Post-surgery sequelae unrelated to disease progression and chemotherapy revealed in follow-up of patients with stage III colon cancer

Alexia Mirandola,[a,j] Andrei Kudriavtsev,[a,j] Catalina Isabel Cofre Muñoz,[b] Raquel Comas Navarro,[c] Marco Macagno,[d] Saidi Daoud,[a] Cynthia Sanchez,[a] Brice Pastor,[a] Ekaterina Pisareva,[a] Mireia Sanchis Marin,[c] Javier Gonzalo Ruiz,[c] Alejandro Piris,[c] Ariadna Garcia Rodriguez,[c] Nadia Saoudi Gonzalez,[c] Ana Vivancos,[c] Virginia Quarà,[d] Alfredo Mellano,[d] Felice Borghi,[d] Giorgio Corti,[d] Caterina Marchiò,[d,e] Anna Sapino,[d,e] Alice Bartolini,[d] Giovanni Crisafulli,[f,g] Alberto Bardelli,[f,g] Massimo Di Maio,[g] Gerald Lossaint,[i] Florence Frayssinoux,[a] Evelyne Crapez,[a,i] Marc Ychou,[a,i] Ramon Salazar Soler,[b] Elisabetta Fenocchio,[d] Paula X. Fernandez Calotti,[b] Thibault Mazard,[a,i] Cristina Santos Vivas,[b,h] Elena Elez,[c] Federica Di Nicolantonio,[d,g] and Alain R. Thierry[a,i,*]

[a]IRCM, Montpellier Cancer Research Institute, INSERM U1194, Montpellier University, Montpellier, F-34298, France
[b]Medical Oncology Department, Institut Català d'Oncologia (ICO) - IDIBELL, Barcelona, Spain
[c]VHIO Vall d'Hebron Institute of Oncology, Medical Oncology Department, Barcelona, Spain
[d]Istituto di Candiolo - Fondazione del Piemonte per l'Oncologia - IRCCS, Candiolo, Torino, Italy
[e]Department of Medical Sciences, University of Torino, Turin, Italy
[f]IFOM, The AIRC Institute of Molecular Oncology, Milan, Italy
[g]Department of Oncology, University of Torino, Turin, Italy
[h]Universitat de Barcelona, Barcelona, Spain
[i]ICM, Institut Régional du Cancer de Montpellier, Montpellier, F-34298, France

Summary

Background We studied the poorly-known dynamics of circulating DNA (cir-nDNA), as monitored prospectively over an extended post-surgery period, in patients with cancer.

Methods On patients with stage III colon cancer (N = 120), using personalised molecular tags we carried out the prospective, multicenter, blinded cohort study of the post-surgery serial analysis of cir-nDNA concentration. 74 patients were included and 357 plasma samples tested.

Findings During post-operative follow-up, the patients' median cir-nDNA concentration was greater ($P < 0.0001$ in the [43–364 days range]) than both the median value in healthy individuals and the pre-surgery value. These cir-nDNA levels were highly associated with NETs markers (P-value associating MPO and cir-nDNA, and NE and cir-nDNA are 6.6×10^{-17}, and 1.9×10^{-7}), in accordance with previous reports which indicate that cir-nDNA are NETs by-products. Unexpectedly, in 34 out of 50 patients we found that NETs continued to be formed for an extended duration post-surgery, even in patients without disease progression. Given that this phenomenon was observed in patients without adjuvant CT, and in patients >18 months post-surgery, the data suggest that the persistence of NETs formation is not due to the adjuvant CT.

Interpretation (1), Given the inter-patient heterogeneity, the post-surgery cir-nDNA level cannot be considered a reliable value, and caution must be exercised when determining mutation allele frequency or the mutation status; and (2), specific studies must be undertaken to investigate the possible clinical impact of the persistent, low-grade inflammation resulting from elevated NETs levels, such as observed in these post-surgery patients, given that such levels are known to potentially induce adverse cardiovascular or thrombotic events.

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*Corresponding author. IRCM, Montpellier Cancer Research Institute, INSERM U1194, Montpellier University, Montpellier Cancer Institute, Montpellier, F-34298, France.
E-mail address: alain.thierry@inserm.fr (A.R. Thierry).
[j]These authors contributed equally to this work and share first authorship.

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Research in context

Evidence before this study

Only a few study has investigated whether cancer surgery causes long lasting effects of the disease and how this might affect their future care. Circulating DNA (cir-nDNA) is of significant relevance to this point, in that it enables non-invasive monitoring of the disease, identification of specific genetic mutations associated with tumours, and customization of treatment strategies for patients with cancer. Moreover, it has been proven that the degradation of neutrophils extracellular traps (NETs) leads to the release of mononucleosome-associated DNA, which constitute the vast majority of cir-nDNA. Cir-nDNA can therefore be considered as NETs markers, and was found being associated with mCRC at diagnosis.

Added value of this study

In patients with cancer, after tumour resection, the concentrations of cir-nDNA and NETs protein markers remained higher than the median values of HI, regardless of adjuvant chemotherapy or tumour progression. The association between cir-nDNA and NETs protein markers can last for up to 2 years, even in patients without relapse. These observations reveal new and unexpected paradigms concerning cir-nDNA origin and post-surgery follow-up of patients with stage III colon cancer.

Implications of all the available evidence

First, the persistence of NETs formation over an extended post-surgery period in a majority of the patients with stage III CC raises the possibility that unbalanced NETs formation may lead to deleterious effects, such as have been observed in some NETs-related disorders, especially with regard to the emergence of thrombotic events. Larger studies are needed to confirm such post-surgery "sequelae" and therefore to justify the monitoring of markers of NETosis and cir-nDNA in these patients. Second, the inter-individual variations in cir-nDNA concentration arising from differences in NETs release may cause bias in detecting mutations and determining the MAF value.

Introduction

The measurement of circulating DNA (cir-nDNA) has many applications in research, including the investigation of disease mechanisms, the exploration of new biomarkers, and the evaluation of the effectiveness of therapeutic interventions. The measurement of cir-nDNA plasma levels can be a valuable resource in cancer diagnosis and monitoring, prenatal testing, transplant rejection monitoring, trauma assessment, infectious disease detection, genetic disorders and in numerous other fields of research. In oncology, its first clinical implementation was in the theragnostic field, specifically that of targeted therapies, where it was used to guide the clinician's selection of an appropriate treatment of lung and melanoma cancer.[1–4] Such diagnostic potential of this biological source is currently the object of intense study, particularly in the contexts of minimal residual disease detection, treatment efficacy, treatment resistance, recurrence monitoring, and cancer screening and early detection.[1–3,5] While clinical applications of cir-nDNA has been used to aid precision oncology, in particular to direct targeted therapies, notably for lung cancer, it is not currently used in clinical practice for colorectal cancer.[1–3,6,7] Because cir-nDNA[8] analysis in oncology has been compared to tissue biopsy, especially when used as a companion to genetic tests,[9,10] it has been referred to as liquid biopsy. As distinct from other biopsies' targets, however, it should be remembered that cir-nDNA are fragments of the genome released into the bloodstream from the body's cells. Knowledge of cir-nDNA's structure and dynamics is now increasing considerably. Thus, the study of the size and sequence of these DNA fragments (fragmentomics) has made it possible to demonstrate that the vast majority of cir-nDNA are associated with mononucleosomes,[11,12] which could enable the differentiation of individuals with cancer.[13,14] Recent studies of cir-nDNA methylation, notably, have demonstrated an extremely varied but mainly leukocyte cellular origin in healthy individuals, and have suggested the possibility of differentiating the course of the disease.[15]

One of the great technological difficulties in using cir-nDNA in oncology lies in the fact that this DNA comes from various cellular sources. In general, cir-nDNA deriving from normal cells, malignant cells and cells from the tumour microenvironment can be distinguished. The proportion of its different origins will obviously vary according to tumour progression, but also according to individual characteristics, given that the tumour microenvironment depends on multiple factors, in particular on individual immunological factors.[2] DNA extracted from the plasma of a cancer patient is commonly referred to as total cir-nDNA. A very large majority of researchers and clinicians use the term circulating tumour DNA (ctDNA) for DNA containing genetic alterations. The use of this term is not ideal, however, given that the "tumour DNA" (or DNA of the tumour) should logically correspond to the DNA of the

cells which make up the tumour, that is to say mainly the malignant cells, the endothelial cells, the stromal cells and immune cells. Accordingly, the amount of cir-nDNA without genetic alterations, may correspond to the amount of DNA from normal cells (mainly hematopoietic) plus the amount of DNA from cells in the tumour microenvironment.[2]

In the context of oncology, the focus of research to date has been on the cir-nDNA of malignant cells (cir-mutDNA[8]), with the consequence that measurement of total cir-nDNA concentration has been poorly investigated, in particular as to its use as a single marker. Indeed, what investigation of it has taken place has focused on: (1), post-surgery presence of residual or recurrent cancer (2), association of cir-nDNA concentration in post-operative samples with overall survival; and (3), evaluation of treatment response.[1–3]

In our prospective, multicenter, blinded study, which focused on the post-surgery monitoring of personalised molecular tags in patients with stage III colon cancer, we investigated the extent to which cir-nDNA varies over an extended post-surgery period, in comparison with its pre-surgery value. Having previously observed that at diagnosis the plasma of patients with metastatic colorectal cancer (mCRC) at diagnosis shows an association of cir-nDNA level and neutrophil extracellular traps (NETs) formation, in this study we also examined NETs markers both pre- and post-surgery.

Methods

Patients and cohort

In this work, we examined plasma from all stage III colon cancer patients included in the THRuST clinical study (015-FPO18), which was a prospective, multicenter, and blinded observational study. THRuST is an ERC Transcan European project (https://www.transcanfp7.eu/index.php/abstract/thrust.html). The inclusion criteria selected patients aged ≥18 years old with histologically confirmed stage III colorectal adenocarcinoma. The main exclusion criteria were: active viral infection (hepatitis, HPV, HIV), previous systemic or radiation therapy for colorectal cancer, and a history of another neoplastic disease. The study's primary objective was to assess the clinical feasibility of dynamically detecting tumour progression by monitoring a molecular and personalised signature by means of a blood test. The study's secondary objectives included: an examination of the performance of each qualitative and quantitative cir-nDNA parameter, the acquisition of descriptive knowledge about the clonal evolution of driver mutations under standard care, and the comparison of the data with conventional biomarkers and imaging. Patients were screened and included at the IRCC (Istituto di Candiolo- Fondazione del Piemonte per l'Oncologia, Candiolo, Italy), VHIO (Vall d'Hebron University Hospital, Vall d'Hebron Institute of Oncology, Barcelona, Spain), ICO (Catalan Institute of Oncology, L'Hospitalet de Llobregat, Barcelona, Spain) and ICM (Montpellier Cancer Institute, Montpellier, France).

In the THRuST protocol, individual molecular signatures were defined for each CRC patient, based on next-generation sequencing analyses performed on DNA from tumour tissue after resection. Thus, this individual molecular tag consisted of at least one actionable driver, one non-actionable driver and a passenger mutation. The IntPlex method was employed for patient follow-up, enabling simultaneous determination of five parameters: (i) cir-nDNA concentration, (ii) presence of a point mutation, (iii) mutant DNA concentration, (iv) mutant allele fractions of cir-nDNA, and (v) cir-nDNA fragmentation index. In addition, specific cir-nDNA methylation patterns were monitored post-surgery. Blood samples for this study were collected before and after surgery, and at each follow-up visit (every 3 months if possible). The collected data was analysed with reference to clinical observations, standard management care, conventional imaging methods, and conventional CRC biomarkers. In this ancillary study, we only examined the dynamics of cir-nDNA in the course of the THRuST clinical study.

