and MPO levels in prostate cancer patients, with $r = -0.02$ and $r = 0.15$, or in breast cancer patients, with $r = -0.03$ and $r = 0.01$ (Fig. 4E).

Dynamics of cir-mtDNA during the perioperative period

Except for two patients (C3 and C9), data show some homogeneity and a low variation in the dynamics of cir-mtDNA in colon cancer patients (Fig. 5A). Data showed very high heterogeneity and a high variation in cir-mtDNA levels for prostate cancer patients, with a peak still observable at T3–T5 (Fig. 5B). Prostate cancer patients had higher values of cir-mtDNA than patients with colon (Fig. 5A) and breast (Fig. 5C) cancer. Cir-mtDNA levels in breast cancer patients were mostly stable, with the exception of one patient (B3) (Fig. 5C). The dynamics of cir-nDNA and cir-mtDNA levels in colon cancer patients were similar up to 36 h after surgery (T7), but diverged subsequently, with an increase in cir-nDNA. In prostate cancer patients, the dynamics of cir-nDNA and cir-mtDNA levels are comparable with those of colon cancer patients, but show a lower difference after T7 (Fig. 5D). The dynamics of cir-nDNA and cir-mtDNA levels in breast cancer patients are similar up to 72 h after surgery (T9) (Fig. 5F). Data showed an increase in cir-mtDNA levels in prostate cancer patients after 24 h of surgery, while this increase did not exist in patients with other types of cancer (supplementary Fig. S3, see online supplementary material).

No association was seen between the levels of cir-mtDNA and the NETs marker levels in prostate and breast cancer patients during the perioperative period (supplementary Figs S4 and S5, see online supplementary material). In contrast, for colon cancer patients data revealed significant positive associations between cir-mtDNA concentrations and NE and MPO, both before surgery (T1) (with $r = 0.70$ and $r = 0.72$, respectively) and until 72 h after surgery (T9) (with $r = 0.40$ and 0.41, respectively) (supplementary Fig. S6, see online supplementary material). We investigated the cir-mtDNA to cir-nDNA ratio (called MNR) in patients with all three cancer types. Data did not show any positive association between MNR and NE, MPO, and cir-nDNA levels in cancer patients, whether before or after surgery (supplementary Figs S4–S6). However, data showed positive associations between MNR and cir-mtDNA levels in cancer patients, both before and after surgery (supplementary Figs S4–S6).

Fragmentomics analysis of cir-nDNA by sWGS

Patients analyzed by sWGS had low levels of cir-nDNA, NE, and MPO before surgery, but high levels of these markers 72 h after surgery. As determined by sWGS, the length of most cir-nDNA fragments in cancer patients and HI are ~166 bp (supplementary Fig. S7A, see online supplementary material). From T2 to T9, for