The protocol was approved by Ethics Committee of Candiolo Cancer Institute FPO-IRCCS and then by all participating institutions. All patients gave their written informed consent before enrolment. The study was conducted in accordance with the Declaration of Helsinki.

The healthy individual control cohort was composed of samples from blood donors to the Etablissement Français du Sang (EFS, Montpellier, France). EFS blood samples are highly controlled and qualified. They are subjected to the same preanalytical conditions as for patients' blood samples before plasma analysis.

Sequencing analysis

Tumour mutation profiling was performed on the surgical tumour samples by Next Generation Sequencing (NGS). Each clinical center used different NGS assays, using various gene panels dedicated to colorectal cancer. These are described in Supplementary Information 1.

Blood samples collection

Each clinical center followed the same strict and precise guidelines[16] concerning blood collection, tube handling and storage.[16] Each center has a specific certified and registered CC bank, which adheres to the THRuST clinical study protocol (015-FPO18). Ethylene diamine tetraacetic acid tubes (EDTA) plasma samples were stored at –80C° and were periodically sent to both the IRCM (3 mL plasma minimum) and the IRCCS (1 tube, 3 mL plasma minimum). In receiving these samples, the IRCM team used quality controls as defined by existing cir-nDNA preanalytic guidelines.[17] Blood

samples were collected between Avril 2019 and July 2023. In the course of their routine pre- and post-surgical surveillance, patients were submitted to a 10-point blood sampling plan: 1 pre-surgery, 1 post-surgery (15 < d < 40 days), and every 3 months thereafter, concomitant with the obtention of clinical and imaging data, all of which is being collected in a customised eCRF. Each patient will be followed up proactively for a duration of up to 24 months or until disease recurrence, whichever comes first.

Plasma isolation and cir-nDNA extraction

EDTA tubes were centrifuged at 1200*g* for 10 min at 4 °C, within 4 h of collection. Plasma samples were immediately stored at 80 °C and transferred on dry ice from the recruiting institutions to our laboratory. Plasma was stored for a number of months (4–12 months), and centrifuged at 16,000*g* for 10 min at 4 °C. An aliquot of plasma was then used to perform an enzyme-linked immunosorbent assay. Cir-nDNA was extracted from 1 mL plasma (Maxwell_ RSC Instrument) using the cfDNA Plasma Kit (Promega Corporation, Madison, WI, USA) in an elution volume of 130 µL. For our quantification of cir-nDNA, we adhered strictly to the cir-nDNA preanalytical guidelines referred to above (plasma isolation, plasma storage at –80 °C[16,17]). DNA extracts were stored at –20 °C until use. In storage at –80 °C[18] for up to 3 years, no significant variation of cir-nDNA concentration was previously reported as determined by quantification by Q-PCR a WT sequence of *KRAS* (67 bp length) used in this study. In total, 357 serial plasma samples from 74 patients were analyzed.

Quantification of cir-nDNA

Analysis of cir-nDNA was carried out using the multiplexed IntPlex® method which was specifically designed for quantifying cir-nDNA.[19] Briefly, on a CFX96 instrument using the CFX manager software (Bio-Rad), Q-PCR amplifications were conducted in two replicates, with each reaction having a total volume of 25 µL. Each PCR reaction comprised of 12.5 µL of IQ Supermix Sybr Green (Bio-Rad), 2.5 µL of DNase-free water (Qiagen) or specific oligoblocker, 2.5 µL of forward and reverse primers (0.3 pmol/mL), and 5 µL of DNA extract. The thermal cycling process consisted of three repeated steps: a hot-start activation step at 95 °C for 3 min, followed by 40 denaturation–amplification cycles at 95 °C for 10 s, then at 60 °C for 30 s. Melting curves were studied by gradually increasing the temperature from 60 °C to 90 °C, with a plate reading taken at every 0.2 °C increment. With a genomic extract of the DiFi cell line at 1.8 ng/mL of DNA, standards curves were generated for each run, in order to maintain accuracy and consistency in the results. Each PCR run was carried out with template control and positive control for each primer set. Validations of Q-PCR amplification were controlled by melt curve differentiation. To quantify the total circulating nuclear DNA (cir-nDNA) concentration in both CC patients and HI, amplification of a 67 bp-length wild-type sequence of the KRAS gene was performed. The coefficient of variation was determined as 24% for the quantification of cir-nDNA, when considering variation due to the extraction procedure and analysis in the same plate.[20] The accuracy of this study's measurement of cir-nDNA concentration is supported by two assessments: (i) the total cir-nDNA concentration, obtained by targeting a KRAS sequence, was routinely controlled by quantifying a BRAF internal control sequence. In addition, this quality control enabled the detection and exclusion of samples which presented a loss of heterozygosity (LOH) or gene amplification, two phenomena which have been reported in CRC patients.[21] Moreover, since KRAS amplification is an infrequent event in CRC (0.67%),[21] its level did not impact our observations or the values described here; and (ii) this method of quantifying cir-nDNA has undergone rigorous experimental and clinical validation, demonstrating unparalleled specificity and sensitivity, to the point of permitting the detection of a single DNA fragment molecule, as determined under Poisson Law distribution experiment. A healthy cir-nDNA median value (12.6 ng/mL) was determined using a control cohort of 22 healthy individuals (Supplementary Fig. S1). To facilitate our observations, we arbitrarily defined a positivity threshold for cir-nDNA concentration, thus enabling us to rigorously distinguish pathological or abnormal values with control values by adding the standard deviation to the median value of the healthy individuals (12.6 ng/mL). Note, we quantified plasma DNA by targeting a nuclear DNA sequence.

Detection and quantification of mutant cir-nDNA

IntPlex analysis is an allele-specific blocker quantitative PCR (ASB Q-PCR) method. It uses specific primer location within a 220 bp region around the targeted mutation, while keeping amplicon length below 100 bp, thus allowing reliable and sensitive mutation detection. This multiparametric test simultaneously enables determination of the quantity of cir-nDNA and cir-mutDNA, the mutation allele frequency and a fragmentation index. The Intplex system offers unmatched sensitivity (MAF down to 0.003%[22]). Amongst its several uses, the fragmentation index allows control of pre-analytics by estimating blood cell DNA contamination. The cir-nDNA analysis follows the MIQE guidelines[23] and has been clinically validated.[19] This IntPlex system was specifically designed to detect nuclear cir-nDNA. This study benefits from the experimentally, clinically validated IntPlex method, which offers levels of specificity and sensitivity which are unmatched by any other method. This study took its data from the THRuST clinical study, in which several point mutations were selected. A 3-point mutation molecular signature was

defined for each patient, based on the surgical tumour sample by Next-Generation Sequencing. The design of the Q-PCR system had been previously validated under stringent conditions.[18] When available, the quantification of cir-mutDNA concentration was used only to control the kinetics of cir-nDNA. The dynamics of the amount of the different cir-mutDNA selected per patient will be further reported when all the analysis of the THRuST study is complete.

Myeloperoxidase and Neutrophil Elastase assay

Myeloperoxidase (MPO) and Neutrophil Elastase (NE) concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) technique with a Duoset kit from R&D systems. This was used according to the manufacturer's standard protocol (Duoset R&D Systems, DY008, DY3174, and DY9167-05). Both compounds synergistically contribute to Netosis and especially to DNA decondensation following neutrophil activation, and are specifically anchored to NETs filaments following NETs extracellular release. The healthy cir-nDNA concentration median value for MPO and NE markers (12.8 ng/mL and 7.6 ng/mL, respectively) was determined using a control cohort of 22 healthy individuals (Supplementary Fig. S1). Similar to our definition of a positivity threshold for cir-nDNA, we arbitrarily defined a positivity threshold for MPO and NE concentration (17.0 ng/mL and 10.5 ng/mL, respectively), by adding the standard deviation to the median value of healthy individuals (Supplementary Fig. S1), to evaluate more stringently any discrepancies between abnormal/pathological values and healthy control values. A reproducibility test revealed a coefficient of variation of 3.14% and 5.82% for the quantification of MPO and NE carried out in the same respective plate. A reference sample was added in triplicate in each plate to normalise the value obtained, to address potential variations deriving from manipulator or plate variations. All MPO and NE measurements were carried out using the same batch of plate.

Statistical analysis

To investigate the associations of patients' cir-nDNA concentrations at different time points, we utilised the Mann–Whitney test. This was done subsequent to conducting the Shapiro–Wilk test, which confirmed that the sample groups did not adhere to a Gaussian distribution. Spearman correlation evaluates the strength and direction of monotonic associations between two variables. It provides a correlation coefficient to explore the relation between these continuous variables: cir-nDNA, MPO and NE. Our guide for the interpretation of the correlation coefficient is: 0.19, no or negligible relationship; 0.20–0.29, weak but significant relationship if there is a P value < 0.05; 0.30–0.39; moderate but significant relationship if there is a P value < 0.05; 0.40–0.69; strong and significant relationship if there is a P value < 0.05; and, >0.70, very strong and significant relationship if there is a P value < 0.05. All P values reported are two sided. The significance level was set at 5% ($P < 0.05$); *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. Statistical analysis was performed using the Graph Pad Prism 10.0.1 software.

Ethics

Informed consent was obtained from all individuals and/or caregivers, and all clinical procedures and genetic testing, including data collection and report, were in accordance with the declaration of Helsinki and approved by the local ethical committees or followed other local guidelines: protocol 015-FP018 by the Ethics Committee at the Hospital Universitari Bellvitge, protocol ID CE IRCCS n.233/2018 approved by the Ethical Committee of the Candiolo Cancer Institute FPO IRCCS, protocol 2019/34 approved by CPP Ouest II; protocol PR(AG)235/2018 approved by Vall d'Hebron Ethical Committee.

Role of funders

The funders had no role in the conceptualization, study design, data collection, analysis, interpretation of data, in writing the paper, or in the decision to submit the paper for publication.

Results

Study cohort follow-up

A total of 120 patients with stage III colon cancer were enrolled in this study from the IRCCS, VHIO, ICO and ICM from April 2019 to May 2023. Of this number, 74 patients were analysed for cir-nDNA and NETs markers, since 32, 12, 1 and 1 patients showed screening failure, no available NGS data, no plasma available for analysis, and no baseline pre-surgery plasma sample, respectively (Fig. 1). A total of 357 plasma samples were tested for cir-nDNA, MPO and NE (Fig. 1). The median follow-up was 314 days. Given the propensity of inflammation to stimulate NETs formation, and NETs' propensity to incite thrombotic events, in this work we distinguish inflammatory or thrombotic events (ITE) from all other adverse events or CT-related toxicity. The discrimination of patients with ITE helps to identify ITE confounding factors. Similarly, given that NETs may be associated with tumour progression, we also identified those patients who suffered relapse during monitoring. In the course of the post-surgery follow-up, 6 patients (8%) showed relapse, while 5 (7%) showed inflammatory/thrombotic adverse events (Fig. 1). It should also be noted that 12 patients experienced neurotoxicity. Patient characteristics are described in Table 1 and Supplementary Table S1.

Marker data from the colon cancer (CC) patient cohort were compared with the data we obtained on control cohort, with either the median value or a

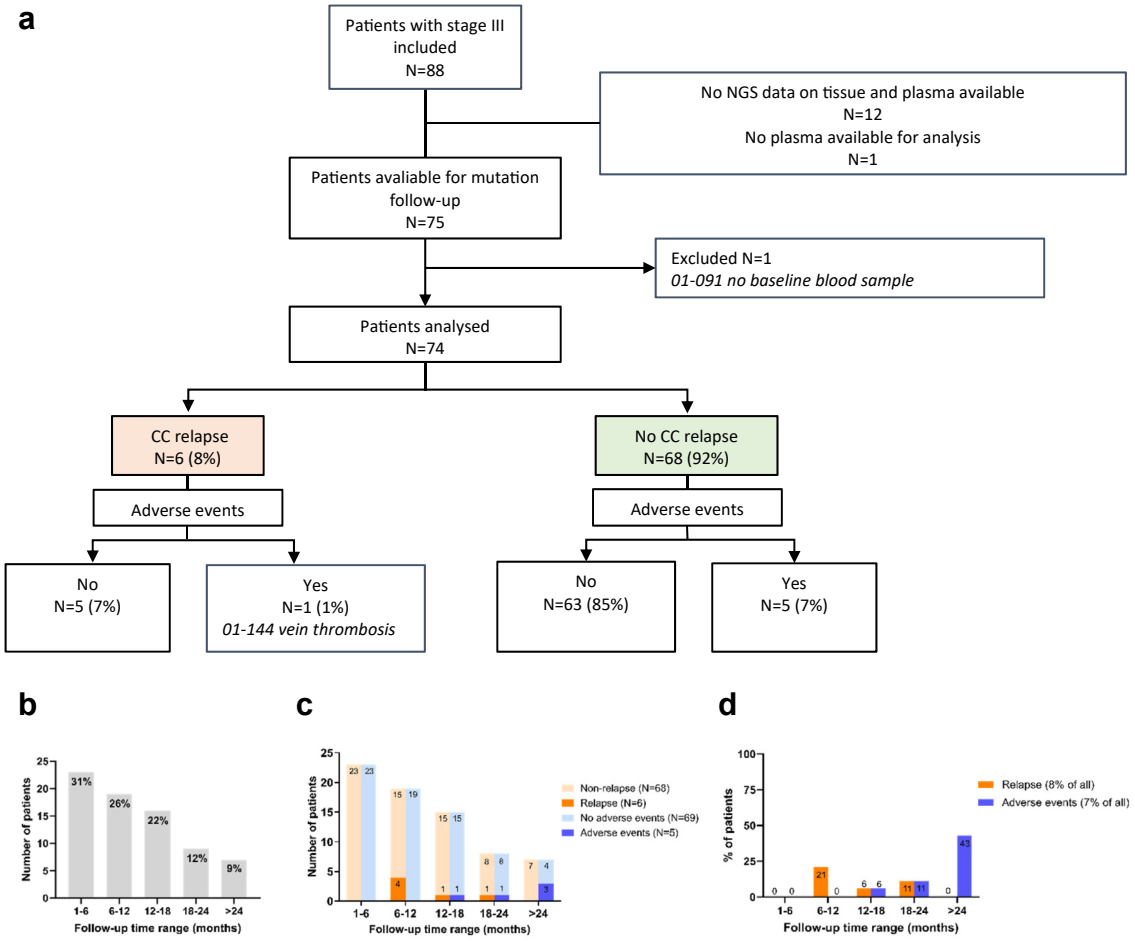


***Fig. 1:* Flow chart of the clinical study (a) and description of the patient cohort (b, c)**. Patient cohort: a: Study cohort flow chart. b: Distribution of the clinical study patients (N = 74) with respect to post-operative follow-up. The number and proportion (%) of patients are grouped according to the follow-up periods in which serial analysis ended: 1–6 (between 1 and 6 months); 6–12 (up to 6 and 12 months range); 12–18 (up to 12 and 18 months range); 18–24 (up to 18 and 24 months range); and >24 months. c: Distribution presented as numbers of patients; d: Distribution presented as proportions, %. (c) Frequency of relapse and adverse events in the study cohort (%). The post-surgery time period follow up is heterogenous due to the late date of inclusion (32–804 days, range) and the end of the clinical study (Fig. 1 and Table 1): >2 months (N = 64), >6 months (N = 50), >12 months (N = 29), >18 months (N = 15), >24 months (N = 9) Note, given that NETs formation is particularly associated with the inflammatory process and thrombosis, this study groups patients experiencing ITE, and excludes patients experiencing neurotoxicity or other adverse events.

positivity threshold (see Methods) being determined with reference to control healthy individuals (HI, N = 22, Supplementary Fig. S1). The cir-nDNA, MPO and NE concentration median in HI was 12.64 (± 7.22, SD), 12.78 (± 4.19, SD) and 7.64 (± 2.81) ng/mL, respectively. Based on this data, the positivity threshold was 19.9, 17.0 and 10.5 ng/mL, respectively. Note, MPO, and NE median values in this short experimental healthy subject cohort were similar to those obtained in other studies which used a higher number of healthy individuals (N = 117).[24,25] However, cir-nDNA values appeared higher than those obtained in our previous study, due to the current study's use of a bead-based extraction robot, instead of the individual column extraction procedure.[26]

Pre- and post-surgery cir-nDNA levels

Pre- and post-surgery cir-nDNA values in patients with stage III colon cancer are shown in Fig. 2. Values are arbitrarily categorised according to pre-surgery period (N = 74) or follow-up period, with the latter divided into periods of 15–42 days (N = 54), 43–364 days (141), 365–729 days (N = 56), and more than 729 days (N = 8). The rationale in selecting a post-surgery period of 15–42 days was, first, to exclude the first two weeks, when the patient is likely to suffer from surgery-related trauma

Patient characteristics	Number
Median age (range), years	69 (36–93)
Sex (%)	
Male	40 (54.1)
Female	34 (45.9)
TNM	
T1	1
T2	9
T3	50
T4	14
N0	2
N1	52
N2	20
Adjuvant chemotherapy	
Complete adjuvant treatment	57
Incomplete adjuvant treatment	5
No adjuvant treatment	11
N/A	1
Xelox	38
Folfox	23
Capecitabine	6
MSS/MSI status	
MSS	39
MSI	5
Follow up median (range), days	314 (4–821)
Relapse	11
Adverse event	5
Neurotoxicity	12
Inflammation/thrombosis event (ITE)	5
Deceased	0

MSS: microsatellite stability. MSI: microsatellite instability.

Table 1: **Patient characteristics.**

and inflammation; and second, because this corresponds to the optimal time period for decisions as to the potential use of deintensification adjuvant therapy in conjunction with minimal residual disease (MRD)-guided targeted therapy based on cir-nDNA,[27–31] as has been suggested by numerous authors. Adjuvant chemotherapy (CT) was administered according to the current guidelines.

The observation of the presence (or not) of the persistence of NETs formation would allow us to predict the existence of at least two subpopulations (75% vs 25%, for instance). However, these values cannot be statistically differentiated.

The patients with the first (lowest 25%) and third (highest 75%) quartile of cir-nDNA values showed no significant clinical differences to the overall patient cohort (Fig. 2). At this time, we are unable to present survival data with sufficient statistical power; data will be reported when all patients have achieved a minimum of two years post-surgery follow-up.

Median cir-nDNA values for each post-operative period (Fig. 2A) are statistically higher than the median HI value (12.6 ng/mL). Except for those values determined more than 2 years post-surgery, the values determined in the 15–42, 43–364, and 365–729 day periods are statistically higher than the pre-surgery values (22.4, 30.8, 23.6, 22.9 and 17.5 ng/mL, respectively). Note, the median value of the 43–364 day period is the highest, being nearly 1.4 times greater than those of the 15–42 day period and the >729 day period, and 1.8 times higher than the pre-surgery median value.

Our data do not allow for a statistical evaluation of the amount of cir-nDNA two years after surgery, given that samples >729 days post-surgery could be obtained from only 7 patients. Nevertheless, it should be noted that, as compared to the pre-surgery (N = 5) and the HI median levels (N = 6), all 7 of those patients showed elevated cir-nDNA levels, which is an unexpected result.

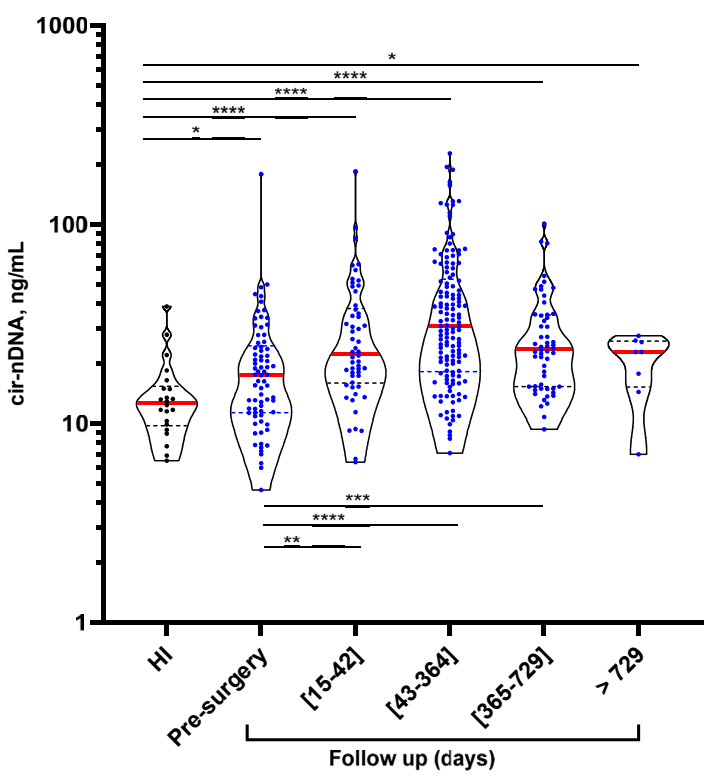
Fig. 2 b and c show the proportion of cir-nDNA values in study cohort patients which are higher than the HI median value (Fig. 2b) and the pre-surgery value (Fig. 2c). 87.5–92.9% of values are higher than the HI median value for each post-surgery period. A lower percentage (67.6%) of values from the pre-surgery period exceed the HI median values. For all post-surgery periods, a large proportion of cir-nDNA values are higher than the pre-surgery values (67.9–75%). When considering only patients who experienced no relapse or ITE, the range is 86.4–100% and 68.2–100%, when comparing post-surgery cir-nDNA values to the HI median and the individual patient pre-surgery value, respectively (Supplementary Table S3D and E).

When using the arbitrarily defined on HI cohort positivity threshold in order to evaluate the data more stringently (see Methods), we observed that 36.5%, 50%, 69.2%, 65.1% and 50% of the plasma samples from patients who suffered no relapse or ITE scored positively in the pre-surgery period, and during the 15–42, 43–364, 365–729, and >729 day follow-up periods, respectively (Supplementary Table S3F). Thus, this sub-group of patients showed a lower proportion of positive plasma samples in the pre-surgery period as compared to the proportion of samples tested during all post-operative periods, when highly stringent evaluation criteria were applied (positive threshold, no relapse or ITE, and comparison with pre-surgery value rather than HI median value) (Supplementary Table S3F).

No statistical evaluation of the impact of the recurrence on cir-nDNA can be made, given that only 6 of the participants had recurrence.