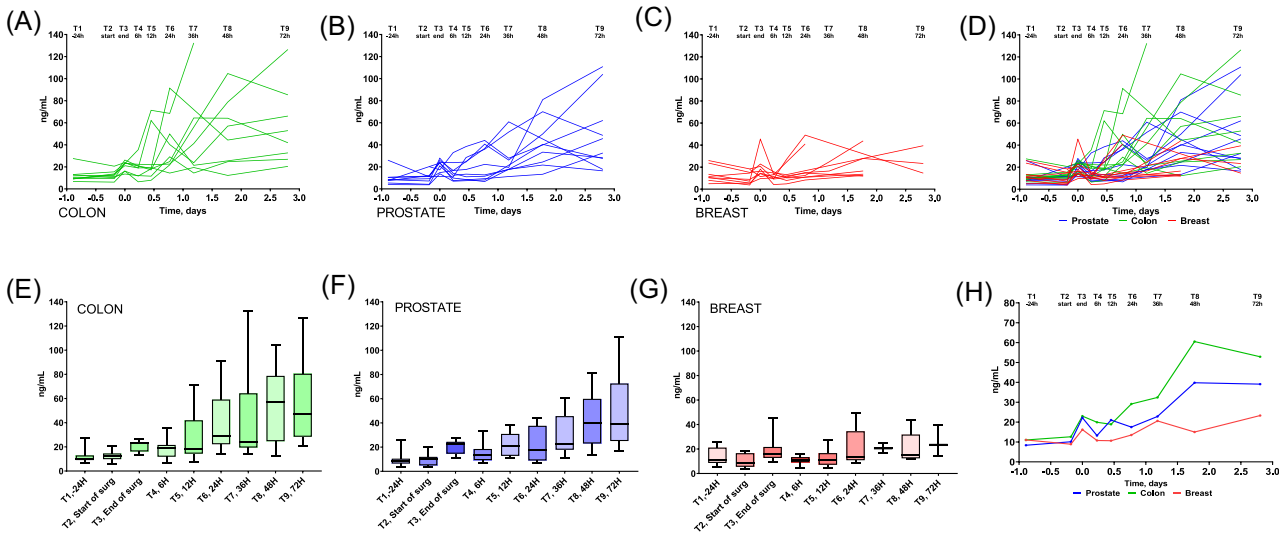

Figure 2. Dynamics of cir-nDNA plasma concentration (ng/ml plasma) before, during, and after surgery. Individual values of cir-nDNA are given for patients with (**A**) colon, (**B**) prostate, (**C**) breast, and (**D**) all types of cancer. Mean values of cir-nDNA are shown for (**E**) colon, (**F**) prostate, (**G**) breast, and (**H**) all types of cancer. The boxplot shows medians with IQR (Q1–Q3), and whiskers represent the minimum and maximum values. IQR: Interquartile range.

the patients C4 and C5, the fraction of short (<151 bp) cir-nDNA fragments increased from 21% to 33% and from 21% to 23%, respectively (supplementary Fig. S7B and C). While the cir-nDNA size profile from plasma samples before surgery (T2) of both these patients had similar characteristics when all parameters studied were taken into account, this was not the case when comparing the cir-nDNA from plasma samples taken 72 h after surgery (T9) (supplementary Fig. S7B and C). Details of the parameters studied are described in Fig. S7B and C. In both illustrative examples, we observed a shift to the lower fragment size in cancer patients, as compared to the HI size profile. This is in accordance with previous reports [52, 53].

Relationship between relapse and increases in cir-nDNA, NE, and MPO during the perioperative period

Supplementary Fig. S8, see online supplementary material, shows longitudinal analyses of cir-nDNA, NE, and MPO from six illustrative patients with a colon cancer: patients C1 (relapsed 1 year after surgery), C4 (relapsed 3 months after surgery), and C7 (relapse 6 months after surgery) experienced a relapse within 2 years of postoperative follow-up. Relapsing patients appear to show higher cir-nDNA, NE, and MPO levels after surgery than patients who do not relapse within the 2 years of postoperative follow-up. Patients who relapsed ($n = 3$) had a ratio of 1.9-fold, 5.5-fold, and 6.8-fold higher cir-nDNA levels at T3, T7, and T9, as compared to pre-surgery levels. By contrast, patients who did not relapse had a ratio of 1.2-fold, 2.4-fold, and 2.8-fold higher cir-nDNA levels at T3, T7, and T9, as compared to pre-surgery levels. Relapsing patients showed a ratio of 1.3-fold, 3.0-fold, and 2.4-fold higher NE levels at T3, T7, and T9, as compared to pre-surgery levels, while patients who did not relapse had a ratio of 1.3-fold, 1.4-fold, and 1.2-fold higher NE levels at T3, T7, and T9, as compared to pre-surgery levels. For MPO levels, relapsing patients showed a ratio of 1.2-fold, 2.0-fold, and 1.9-fold higher levels at T3, T7, and T9, as compared to pre-surgery levels, while patients who did not relapse had a ratio of 1.3-fold, 1.4-fold, and 1.4-fold more at T3, T7, and T9, as compared to pre-surgery levels.

Discussion

In this study, plasma concentrations of cir-nDNA, cir-mtDNA, and the NETs protein markers are higher after surgery than before surgery in most patients with colon, prostate, and breast cancer. In addition, data revealed that (i) NETs formation is implicated in post-surgery conditions; (ii) post-surgery cir-nDNA levels were associated with NETs proteic markers in colon cancer, but not associated with NETs markers 36 h after surgery in prostate and breast cancer; and (iii) each tumor type showed a specific pattern of cir-nDNA and NETs marker dynamics, with breast cancer differentiating the most, showing a low increase with time of cir-nDNA, NE, and MPO, 6 h after surgery (supplementary Fig. S10, see online supplementary material). In addition, both pre- and post-surgery median values of cir-nDNA, NE, and MPO were significantly higher in cancer patients than in HI ($n = 114$).

Numerous previous studies have shown that the concentration of cir-nDNA is lower in healthy individuals than in cancer patients at diagnosis [4, 44, 46, 54, 55]. However, several studies have shown that cir-nDNA can also be elevated in HI as a result of physical activities [56]. Meddeb *et al.* showed that cirDNA levels in healthy individuals can also vary with age and gender. cir-nDNA levels may be elevated in pathological conditions such as cancer [46, 47], infectious diseases, and inflammatory diseases [53, 57]. Over the past several decades, it has been widely demonstrated that circulating DNA derived from tumors carries genetic and epigenetic alterations of the primary tumor and associated metastases. Building on this, the detection of cir-mutDNA in plasma could serve as a liquid biopsy in oncology, with numerous potential diagnostic applications. Among such potential applications in oncology, the detection of MRD through cirDNA analysis would appear to be a promising strategy towards a guided deintensification of chemotherapy, as has been previously reported [58, 59].