When considering the sub-group composed of patients who experienced no relapse or ITE, and who were monitored for more than 2 months (>75 days), 29 out of 50 (58%) patients scored positive at the last follow-up point (Supplementary Table S2A and B). From this sub-group, 14 out of 25 patients (56%) who were monitored for at least one year scored positive at the last

a



b

		Pre-surgery	Follow-up (days)			
			[15-42]	[43-364]	[365-729]	> 729
Total		74	54	141	56	8
Patients with cir-nDNA conc > median of HI	Number	50	48	129	52	7
	Percentage	67.6	88.9	91.5	92.9	87.5

c

		Follow-up (days)			
		[15-42]	[43-364]	[365-729]	> 729
Total		54	141	56	8
Patients with cir-nDNA conc > pre-surgery value	Number	38	105	38	5
	Percentage	70.4	74.5	67.9	75.0

***Fig. 2:* Pre- and post-surgery cir-nDNA values in stage III colon patients with cancer** (a). Values are arbitrarily categorised according to pre-surgery period or these follow-up periods: 15–42 days; from 42 days up to one year; between one and two years; more than 2 years. The dotted line corresponds to the median value obtained in healthy individuals. Proportion of cir-nDNA values being higher than the healthy individual median value (b) or higher than the pre-surgery value (c). *Mann–Whitney test.

monitoring point. Note, we identified patients who were monitored for more than 2 months, on the assumption that no additional or only very minor surgery-related confounders occur after this period. In addition, 47 out of 50 (94%) patients of this sub-group scored positive at least once during follow-up (Supplementary Table S2A and B). With respect to pre-surgery individual values, during the 15–42 day post-surgery period 29.6%, 64.8%, and 5.6% of cir-nDNA concentrations decreased, increased, or remained relatively stable

(± 5%), respectively (Supplementary Fig. S2). Consequently, to avoid any confounding factors which could be attributed to peri- or post-operative trauma, for the most part we excluded cases (N = 13) where post-surgery serial analysis ended within 2 months (61 days).

Whereas cir-nDNA from healthy individuals was found to vary with age and sex, cir-nDNA amount (as exactly determined in this work) in patients with metastatic colorectal cancer from metastatic colorectal cancer was independent of these parameters, and only slightly influenced by the delay between food intake and blood collection (less than 1.7-fold).[18] The high concentrations which our study observed at different post-operative time points generally represent a 2 to 20-fold increase, as compared to healthy controls or pre-surgery values. Given the high standardization of the preanalytics we used throughout this study, these cannot be attributed to pre-analytical factors.

Altogether, the data of patients who experienced no relapse or adverse event, and who were monitored for more than 2 months post-surgery showed: (i), 85.4–100% and 70.7–100% proportion ranges of patients show elevated cir-nDNA levels during the post-operative period, when cir-nDNA values are compared to the HI median and to the individual patient's pre-surgery value, respectively; (ii) of a sub-group of patients who were monitored for at least one year, more than half showed elevated cir-nDNA levels at the end of the monitoring; and (iii), in this sub-group of patients, a lower proportion of positive plasma samples was found in the pre-surgery range than during all subsequent post-operative periods.

Pre- and post-surgery serial analysis of NETs proteic markers

Fig. 3 and Supplementary Table S3 compare all cir-nDNA, MPO and NE concentration values from the 357 plasma samples obtained from the study cohort patients with healthy median values or with pre-surgery values, for the various pre- and post-surgery periods (15–42, 43–364, 365–729, and >729 days). The cir-nDNA, MPO and NE median values determined for these periods are all higher than the respective healthy median values, and are all similar to their counterpart individual pre-surgery values (Fig. 3). Such similarity is also observed in patients who experienced no relapse or ITE only (Supplementary Table S3D). Similar observations are inferred when examining cir-nDNA amount values using a positivity threshold (Methods, Supplementary Fig. S3).

We observed in patients with a long follow-up period (18 months) who received standard CT (N = 57), or of those who received no (N = 11) or one or two CT (N = 5): (i), only 18% of patients (N = 2) who experienced no relapse or ITE, who received standard CT, and who were monitored over 18 months post-surgery, showed marker values similar to control values; (ii), in 54.5% of patients from the former sub-group (N = 11), all three markers were elevated up to the end of their follow-up, while both NE and MPO NETs markers were elevated in 73% of those cases (N = 8); (iii), 20% of patients who were administered no or only one cycle of adjuvant CT (N = 10) showed marker levels comparable to control values; and (iv), 80% of those who did not receive CT (N = 8) scored positive for at least two markers (Supplementary Table S4).

58.1%, 70.4%, 73.8%, 80.4% and 50% of plasma samples showed MPO, NE and cir-nDNA concentration higher than the HI median value, as determined during the pre-surgery, 15–42, 43–364, 365–729, and >729 day periods, respectively. These percentages were 55.6%, 65.9%, 70.1%, 81.4% and 50% when taking into account patients not experiencing relapse nor ITE, respectively (Supplementary Table S3D). Note, in both cohorts, the percentages increased from pre-surgery to 729 days post-surgery, and declined down to pre-surgery period levels in patients with over 2 years post-surgery monitoring. 31.5%, 28.4%, 23.2% and 12.5% of plasma samples showed MPO, NE and cir-nDNA concentrations higher than the pre-surgery values as determined during the 15–42, 43–364, 365–729, and >729 day periods, respectively (29.5%, 22.2%, 27.9%, and 25%, respectively, when taking into account patients who experienced no relapse or ITE) (Supplementary Table S3B). Note, in both cohorts the percentages decreased slightly from the 15–42 to the >729 day post-surgery periods.

In part of our analysis, the following, more stringent criteria were applied in the evaluation of the MPO, NE and cir-nDNA concentrations:, first, by using the positivity threshold; second, by including only cases where all three markers were positive; and third, by taking into account patients who experienced no relapse or ITE (Supplementary Table S2F). When such criteria were applied, 67.9%, 85.7%, 94.2%, 100% and 100% plasma samples were found to be positive during the pre-surgery, 15–42, 43–364, 365–729, and >729 day follow-up periods, respectively. Supplementary Tables S1 and S2 offers distinctions between: patients who were monitored for no more than 2 months (N = 13, 17.6%); patients who experienced relapse (N = 6, 8.1%); patients who experienced an ITE (N = 5, 6.8%); patients who experienced no relapse or ITE, were monitored for at least two months, and showed values above the HI median value at the end of follow-up (N = 42, 56.7%); and patients who experienced no relapse or ITE, were monitored for at least two months, and showed values similar (± 20%) to the HI median values at the end of follow-up (N = 8, 10.8%). Among those who were monitored for more than 2 months (>75 days) and who experienced no relapse or ITE, only 16% showed values for all three markers similar to control values at the end of follow-up (Supplementary Table S2A). When using the positivity threshold for higher stringency in

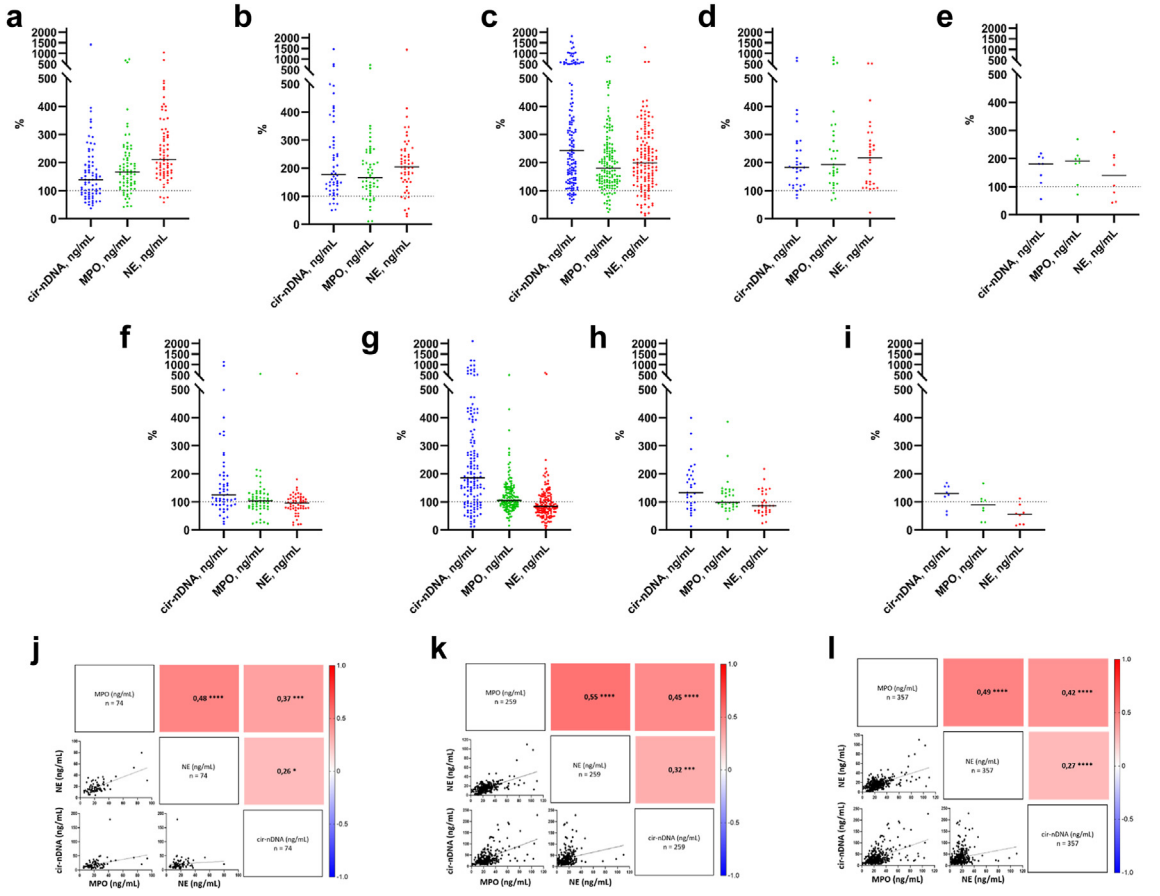


***Fig. 3:* Evolution of all the values of cir-nDNA, MPO and NE markers determined in the cohort study patients upon follow-up period ranges**. Comparison of cir-nDNA, MPO and NE values with healthy median values or pre-surgery values. Healthy subjects' median values vs pre-surgery (a); vs 15–42 days post-surgery (b); vs follow-up between 43 days and one year (c); vs follow-up between one to two years (d); and vs follow-up of more than 2 years (e); pre-surgery vs 15–42 days post-surgery (f); vs follow-up between 43 days and one year (g); vs follow-up between one and two years (h); and vs follow-up of more than 2 years (i). Data are expressed as the ratio of the post-surgery value over healthy individual median value, or over the pre-surgery value; samples with value over HI median or pre-surgery value are over 100%. Spearman correlation study between all MPO, NE and cir-nDNA values. (j), pre-surgery values; Spearman P-value associating MPO and NE, MPO and cir-DNA and NE and cir-nDNA are 1.6×10^{-5}, 1.0×10^{-3} and 2.3×10^{-2}; (k), post-surgery values. Spearman P-value associating MPO and NE, MPO and cir-DNA and NE and cir-nDNA are 1.7×10^{-21}, 1.5×10^{-14} and 2.1×10^{-7}; and (l), pre- and post-surgery values. Spearman P-value associating MPO and NE, MPO and cir-DNA and NE and cir-nDNA are 1.5×10^{-22}, 6.6×10^{-17}, and 1.9×10^{-7}. Regression curves of the association between all MPO, NE and cir-nDNA values. MPO vs NE, MPO vs cir-nDNA, and cir-nDNA vs NE. R values are (j) 0.44, 0.11, 6.1×10^{-3}; (k) 0.36, 0.27 and 0.05; (l) 0.35, 0.23 and 0.03, respectively.

evaluating discrepancies, we found that within the same group of patients, of those who scored positive for all three markers, the marker values of one out of 8 (12.5%) returned to the control level, and 27 out of 42 (63.3%) persisted at a high level (Supplementary Table S2F). Using the same stringent criteria for patients who experienced no relapse or ITE and who were monitored for at least 2 months (>75 days), 13 (26%), 26 (52%), 42 (8%) and 8 (16%) out of 50 patients showed positive values for all three markers, two markers and one marker, respectively; while 8 (16%) patients showed negative values for all three markers at the end of the follow-up period (Supplementary Table S2A and B). In the same sub-group, 30 (60%) and 43 (86%) out of 50 patients showed positive values for all three and two markers at one point at least during follow-up, respectively (Supplementary Table S2A and B, and Supplementary Table S3).

Association of cir-nDNA concentration with NETs protein markers

Fig. 3j–l shows the Spearman correlations among all MPO, NE, and cir-nDNA values. All markers exhibited statistically significant positive coefficients of correlations (R values). Specifically, for the pre-surgery values, the R values were 0.48 for MPO vs NE, 0.37 for MPO vs

cir-nDNA, and 0.26 for cir-nDNA vs NE. In the post-surgery values, these R values were 0.55, 0.45, and 0.32, respectively. For the combined pre- and post-surgery values, the R values were 0.49, 0.42, and 0.27, respectively. Notably, among these associations, the highest R value for positive correlation was observed between MPO and NE (R = 0.48–0.55) in both pre- and post-surgery and in their combination, followed by the correlations between MPO vs cir-nDNA (R = 0.37–0.45) and NE vs cir-nDNA (R = 0.26–0.32). The linear regression curves and their corresponding R^2 values illustrate the variance among the individual values used to compute the aforementioned Spearman correlations, with the lowest variance being observed for the MPO vs NE correlations.

Description of the follow-up data from illustrative clinical cases

To better analyse the evolution of marker levels throughout patient follow-up, we describe in detail a number of patients whose clinical conditions are illustrative.

Description of the follow-up data from illustrative examples of patients who experienced no relapse or ITE

Fig. 4 shows illustrative examples of post-operative monitoring of MPO, NE and cir-nDNA concentrations in patients who to date have shown no disease progression. Observation of all individual cases (Supplementary Fig. S4) confirmed the association of MPO, NE and cir-nDNA concentration values, which association is also confirmed by the similar variation of their respective kinetic curves. As shown in Fig. 2 and Supplementary Tables S2 and S3, nearly all patients showed elevated levels of MPO, NE and cir-nDNA at pre-surgery time points. Note, in most patients, the three marker values peaked during the 100–200 day post-surgery period. In addition, two categories of patient who experienced no relapse or adverse events could be distinguished: (1), those showing cir-nDNA, MPO and NE concentrations which persist above the healthy control median and mostly remain above pre-surgery values; and (2), those showing elevated levels of MPO, NE and cir-nDNA at pre-surgery time points, followed by a sharp decrease of marker values to control levels in accordance with tumour resection and/or adjuvant CT efficacy. Contrary to what we expected, the vast majority of patients were assigned to the first category, with characteristics comparative to the clinical cases we selected for the purposes of illustration (Fig. 4a–c). Fig. 4d–f show examples which, prior to our findings, we would have expected to be illustrative of the majority of patients (i.e., corresponding to category (2) above): that is, showing elevated levels of MPO, NE and cir-nDNA (as previously shown in mCRC) at pre-surgery time points, followed by a sharp decrease towards normal marker value levels, in accordance with tumour resection and treatment. All 74 patients' follow-up data is presented in Supplementary Fig. S4. The relative proportions of both groups is described in Supplementary Table S2. Among the plasma of the patients who experienced no relapse or ITE, at one time point at least in the >2 months post-surgery period, 78%, 36% or 48% showed levels of the three markers which were more elevated than the HI median, the individual pre-surgery value or the positivity threshold, respectively.

Description of the follow-up data from illustrative examples of patients experiencing relapse

Fig. 5a–d show illustrative clinical cases from the follow-up of patients with relapse. The dynamics of the markers are detailed in Supplementary Information 2. Overall, cir-nDNA, MPO, and NE concentration were elevated pre-surgery and increased in the first months post-surgery, with all concentrations then decreasing to values above control values post-surgery. Concentration values of the three markers are all higher than the HI median values at all time points. They increased again at the visit just before and just after the relapse diagnosis. All three markers' values similarly varied from the pre-surgery time point: first, they increased during the first months post-surgery; second, they decreased at the end of treatment; third, they increased again at the time point just before and after tumour relapse diagnosis. It should be noted that the profile of the curve of some passenger mutations bearing cir-mutDNA matched that of cir-nDNA. It is also notable that driver mutation bearing cir-mutDNA was detected pre-surgery, but not in post-surgery follow-up. The three markers may thus be regarded as correlated with the clinical conditions of the patients.

Description of the follow-up data from illustrative examples of patients experiencing ITE

Fig. 5e–h show illustrative cases of patients who experienced ITE (microbial infection or thrombotic events) during follow-up. The relevant marker dynamics are detailed in Supplementary Information 2. Overall, the levels of all tested markers varied equivalently over the course of post-operative monitoring. They peaked in the first months post-surgery, then returned to levels at or above basal levels. Following ITE diagnosis, they all peaked at their highest values, then returned to lower values under antibiotic or anticoagulant therapies, thus pointing to the association of the NETs markers' levels with microbial infection and thrombus formation. The three markers may thus be regarded as correlated with the clinical conditions of the patients.

Specific analysis of the data with regard to potential confounding effects of adjuvant CT

In order to specifically address this issue, we analysed the data obtained from patients who were monitored for

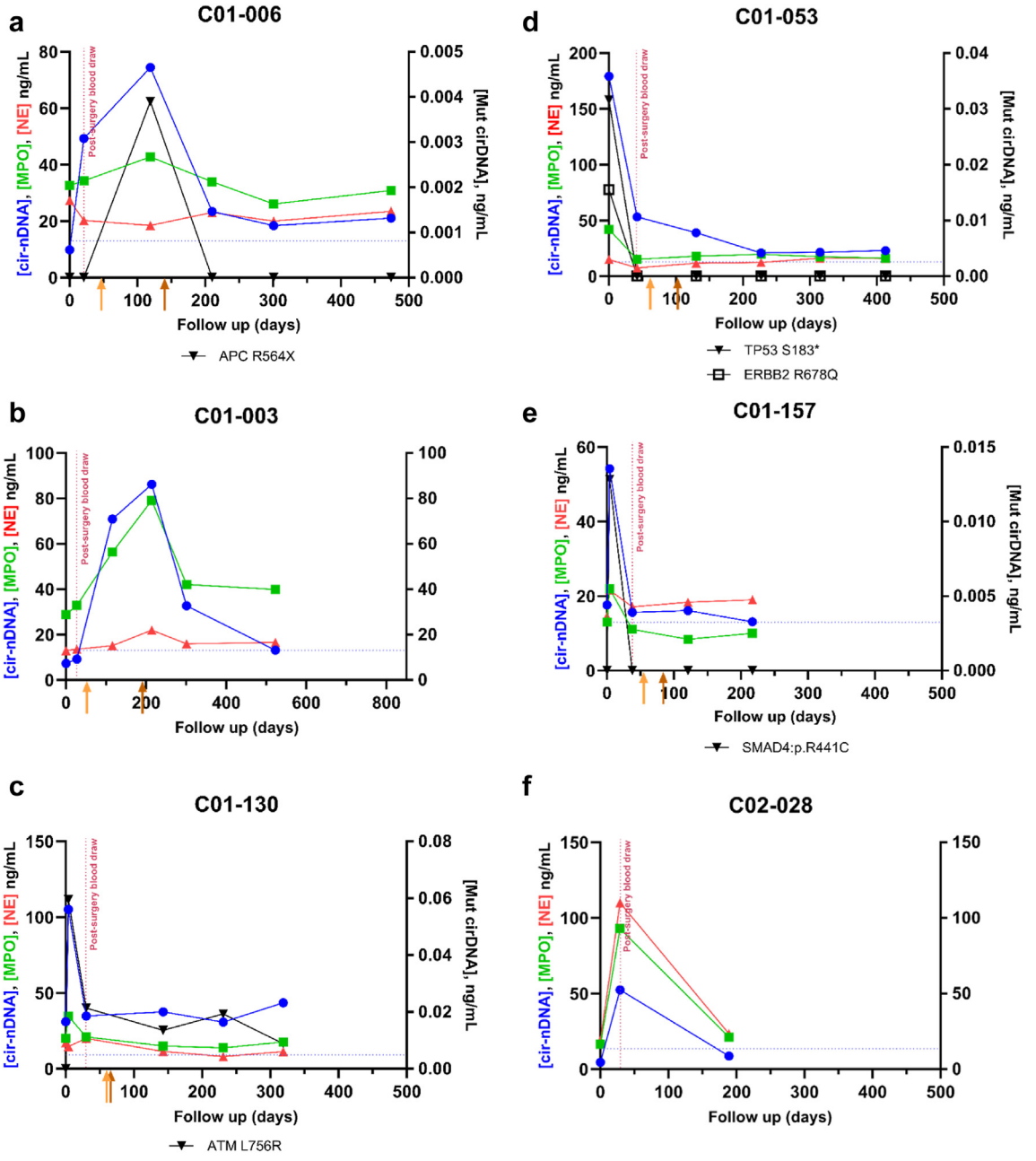


Fig. 4: **Illustrative examples of clinical cases**. Patients with two types of marker dynamics during follow-up of patients experiencing no relapse or ITE, and showing MPO, NE, and cir-nDNA at a persistently elevated level (a, b, and c), or returning to control levels (d, e, and f). Orange arrows: start of Adjuvant CT; Brown arrows: end of Adjuvant CT.

more than 18 months post-surgery, on our arbitrary assumption that the standard adjuvant CT administered to stage III CC patients would not or would only infrequently produce acute secondary effects after a lapse of 10 months after treatment (Supplementary Table S4A). This sub-cohort (N = 11) of patients who received standard CT and whose follow-up ranged from 555 to 804 days, does not contain patients who experienced relapse or ITE. To be even more stringent, we numbered the values of MPO, NE and cir-nDNA concentrations above their respective positivity thresholds (see Methods section). All three markers' levels remained elevated up to the end of follow-up in 6 out of 11 patients (54.5%), while the MPO and NE levels were