While surgery is generally considered a curative intervention, the perioperative phase nonetheless represents a window of vulnerability which offers residual disease opportunities to spread. Recurrent disease occurs in up to 30% of patients with colorectal cancer after initial curative surgery, with 70% of these cases typically occurring within 2 years of surgery; current understanding

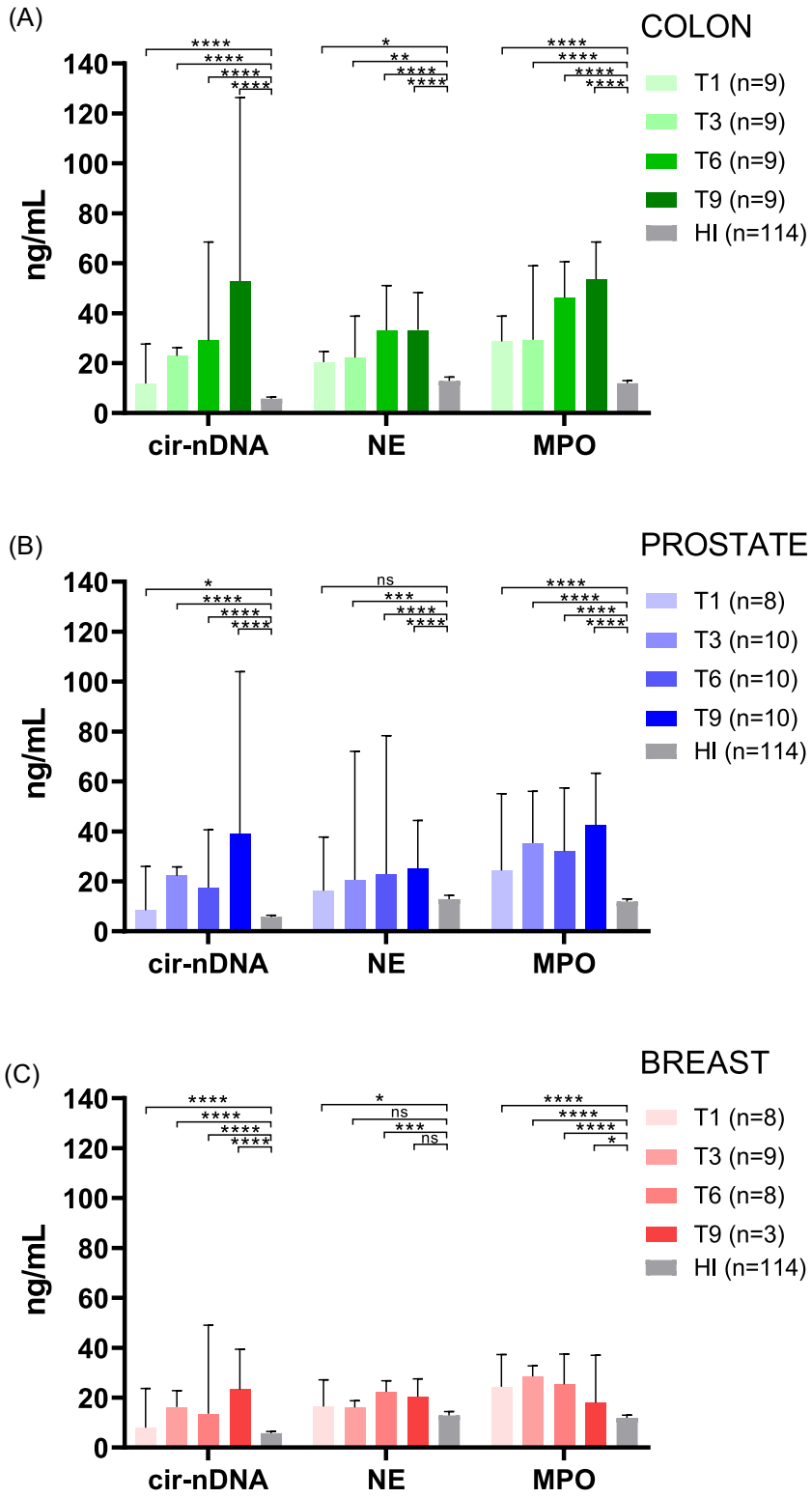

Figure 3. Comparison of cir-nDNA, NE, and MPO concentrations before surgery (T1), at the end of surgery (T3), and after surgery (T6, T9), of patients with cancer, with values of HI ($n = 114$). Histograms represent medians with the 95% confidence interval (CI) of HI and for patients with (**A**) colon , (**B**) prostate , (**C**) breast cancer. Test for comparison of median between groups is done by the Mann–Whitney test. A probability < 0.05 was considered statistically significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$; ns: non-significant.