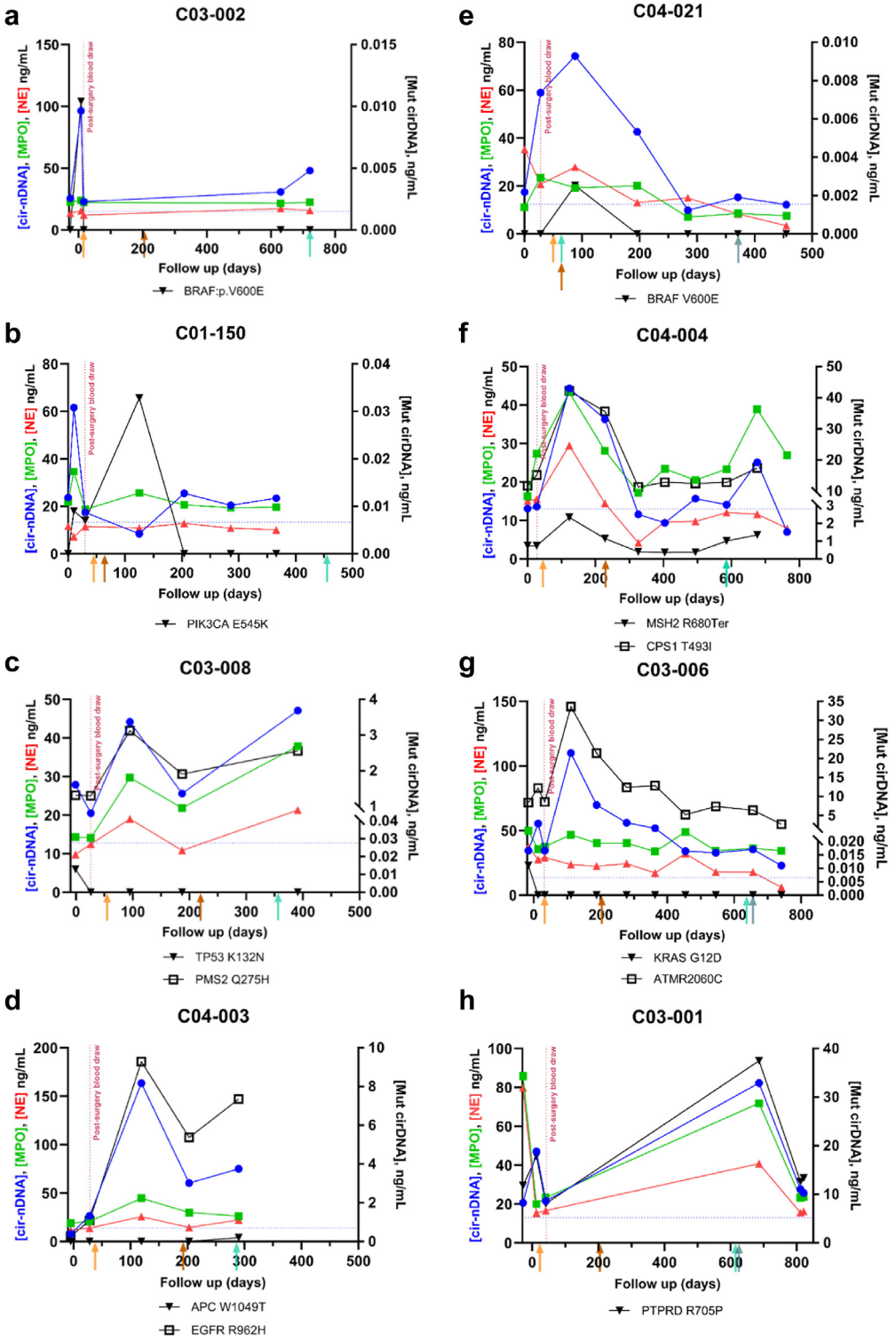


Fig. 5: **Illustrative examples of clinical cases**. Monitoring of patients experiencing relapse (a, b, c and d) or ITE (e, f, g and h). Orange arrows: start of Adjuvant CT; Brown arrows: end of Adjuvant CT; Cyan arrows: relapse or ITE; Grey arrows: end of ITE.

both elevated in 8 out of 11 patients (73%). Thus, only 18% of patients who received standard CT and monitored over 18 months post-surgery showed marker values similar to control values within this time period (Supplementary Table S4A).

In addition, with regard to patients who experienced no relapse or ITE, we analysed the data of a subgroup of patients to whom no adjuvant CT was administered, combined with patients to whom only one or two adjuvant CT cycles were administered (N = 15, Supplementary Table S4B). To avoid any potential trauma confounding factor, five patients were not included in this sub-group because their serial blood collection stopped before 8 weeks post-surgery. The data available for the 10 other patients extends for 4 months post-surgery. 6 patients were administered no CT, 3 received only one CT cycle, and one received only two CT cycles. 8 out of 10 (80%) patients showed elevated levels for at least two of the three markers, while 6 out of 10 (60%) patients showed elevated levels for all three markers, when the stringent positivity threshold was applied. Overall, in this subgroup of patients, only two out of 10 (20%) showed marker concentrations similar to control values, as was the case in the subgroup of patients receiving standard CT. 8/10 (80%), 6/7 (86%), and 4/4 (100%) patients were positive for at least two markers in periods >4, >6, and >12 months post-surgery, respectively; 6/10 (60%), 5/7 (71%), and 3/4 (75%) were positive for all three markers in periods >4, >6, and >12 months post-surgery, respectively (Supplementary Table S4B). Among the plasma of the patients who received no or at the most one CT cycle, at one time point at least in the >2 months post-surgery period 70%, 30% or 40% showed the three markers as more elevated than the HI median, the individual pre-surgery value or the positivity threshold, respectively.

Discussion

Conventionally, cir-nDNA levels in plasma have been shown (i), to be generally higher in patients with cancer than in healthy subjects[18,32–34] (ii), to vary according to cancer type[35]; (iii), to increase with cancer staging[36]; and (iv), to increase in patients with cancer recurrence.[31] That said, the literature is replete with conflicting data on cir-nDNA values, because of a lack of standardization of preanalytical conditions, and because of the variety of quantification methods previously used.

Challenging a number of significant assumptions currently made in the literature,[37–39] our study demonstrates that elevated levels of cir-nDNA persist in a majority of stage III CC patients long after surgery. In this regard, some of our observations are striking, especially as to the data of patients who experienced no relapse or adverse event, and who were monitored for more than 2 months post-surgery. In addition to these findings, our work clearly showed the occurrence of NETs formation in stage III CC in both pre- and post-surgery conditions, irrespective of the disease progression associated with increased cir-nDNA concentrations. A clear return to normal cirDNA values following the peri-surgery period, in accordance with tumour resection, thus confirming previous assumptions, was observed in only a minor fraction of patients.

This postulate is principally based on solid observations: (i), MPO, NE and cir-nDNA concentration values showed a similar variation at most post-surgery time points; (ii), when taking account of patients who experienced no relapse or adverse event and who were monitored for more than 2 months post-surgery, 47.9% and 70.8% of patients scored positive for all three and two markers at one time point at least during follow-up, respectively; (iii), examination of the clinical cases of patients who experienced a relapse or an adverse event showed that the variations of concentrations of all three markers correlated with the occurrence and cure of clinical events in particular with respect to tumour progression or adverse events such as an inflammatory process; and (iv), in two clinical cases, anti-coagulant therapy was associated with a decrease in concentration of all three markers.

Previous investigation in this area focused almost exclusively on the circulating DNA of malignant cells (or mutant cir-nDNA, cir-mutDNA). Because of the clinical necessity of determining the molecular profile for predictive information, nearly all initial efforts focused on the cir-mutDNA. For these reasons, over the past decade the agnostic cir-nDNA approach has been neglected, despite intriguing observations of (i), the high variation of cir-nDNA in patients with the same malignancy and the same imaging/TNM staging,[22] and (ii), its prognostic value.[40–42] To date, there has been no large prospective study on the cir-nDNA concentration dynamic during patient follow-up.

As for cir-mutDNA, cir-nDNA was thought to decrease following surgery in cases where no relapse occurs.[1,3] Our observations challenge that paradigm. Agreement with these observations can be found only in a small number of previous studies, whose monitoring of cir-nDNA post-surgery was limited to a short post-surgery period (mainly peri-surgery), or to a small patient cohort.[32,43,44] For instance, using an advanced digital PCR method, Diehl et al.'s milestone study[32] pointed to no direct relationship between the level of cir-nDNA (total APC fragments) and the mutational load in stage I-III CRC at diagnosis. They also asserted that the observed increase in cir-nDNA concentration in metastatic colorectal cancer (mCRC), as compared to stage I-III, did not derive from neoplastic cells themselves. Further to this point, Wei et al.[44] showed that the level of cir-nDNA is stable at different time points up to 6 months post-surgery, while remaining higher than those of high-risk healthy individuals.

In the literature to date, the elevated cir-nDNA concentrations observed in most patients with cancer at diagnosis or under disease progression has been attributed mainly to their release from tumour cells.[1,34,35,45] Our work challenges this assumption. A tumour is constituted of malignant cells and of non-malignant cells from the tumour microenvironment, the latter being itself composed of a large variety of cells, namely endothelial cells, stromal cells and immune cells. It was previously assumed that cir-nDNA from normal cells outside the tumour mass would represent a low proportion of the total cir-nDNA amount. We recently demonstrated that the cir-nDNA level is associated with NETs markers in mCRC patients at diagnosis.[46] Margraf et al. correlated the release of cir-nDNA with NETs in post-traumatic inflammation and sepsis.[47] In 2015, we were among the first to postulate that a significant portion of the cir-nDNA found in cancer patient plasma may originate from NETs.[2] Subsequently, we directly proved that NETs degradation leads to the release of mononucleosomes, which constitute the vast majority of cir-nDNA-associated structures.[24] The historical observation of mononucleosomes (and to a lesser extent dinucleosomes) as the principal structures associating cir-nDNA led to apoptosis being considered the main source of cir-nDNA.[48] Our previous direct observations revealed the importance of NETs formation (Netosis) as a mechanism of cir-nDNA release. Among other mechanisms such as apoptosis, necrosis, and active cellular and microvesicular release, its precise contribution has yet to be elucidated, but it may certainly vary according to individual physiopathology.

Our results clearly show that the increase in cir-nDNA amount is associated with an increase in NETs proteic markers, thus suggesting that NETs may be a source of cir-nDNA, at least post-surgery. The significance of granulocytes as a the most important source of cir-nDNA appears to be supported by the study of the cir-nDNA fragmentome and methylome.[11,15,49–51] Post-surgery serial analysis of cir-nDNA methylation in patients with cancer is needed to confirm our observation, and could potentially offer the clinician a wider scope of patient clinical conditions on which to base a personalised medicine solution. The preponderance of cir-nDNA of neutrophil origin, despite some minor variation, is seen in all physiological and physio-pathological conditions, especially in inflammatory diseases such as sepsis and COVID-19,[52] as well as in cancer. Elevated levels of cir-nDNA have been particularly observed in numerous sterile and non-sterile inflammatory diseases, strongly evidencing a link between inflammation and cir-nDNA release. It is well established that fragments of DNA or circulating DNA, whether of nuclear or mitochondrial origin, are immunogenic, particularly through the TLR9 signaling pathway, but also by activating endothelial cells of the vascularization.[53] In this way, they lead to the production of inflammation, which by means of a positive feedback loop in turn leads to the stimulation of neutrophils, and therefore the formation of NETs.[54] It would also appear that another positive feedback loop is established by the NETs' activation of platelets, leading to a state of hypercoagulation and the creation of clots.[55,56] These can activate the vessels' endothelial walls, leading in turn to inflammation and the stimulation of neutrophils. It is possible that a third positive feedback loop occurs through the creation of autoantibodies, which also activate the immune system.[57] The hypothesis of the presence of these three positive feedback loops, and the constant self-stimulation of neutrophils which results, has been proposed for the case of long COVID[58](under review).