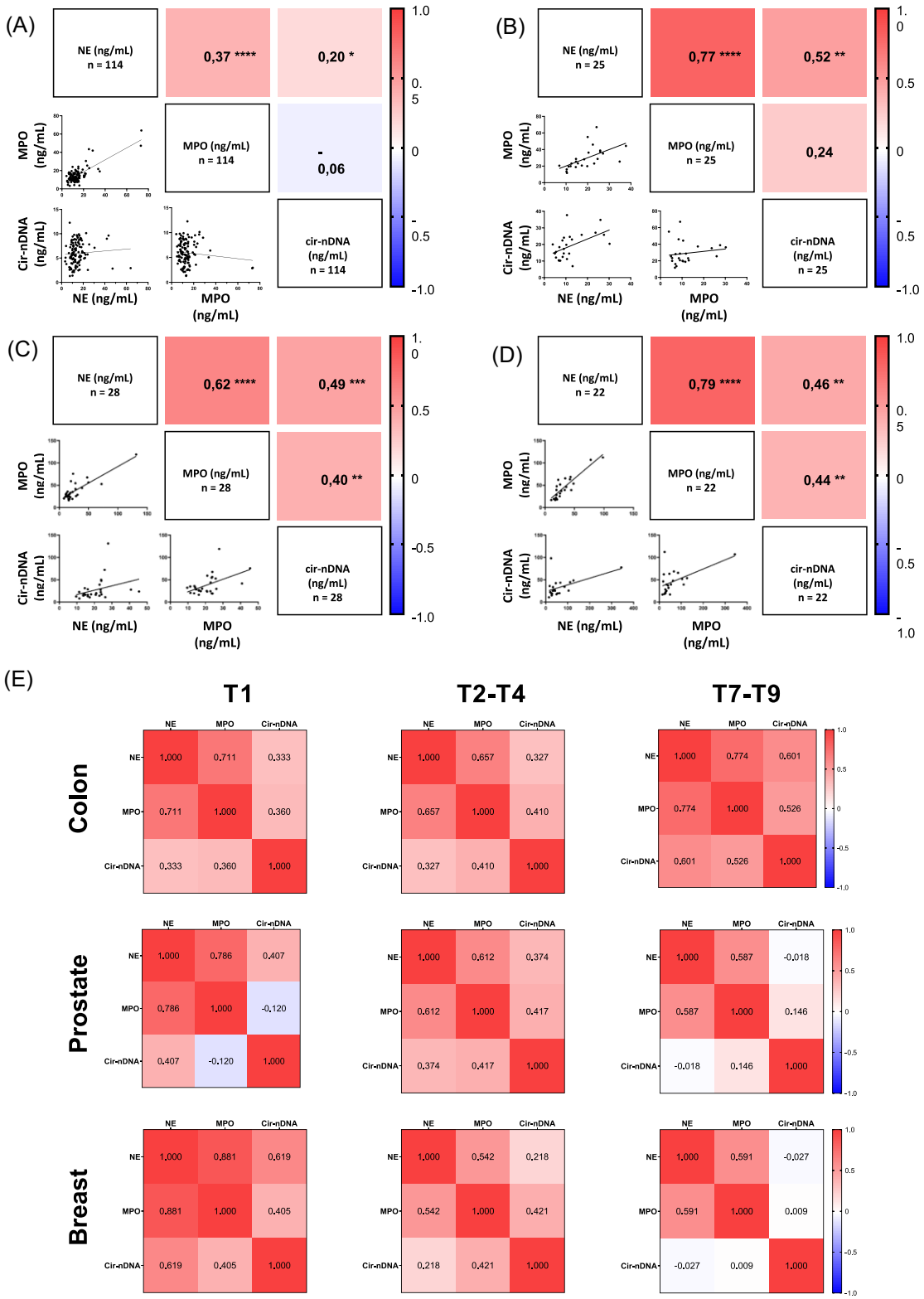

Figure 4. Correlation matrix of cir-nDNA, MPO, and NE concentrations (ng/ml plasma) in cancer patients ($n = 29$) and in HI ($n = 114$). Spearman correlation matrix of cirDNA, MPO, and NE concentrations (ng/ml plasma) in (**A**) HI ($n = 114$), (**B**) in all cancer patients before surgery (T1, $n = 25$), (**C**) in all cancer patients at the end of surgery (T3, $n = 28$), (**D**) in all cancer patients 72 h after surgery (T9, $n = 22$), and (**E**) in colon, prostate, and breast cancer patients before surgery (T1), from the implementation of the anesthesia to 6 h after surgery (T2–T4), and from 36 to 72 h after surgery (T7–T9). Heatmap shows the strength of relationships by Spearman's correlation analysis (red: positive correlation; blue: negative correlation). A probability < 0.05 was considered statistically significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

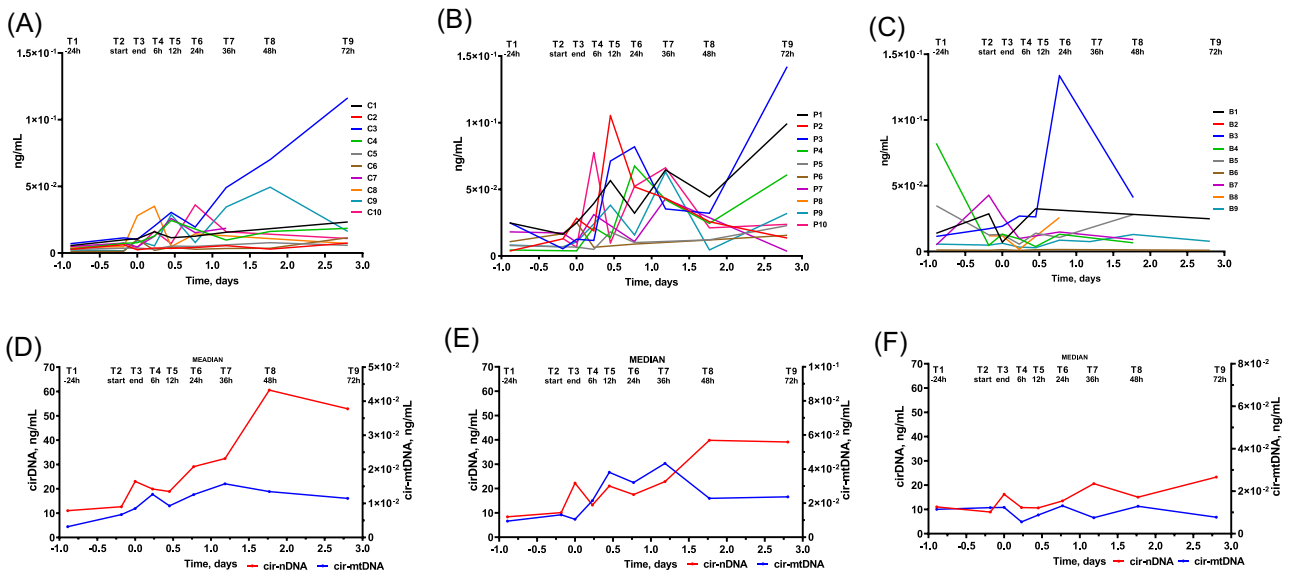

Figure 5. Dynamics of cir-mtDNA plasma concentration (ng/ml plasma) before, during and, after surgery. Individual values of cir-mtDNA are given for patients with (**A**) colon, (**B**) prostate, and (**C**) breast cancer. Median values of cir-mtDNA (blue lines) and cir-nDNA (red lines) are shown for (**D**) colon, (**E**) prostate, and (**F**) breast cancer patients.

points to the acceleration of this phenomenon by perioperative inflammation [60]. Higher concentrations of cirDNA have been observed in those with early disease recurrence as compared to those who were disease-free at 2 years. To reduce the incidence of recurrence, a strategy has been proposed which includes: (i) the use of regional rather than general anesthesia, to attenuate the sympathetic nervous system's response during surgery; (ii) a reduction of opioid requirements, thus diminishing their direct immunosuppressant effects (natural killer cells), and (iii) the provision of anti-tumor and anti-inflammatory effects by means of systemic local anesthetic action, specifically through the use of lidocaine, ropivacaine, and bupivacaine [61].