The post-surgery treatments administered to the patients in our multicenter and prospective study were generally homogenous under stage III CC standardised management care when no relapse or adverse event occurred. Consequently, the reported differences in marker concentrations, especially when comparing patients with and without post-surgery elevated levels, cannot (or can only slightly) be attributed to variations in post-surgery treatment.

That said, it must be acknowledged that adjuvant CT probably causes cir-nDNA release or inflammation. Longitudinal cir-nDNA quantification performed post-surgery or during chemotherapy has been poorly reported in the literature, and where such reports do exist there are discrepancies with respect to the influence of chemotherapy.[33,59–61] Although no prospective, blinded, large clinical study has been established towards this goal, most of the literature on this point reports a positive association of cir-nDNA concentration with tumour burden.[33,59] The paradigm of the cir-nDNA concentration-tumour burden correlation may have induced researchers to neglect observations of the persistence of a high level of cir-nDNA during treatment irrespective of progression-free survival.[44,60,62] For instance, Wei et al. concluded that the dynamics of cir-nDNA concentration correlated with pancreatic ductal adenocarcinoma tumour burden following FOLFIRINOX chemotherapy.[44,60] A small number of other authors have admitted that no clear association between cir-nDNA levels and chemotherapy has been found, and that cir-nDNA concentration does not appear to predict treatment response.[62] Consequently, the effect of chemotherapy would appear to be a plausible explanation of the persistence of cir-nDNA levels, which are released from dying cells, but also of the persistence of NETs formation, given that persistent local and systemic inflammation may be generated by widespread chemotherapy-induced senescence.[61] However, we may assume that the standard adjuvant CT administered to stage III CC patients would not or would only infrequently produce secondary effects 10 months following treatment. The findings of this study, likewise, do not

support the conclusion that CT causes persistent NETs formation and cir-nDNA release, given that these were observed for an extended period after the completion of the CT regimen; all the patients included in our study, that completion occurred 4–7 months post-surgery. Our postulate relies in part on the study of patients with a long follow-up period (18 months) who received standard CT (N = 57), or of those who received no (N = 11) or one or two CT (N = 5).

Among the six patients in our study who experienced relapse, five suffered relapse at least 6 months post-surgery, and one at least 12 months post-surgery. Despite the relatively low number of the patients included in our study, and the follow-up median (314 days), our data are generally in line with the OS mentioned above. Our data shows that, for the vast majority of patients in whom cir-nDNA bearing a CC driver mutation had been detected pre-surgery, this mutation was no longer detected post-surgery, despite those patients showing high post-surgery levels of cir-nDNA and NETs markers. This is in stark contrast with the theory that cir-nDNA levels should decrease with the resection or progression of a tumour. By contrast, our data seem to reveal the persistence of phenomena underlying the systemic release of these markers. In addition, our observations that the highest levels of cir-nDNA concentration appear between 45 days and one year, and that the phenomenon is of prolonged duration, eliminate the possibility that this is the result of surgery-induced trauma. We therefore suggest that elevated levels of NETs formation may be mostly correlated with surgical trauma in the days immediately following surgery, and with surgical stress in the ensuing months. Our results have been replicated in a cohort of stage II-III small cell lung cancer (SCLC) patients (Data not shown).

NETs formation and elevated levels of cir-nDNA are not specific to cancer. Comorbidity or adverse events may be confounding factors. Generally, during post-surgery follow-up, a significant proportion of patients with cancer experience adverse events related to the cancer and/or its treatment; these include fatigue, bowel dysfunction, abdominal pain, anxiety about health, limitation of activities, and so on, and can persist long after the end of initial care management.[63] Among such adverse effects, a major concern remains the cumulative and long-lasting neurotoxicity induced by the use of oxaliplatin as adjuvant chemotherapy. In our study, 28.4% of patients experienced adverse events (15 with neurotoxicity, 3 with infection, and 3 with thrombosis). The proportion of these patients is too low to account for the persistence of cir-nDNA and NETs markers levels in the vast majority of our study's patients. Nevertheless, the study of individual cases allows us to better delineate our global observations in light of the marker levels and the medical response to ITE. It is beyond the scope of this study to speculate whether thrombosis is a cause or a consequence of NETs formation. More work is needed on the question of whether or not elevated NETs markers indicate a risk of venous thromboembolism (VTE). In addition to the demonstrated link between malignant diseases and venous thromboembolism, the incidence of deep vein thrombosis (DVT) in post-operative patients with malignancy was found to be significantly higher than in patients with non-malignant diseases. Surgery, especially abdominal, may induce surgical trauma and surgical stress, resulting in short and long term effects, respectively. It may also induce immunothrombosis, in which NETs are implicated, which leads to the formation of clots of all kinds, including thrombi and in particular microclots. We should differentiate but not oppose the persistent observation of NETs formation observed here with the hyper-coagulation state which follows abdominal surgery, and which is conventionally prevented by the use of anti-coagulants during the first three weeks post-operation. The latter well-established phenomena may lead to post-operative thromboembolism, and may be due to inflammation resulting from surgical trauma. We postulate that NETs formation may be amplified and prolonged by the auto-stimulation of platelets and neutrophils, and that this potentially (1), contributes slightly to thromboses in the peri- and post-surgery period; and (2), stimulates the production of circulating microclots. We also postulate that the emergence of thrombotic events (DVT, VTE,...) is modulated by genetic and/or epigenetic factors, which would explain for instance why a significant part of the patients in our study (16%) exhibited no such post-surgery biological sequelae. Our observation could also challenge the widely-adopted use of short course anticoagulation therapy.

About one out of 7 patients with cancer suffer from both venous thromboembolism (VTE) and arterial thromboembolism.[64,65] Cancer represents a risk factor for VTE, with a five times higher risk compared to the general population.[66] Thrombosis is particularly frequent in lymphomas and cancers of the pancreas, the digestive tract, the ovaries and the lungs.[67] Several reports have demonstrated that NETs are involved in the coagulation cascade. Hisada et al.[66,68] found that the plasma level of biomarkers of NETs formation was associated with VTE in patients with pancreatic or lung cancer. A recent study revealed that the detection of micro-emboli may help to flag CRC recurrence after medical treatment.[69] Our present work may explain the frequency with which VTE occurs in patients with cancer. Furthermore, considering the high level of NETs markers post-surgery in most of the patients with cancer included in this study, our work reveals the need for higher scrutiny of post-surgery VTE detection[66] over an extended period, and also raises questions about the benefit of prophylaxis. Note, excessive NETs formation is associated with various symptoms in different pathologies that might occur post-surgery in

patients with cancer[64,65,70] but which were previously assumed to derive from CT.[71]

Our observations find echoes in two previous works. El Messaoudi et al.[41] reported a negative correlation between the level of cir-nDNA concentration at mCRC diagnosis and overall survival duration. Likewise, Mattox et al.[51] recently revealed that the presence of excess cir-nDNA at diagnosis does not derive predominantly from malignant or tumour microenvironment cells, but originates rather from leukocytes, implying a systemic release.[51] Our data at least partially explain the origin of such a systemic effect as deriving from tumour-associated inflammation, which would induce the stimulation of neutrophils and consequently the formation of NETs (Netosis) that leads to the release into the circulation of NETs byproducts, principally cir-nDNA. An alternative explanation would be that cancer cells may produce soluble factors able to "educate" neutrophils toward an activated functional state, as has been suggested in a recent *in vitro* study which used melanoma cell lines.[72] Investigation of the post-surgery persistence of NETs formation due to the existence of tumour-educated neutrophils should be further investigated. Despite such lacunae, taken together these reports point to a new paradigm on the post-operative cir-nDNA origin in patients with cancer,[73] at least in patients with stage III colon cancer. In addition, they highlight the strong clinical potential of cir-nDNA agnostic parameters, whose combination with NETs markers could significantly improve the identification of patients at high risk of relapse.

Our observations appear to preclude or at least to limit the exploitation of cir-nDNA as a stand-alone biomarker in patients with cancer undergoing chemotherapy as a means of assessing treatment response, monitoring disease, predicting treatment outcomes, or determining personalised treatment. Our observations appear to cast significant doubt on those four potentials, given our conclusion that NETs formation is a confounding factor. Given that these studies, such as the detection of MRD,[31] are based on the detection of cir-nDNA bearing a mutation which shows the highest mutation allele frequency (MAF, proportion of mutant DNA among total DNA) as determined from blood sample by NGS, subsequent analysis of cir-mutDNA bearing such mutation may not be always reliable with respect to the level of WT cir-nDNA, which may vary according to the patient. We believe it is ineffective and misguided to define an optimal post-surgery blood collection time for MRD detection in all patients. Furthermore, such use of MAF from plasma may call its reliability into question, notably the use of a MAF threshold for the selection of patients for targeted therapy according to tumour mutation status.[28,29,74–76] Our data support the following recommendationfor MRD detection, using the absolute mutation quantification (concentration of cir-mutDNA), rather than the relative MAF.

This work has several limitations, arising principally from the fact that this is an *ad hoc* ancillary study dependent on a clinical study designed towards different objectives (Supplementary Information 3). In addition, we eliminated the large majority of potential confounding factors but not all of them (Supplementary Information 3).

Because our study is a prospective, multicenter and blinded study on the dynamics of cir-nDNA and NETs markers, as monitored over an extended post-surgery period, the observations it makes, along with the conclusions it draws, offer a fundamental challenge to existing paradigms. Indeed, they have profound implications for cancer research generally, and particularly to the clinical application of cir-nDNA analysis. They clearly show the need for a reassessment of the optimal cir-nDNA technologies, and the need to identify patients at high risk of relapse or ITE such as VTE. The persistence of NETs markers and the elevated cir-nDNA concentration levels observed in a significant proportion of Long COVID patients (Post-acute sequelae of COVID-19) invites comparison with our study's observations, particularly in light of inter-individual disparities. Further investigation is required to confirm our observations in various malignancies of various cancer staging, and more specifically on the use of genomics, transcriptomics and methylomics for post-surgery conditions. All would contribute to the definition of a "Post-surgery sequelae of cancer" condition.

Contributors

Conceptualization: AM, AK, RSS, TM, CSV, EE, FdN, ART. Data curation: AM, AK, CICM, RCN, MM, SD, CS, BP, EP, MSM, AGR, NSG, VQ, AlfM, FB, GrC, CM, AS, AiB, GvC, AbB, MDM, GL, EC, RSS, EF, PXFC. Formal Analysis: AM, AK, CICM, RCN, MM, SD, CS, BP, EP, NSG, VQ, AlfM, FB, GrC, CM, AS, AiB, GvC, AbB, MDM, RSS, EF, PXFC, ART. Funding acquisition: JGR, MY, CSV, EE, FdN, ART. Investigation: AM, AK, CICM, MM, SD, CS, BP, EP, NSG, RSS, EF, PXFC, TM, CSV, EE, FdN, ART, AV. Methodology: AM, AK, CICM, FF, MM, SD, CS, BP, EP, AGR, NSG, VQ, AlfM, FB, GrC, CM, AS, AiB, GvC, AbB, MDM, AV. Project administration: AK, MSM, JGR. Resources: RCN, MM, MSM, JGR, AP, AGR, GL, MY, RSS, PXFC, TM, CSV, EE, FdN, ART, AV. Supervision: AP, MY, RSS, TM, CSV, EE, FdN, ART. Validation: AM, AK, CICM, MM, SD, CS, BP, EP, TM, CSV, EE, FdN, ART, AV. Writing—original draft: AM, AK, CICM, MM, EP, EC, TM, CSV, EE, FdN, ART, NSG. Writing—review & editing, AM, AK, CICM, MM, EP, EC, TM, CSV, EE, FdN, ART.