Previous studies have generally operated under the assumption that, excepting cases of recurrence, cir-mutDNA and cirDNA decreases following surgery [2, 4]. Nonetheless, Wei *et al.* showed that the cirDNA levels are stable at different time-points up to 6 months after surgery, and remain at levels higher than those of high-risk healthy subjects [62]. The perioperative phase represents a vulnerable state where minimal residual disease (residual circulating cancer cells or residual cir-mutDNA) may be inadequately eliminated by the host immune system, which may be weakened by surgical stress and the pharmacologic effects of various cancer-fighting drugs [63, 64]. As a result, the perioperative period is considered to be a crucial juncture at which tumor cells may be released into the bloodstream, which may in turn result in metastasis development. In addition, surgery-induced trauma could provoke an immediate and significant immune response [15]. Studies have shown that activated platelets may coat tumor cells and protect them from detection and clearance by NK cells [65, 66]. Activated platelets are not the only blood cells that could capture the tumor cells and/or circulating tumor DNA in the bloodstream. Systemic immune inflammation, as determined by the neutrophil–lymphocyte ratio (NLR), has been found to predict survival outcome in patents with colorectal or breast cancer [67]. NLR may have prognostic value regarding overall, cancer-free and cancer-specific survival [68].

Moss *et al.*'s methylome study showed for the first time that ~80% of cirDNA is derived from white blood cells in healthy subjects [69]. They postulated that in cancer conditions, by contrast, cirDNA is mainly released from the tumor itself and by the tumoral microenvironment. In a study that contradicted such expectations, Mattox *et al.* found no evidence that neoplastic cells or surrounding non-neoplastic epithelial cells are major sources of elevated cirDNA in cancer patients [26]. In 2015, our own team was among the first to postulate that cir-nDNA found in cancer patient plasma may significantly originate from NETs [3]. We subsequently observed that the concentrations of cir-nDNA in newly diagnosed mCRC patients were positively associated with NETs protein markers (NE and MPO) [21]. This work showed that a proportion of cirDNA in cancer patients may derive from immune cells, in particular neutrophils. Two further recent studies have employed the analysis of cir-nDNA methylation to confirm that three-quarters of the cir-nDNA found in patients with different types of cancer derives from white blood cells with a predominance of granulocytes (particularly neutrophils) and erythrocyte progenitors. [26, 70]. It should be noted, furthermore, that the tumor tissue environment of the breast, the colon, and the pancreas are significantly different. Thus, the much lower cirDNA or NETs protein marker levels found in certain types of cancer could be explained in part by the smaller proportion of vascular or connective tissues, which might influence the inflammatory process, for instance.

In light of these findings, we suggest that a great part of the cir-nDNA found immediately after surgery in colon, prostate, and breast cancer comes from NETs, and so from activated neutrophils. We also found that cir-nDNA levels in HI was not associated with NETs markers. These results confirm our previous work on the origin of cir-nDNA in cancer patients and HIs [21]. To date, this is the first study to show that elevated cir-nDNA levels may be derived from NETs in the perioperative period. A comprehensive understanding of cirDNA dynamics and origins in the perioperative period is indispensable to identify the optimal time-point at which blood samples should be taken for further cirDNA analysis (for MRD detection, for instance). We have also demonstrated that NETs may persist after the acute infection of COVID-19, augmenting disease severity where the level of NETs is elevated [53].

While this study focuses on the immediate perioperative period, an investigation that extends over a period of 1 month is needed to examine more fully the dynamics of this biomarker. Numerous studies have shown the relevance (prior to the advent of imaging techniques) of cir-mutDNA as a postoperative marker of the MRD, in order to predict potential recurrence [58, 71–73]. To prevent the dilution of cir-mutDNA among total cirDNA, which might impact the capacity of next generation sequencing to identify cir-mutDNA, Henriksen *et al*. recommended performing cir-mutDNA for MRD detection in the fourth week post-surgery [20]. Currently, no standardized procedures or guidelines exist that suggest the ideal time-point at which blood should be collected for a postoperative evaluation of total cirDNA, even if the required postoperative delay for blood collection can be inferred from the negative detection of cir-mutDNA. Given that NETs have been implicated in cancer progression [32, 35, 74], we analyzed those patients in our study who suffered relapse. Thus, as mentioned above, there is a need to investigate further the dynamics of cirDNA release and NETs formation in order to better characterize postoperative inflammation and circumscribe the prognostic potential of those markers regarding recurrence in non-metastatic solid cancers. One of the goals of this study is to initiate experimental groundwork that would improve assessments of the impact of perioperative anesthesia on tumor recurrence and metastasis. In that context, this study has shown that the cir-nDNA release begins just after surgery and continues to gradually increase for at least 3 days. Furthermore, clear and specific patterns were revealed for each tumor type when post-surgery levels of cir-nDNA, NE, and MPO were compared.

Four (13.8%) out of the 29 patients analyzed in this study relapsed within 2 years of postoperative follow-up (3 colon cancer patients and 1 prostate cancer patient). These two types of cancer showed higher levels of the three markers, a similar increase in the levels of these markers with time, and a continuous increase in NETs formation for up to 36 h (for prostate) and 72 h (for colon) after surgery. Patients with colon cancer who experienced early relapse (patients C4 and C7, who did so within 3 and 6 months of surgery, respectively) had higher cir-nDNA, NE, and MPO levels at 72 h following surgery than patients who experienced a late relapse (patient C7, relapse after 1 year of follow-up) and patients without relapse within 2 years of post-operative follow-up. Due to the limited sample sizes, extreme caution must be exercised in the interpretation of these findings.

Data revealed a peak in cir-DNA levels immediately at the end of surgery (T3), for all three cancer types. Since this peak is readily observable, it is reasonable to think that the mechanism of the release of extracellular DNA in the blood circulation at the end of the intervention differs from that occurring between 6 h and 3 days post-surgery. It might be explained by an early cirDNA release provoked by cell degradation resulting from trauma, or by tissue damage, or by some unknown effect of anesthesia. It could alternatively be explained by the duration of the various surgical operations. In this study, the median duration of the intervention was ∼2.5 h for patients with breast cancer (quadantectomy, mastectomy, and nodal picking), 4 h for colon cancer patients (laparoscopic left or right hemicolectomy), and 5 h and 42 min for prostate cancer patients (robotic radical prostatectomy) (supplementary Fig. S9, see online supplementary material). Prolonged operative duration increases the risk of SSIs. In a meta-analysis of the literature, Cheng *et al*. showed that for every 15, 30, and 60 min of surgery, the likelihood of SSI increases by about 13, 17, and 37%, respectively [16]. Moreover, surgery induced-trauma provokes an important immune response [15]. In addition, Margraf *et al*. showed that the release of cirDNA was correlated with NETs in posttraumatic inflammation and sepsis [19]. In this context, it should be noted that NETs are released by two distinct types of NETosis: early vital NETosis and late suicidal NETosis [74]. Vital NETosis occurs in the 5–25 min after activation of neutrophils, whereas late suicidal NmETosis releases NETs within the 2–5 h period following neutrophil activation. Consequently, the longer duration of the intervention in colon and prostate cancer could induce a greater immune response and, therefore, a greater production of NETs than is the case for breast cancer surgery. This could explain the higher increase of cir-nDNA, MPO, and NE levels after colon and prostate surgery than in breast cancer surgery. Overall, our study indicates that the combination of data from the three cancer types may be misleading, since the correlation of data from breast and, to a lesser extent, prostate cancer exhibit clear differences with colon cancer data, especially with respect to the correlation of cir-nDNA with the NET marker, as highlighted in Fig. 4.

Various explanations of these discrepancies are possible: first, the surgical procedure and surgical duration; second, the tumor type biology; and third, anesthesia. Anesthesia may be ruled out, since the compounds and procedure are the same for all three cancer types. Regarding tumor type biology, we can observe that the pre-surgical NET and cirDNA marker levels for the three cancer types varied significantly in contrast to NETs markers values that showed high homogeneity in HI (supplementary Fig. S2). We are more inclined to believe that surgery procedures and timing lead to NETs formation, and may affect in different ways both the magnitude of NETs formation and their degradation, leading in turn to variations in cir-nDNA production. Consequently, the various forms of malignancy and of operation types must all together be taken into account when determining the best time frame for MRD identification.

Administered therapeutics and anesthetics may have an immunomodulatory effect [75]. Thoracic propofol–epidural anesthesia has been shown to reduce the expression of NETosis (MPO and citrullinated histone H3 (H3Cit)) in serum during colorectal cancer surgery [76]. In contrast to this finding, Kim has shown that a sharp increase in cirDNA between the start and the end of the intervention coincided with the administration of anesthesia [17]; a sharp decrease in cirDNA level was then observed by the end of the intervention, due to the elimination of anesthesia. Given such discrepancies in the literature, one can speculate that different types of anesthesia as well as their different delivery protocols may have contrasting effects on cirDNA release. In our study, the induction of anesthesia was performed in the same way for all cancer types, and was performed with intravenous propofol (2–3 $mg.kg^{-1}$), cisatracurium (0.6–1 $mg.kg^{-1}$), sufentanil (0.25 $\mu g.kg^{-1}$), and maintained with sevoflurane 1–2% and additional IV sufentanil. In our study, patients with colon and prostate cancer received enoxaparin after surgery, but patients with breast cancer did not. Enoxaparin is not an anesthetic compound but a low-molecular-weight heparin used to prevent blood clots and thrombosis. Saithong *et al*. showed that enoxaparin inhibits the formation of NETs in COVID-19 disease [39]. Interestingly, in our study, patients with enoxaparin intake 6 h after surgery showed increased and higher cir-nDNA, NE, and MPO levels than patients with no such intake (breast cancer patients). The most obvious explanation of this counterintuitive result would be that enoxaparin could also prevent NETs degradation by inhibiting DNases and proteases in the bloodstream (like EDTA), resulting in the accumulation of NETs until their degradation (fragmentation of the chromatin into cir-

nDNA fragments). However, we are inclined to believe that it is, rather, the shorter duration of the breast cancer operation that reduces the formation of NETs and thus diminishes the level of cir-nDNA.

cir-nDNA is highly fragmented, and the size profile pattern corresponds to the size of the DNA packed mostly in mono-nucleosomes (~180 bp) and, to a much lesser extent, in di-nucleosomes [77, 78]. Observations of mono- and oligo-nucleosomes have long suggested that the main mechanism of cirDNA release is apoptosis [3, 77, 78]. Recently, however, our team have shown that chromatin and NETs degradation leads to the release of mono-nucleosomes [40]. In this study, the size profiles of cir-nDNA from two colon cancer patients after surgery (T9) showed a clear shift to shorter fragments, as compared to the cir-nDNA size profile before surgery and the size profile in HI. It should be noted that this shift toward a lower fragment size has also been observed in DNA derived from NET degradation [40], pointing to the release of cir-DNA deriving from NETs during the post-operative period. This finding needs to be confirmed by a larger set of samples.

Activated neutrophils may release mitochondrial DNA into NETs. This later kind of NETosis occurs 30 min after neutrophil stimulation, like early vital NETosis. Our data here showed a weak increase in cir-mtDNA level over time for prostate and colon cancers, but it appeared that cir-mtDNA amounts are not associated with cir-nDNA amounts. A different cir-mtDNA dynamic was also observed in each of the three cancers. One possible explanation of this phenomenon would be that the production of NETs with a part of mitochondrial DNA occurred differently in these three types of cancer [79]. In this study, it is difficult to determine if cir-mtDNA is associated with NETs formation. Moreover, we have previously shown that our standard cirDNA extract is composed in healthy individuals of ~24.3% cir-mtDNA which mostly derives from circulating cell-free mitochondria and to a much lesser extent from small extracellular vesicules, exosomes, and proteins complexed with mitochondrial DNA [51]. We are therefore inclined to think that surgery or administered therapeutics or anesthetics could induce mitochondrial injury or mitochondrial dysfunction.

Conclusions

This is the first study of the immediate perioperative follow-up of cirDNA (i.e. within 72 h) and of the causes of post-surgery cirDNA increase. Taken as a whole, our data suggest that (i) NETs are associated with cir-nDNA pre-surgery and are elevated in cancer; (ii) the immediate inflammation after curative surgery in cancer patients leads to neutrophil stimulation and NETosis, subsequently producing cirDNA; and (iii) NETs are formed during the perioperative period. This challenges the current paradigms [80]. This study has several limitations. First, the data is limited to infer the impact on NETs formation or cirDNA release of anesthesia and other drugs administered during and after surgery. However, as to the dynamics of cirDNA and NET markers, our work revealed clear differences between colon, breast, and prostate cancer, which were certainly due to the surgery protocol, the variation of tumor vascularization of the cancerous cell/tissue and tumor micro-environment, and of the location/surrounding organ. These differences should be taken into consideration when selecting the appropriate time-frame for blood drawing for cirDNA-guided adjuvant therapy deintensification, or when inferring the threshold positivity for biomarkers, or when investigating tumor biology with DNA analysis. Furthermore, as an exploratory study, the perioperative period is a complex and extensive scene of multiple factor interventions. Consequently, its data should be interpreted with caution and should be confirmed with a large cohort of patients with stages I–III and with patients with other types of cancer. This study focuses on the immediate perioperative period. Investigation over a period of up to 1 month is consequently needed to examine the dynamics of these biomarkers, especially with respect to MRD detection. The role of anesthesics, in particular with regard to the association of cirDNA release and immune-suppression or -protection could not be circumscribed.

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Author contributions

Conceptualization and Funding acquisition: P.C. and A.R.T. Methodology: A.K., B.P., and A.R.T. Investigation: A.K.,B.P., E.P., A.M., Y.G., X.C. Supervision: A.R.T. Formal Analysis: A.K. and B.P. Writing – original draft, review & editing: A.K., B.P., P.C. and A.R.T. All of the authors discussed the results and approved the manuscript.

Supplementary data

Supplementary data are available at *PCMEDI Journal* online.

Conflict of interest

None declared. As an Editorial Board Member of *Precision Clinical Medicine*, the corresponding author Alain R. Thierry was blinded from reviewing and making decisions on this manuscript.

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