All authors have read and approved the final version of the manuscript.

Data sharing statement

The data that support these findings of the study are available upon request from the corresponding authors.

Declaration of interests

TM has received personal fees from AMGEN, PIERRE FABRE, MSD, GALAPAGOS, SERVIER, MERCK SERONO and SANOFI, and TM declare participation on DSMB of the ongoing study PRODIGE 70 from FFCD, outside the submitted work. CICM has received personal fees from Instituto de Salud Carlos III/ISCIII and the Asociación Española Contra el Cancer/AECC, outside the submitted work. PXFC and CSV had received personal fees from Merck, Merck KGaA and Amgen, outside the submitted work. RSS is an advisor for GSK, Sanofi Genzyme

and an expert testimonyEsteve, Laboratorios Servier, also has received personal fees from SACE Medhealth and WNT Pharma, outside the submitted work. AbB has received personal fees from Neophore, AstraZeneca Guardant Health, Kither Biotech and he is an advisor for Neophore, Roche/Genentech Global CRC, outside the submitted work. FdN has received personal fees from Illumina and Pierre Fabre, outside the submitted work. EF has received personal fees from Amgen and Pierre Fabre and Merck, outside the submitted work. MDM reports grants from GlaxoSmithKline, Exelixis, Roche, BeiGene, Novartis, Merck Sharp & Dohme, Pfizer; and he has received personal fees from Amgen, Merck, Ipsen, Viatris, Janssen, Astellas, AstraZeneca, Boehringer Ingelheim, Roche, Novartis, Servier, outside the submitted work. CM has received personal fees from Menarini, Roche, Veracyte, Illumina, Bayer and Daiichi Sakyo, outside the submitted work. AV reports grants from AVINCYTE, ROCHE and is an advisor for INCYTE, ROCHE, and BAYER, outside the submitted work. EE has received personal fees from Amgen, Bayer, Cure Teq, AG Hoffmann-La Roche, BMS, Boehringer Ingelheim, Janssen, Lilly, Medscape, Merck Serono, MSD, Novartis, Organon, Pfizer, Pierre Fabre, Repare Therapeutics Inc., RIN Institute Inc., Sanofi, Seagen, Servier and Takeda, outside the submitted work. NSG has received personal fees from AMGEN, MERCK, outside the submitted work, outside the submitted work. ART reports grants from SIRIC Montpellier Cancer Grant INCa_Inserm_DGOS_12553 and Société Française des Acides Nucléiques Circulants (SFAC), outside the submitted work.

This does not alter our adherence to journal policies on sharing data and materials. All authors critically reviewed and approved the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2024.105352.

References

1 Diaz LA, Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. *J Clin Orthod*. 2014;32(6):579–586.
2 Thierry AR, El Messaoudi S, Gahan PB, Anker P, Stroun M. Origins, structures, and functions of circulating DNA in oncology. *Cancer Metastasis Rev*. 2016;35(3):347–376.
3 Wan JCM, Massie C, Garcia-Corbacho J, et al. Liquid biopsies come of age: towards implementation of circulating tumour DNA. *Nat Rev Cancer*. 2017;17(4):223–238.
4 Heitzer E, Haque IS, Roberts CES, Speicher MR. Current and future perspectives of liquid biopsies in genomics-driven oncology. *Nat Rev Genet*. 2019;20(2):71–88.
5 Dasari A, Morris VK, Allegra CJ, et al. ctDNA applications and integration in colorectal cancer: an NCI Colon and Rectal–Anal Task Forces whitepaper. *Nat Rev Clin Oncol*. 2020;17(12):757–770.
6 Elez E, Chianese C, Sanz-García E, et al. Impact of circulating tumor DNA mutant allele fraction on prognosis in *RAS* -mutant metastatic colorectal cancer. *Mol Oncol*. 2019;13(9):1827–1835.
7 Ros J, Matito J, Villacampa G, et al. Plasmatic BRAF-V600E allele fraction as a prognostic factor in metastatic colorectal cancer treated with BRAF combinatorial treatments. *Ann Oncol*. 2023;34(6):543–552.
8 Bronkhorst AJ, Ungerer V, Diehl F, et al. Towards systematic nomenclature for cell-free DNA. *Hum Genet*. 2021;140(4):565–578.
9 Van Emburgh BO, Arena S, Siravegna G, et al. Acquired RAS or EGFR mutations and duration of response to EGFR blockade in colorectal cancer. *Nat Commun*. 2016;7(1):13665.
10 Montagut C, Argilés G, Ciardiello F, et al. Efficacy of Sym004 in patients with metastatic colorectal cancer with acquired resistance to anti-EGFR therapy and molecularly selected by circulating tumor DNA analyses: a phase 2 randomized clinical trial. *JAMA Oncol*. 2018;4(4):e175245.
11 Snyder MW, Kircher M, Hill AJ, Daza RM, Shendure J. Cell-free DNA comprises an in vivo nucleosome footprint that informs its tissues-of-origin. *Cell*. 2016;164(1–2):57–68.
12 Jiang P, Chan CWM, Chan KCA, et al. Lengthening and shortening of plasma DNA in hepatocellular carcinoma patients. *Proc Natl Acad Sci U S A*. 2015;112(11):E1317–E1325.
13 Sanchez C, Roch B, Mazard T, et al. Circulating nuclear DNA structural features, origins, and complete size profile revealed by fragmentomics. *JCI Insight*. 2021;6(7):e144561.
14 Cristiano S, Leal A, Phallen J, et al. Genome-wide cell-free DNA fragmentation in patients with cancer. *Nature*. 2019;570(7761):385–389.
15 Moss J, Magenheim J, Neiman D, et al. Comprehensive human cell-type methylation atlas reveals origins of circulating cell-free DNA in health and disease. *Nat Commun*. 2018;9(1):5068.
16 El Messaoudi S, Rolet F, Mouliere F, Thierry AR. Circulating cell free DNA: preanalytical considerations. *Clin Chim Acta*. 2013;424:222–230.
17 Meddeb R, Pisareva E, Thierry AR. Guidelines for the preanalytical conditions for analyzing circulating cell-free DNA. *Clin Chem*. 2019;65(5):623–633.
18 Meddeb R, Dache ZAA, Thezenas S, et al. Quantifying circulating cell-free DNA in humans. *Sci Rep*. 2019;9(1):5220.
19 Thierry AR, Mouliere F, El Messaoudi S, et al. Clinical validation of the detection of KRAS and BRAF mutations from circulating tumor DNA. *Nat Med*. 2014;20(4):430–435.
20 Mouliere F, El Messaoudi S, Pang D, Dritschilo A, Thierry AR. Multi-marker analysis of circulating cell-free DNA toward personalized medicine for colorectal cancer. *Mol Oncol*. 2014;8(5):927–941.
21 Valtorta E, Misale S, Sartore-Bianchi A, et al. KRAS gene amplification in colorectal cancer and impact on response to EGFR-targeted therapy. *Int J Cancer*. 2013;133(5):1259–1265.
22 Mouliere F, El Messaoudi S, Gongora C, et al. Circulating cell-free DNA from colorectal cancer patients may reveal high KRAS or BRAF mutation load. *Transl Oncol*. 2013;6(3):319–328.
23 Bustin SA, Benes V, Garson JA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem*. 2009;55(4):611–622.
24 Pisareva E, Mihalovičová L, Pastor B, et al. Neutrophil extracellular traps have auto-catabolic activity and produce mononucleosome-associated circulating DNA. *Genome Med*. 2022;14(1):135.
25 Pisareva E, Badiou S, Mihalovičová L, et al. Persistence of neutrophil extracellular traps and anticardiolipin auto-antibodies in post-acute phase COVID-19 patients. *J Med Virol*. 2023;95(1):e28209.
26 Thierry AR, Pastor B, Jiang ZQ, et al. Circulating DNA demonstrates convergent evolution and common resistance mechanisms during treatment of colorectal cancer. *Clin Cancer Res*. 2017;23(16):4578–4591.
27 Moding EJ, Nabet BY, Alizadeh AA, Diehn M. Detecting liquid remnants of solid tumors: circulating tumor DNA minimal residual disease. *Cancer Discov*. 2021;11(12):2968–2986.
28 Parikh AR, Van Seventer EE, Siravegna G, et al. Minimal residual disease detection using a plasma-only circulating tumor DNA assay in patients with colorectal cancer. *Clin Cancer Res*. 2021;29:5586–5594.
29 Ma Y, Gan J, Bai Y, Cao D, Jiao Y. Minimal residual disease in solid tumors: an overview. *Front Med*. 2023;17(4):649–674.
30 Naidoo M, Gibbs P, Tie J. ctDNA and adjuvant therapy for colorectal cancer: time to Re-invent our treatment paradigm. *Cancers*. 2021;13(2):346.

31 Tie J, Cohen JD, Wang Y, et al. Circulating tumor DNA analyses as markers of recurrence risk and benefit of adjuvant therapy for stage III colon cancer. *JAMA Oncol*. 2019;5(12):1710–1717.
32 Diehl F, Li M, Dressman D, et al. Detection and quantification of mutations in the plasma of patients with colorectal tumors. *Proc Natl Acad Sci U S A*. 2005;102(45):16368–16373.
33 Mehra N, Dolling D, Sumanasuriya S, et al. Plasma cell-free DNA concentration and outcomes from taxane therapy in metastatic castration-resistant prostate cancer from two phase III trials (FIRSTANA and PROSELICA). *Eur Urol*. 2018;74(3):283–291.
34 Fettke H, Kwan EM, Bukczynska P, et al. Prognostic impact of total plasma cell-free DNA concentration in androgen receptor pathway inhibitor–treated metastatic castration-resistant prostate cancer. *Eur Urol Focus*. 2021;7(6):1287–1291.
35 Bettegowda C, Sausen M, Leary RJ, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med*. 2014;6(224):224ra24.
36 Phallen J, Sausen M, Adleff V, et al. Direct detection of early-stage cancers using circulating tumor DNA. *Sci Transl Med*. 2017;9(403):eaan2415.
37 Meng P, Wei J, Geng Y, et al. Targeted sequencing of circulating cell-free DNA in stage II-III resectable oesophageal squamous cell carcinoma patients. *BMC Cancer*. 2019;19(1):818.
38 Schraa SJ, van Rooijen KL, van der Kruijssen DEW, et al. Circulating tumor DNA guided adjuvant chemotherapy in stage II colon cancer (MEDOCC-CrEATE): study protocol for a trial within a cohort study. *BMC Cancer*. 2020;20(1):790.
39 Taniguchi H, Nakamura Y, Kotani D, et al. CIRCULATE-Japan: circulating tumor DNA-guided adaptive platform trials to refine adjuvant therapy for colorectal cancer. *Cancer Sci*. 2021;112(7):2915–2920.
40 Spindler KLG. Methodological, biological and clinical aspects of circulating free DNA in metastatic colorectal cancer. *Acta Oncol*. 2017;56(1):7–16.
41 El Messaoudi S, Mouliere F, Du Manoir S, et al. Circulating DNA as a strong multimarker prognostic tool for metastatic colorectal cancer patient management care. *Clin Cancer Res*. 2016;22(12):3067–3077.
42 Tanos R, Tosato G, Otandault A, et al. Machine learning-assisted evaluation of circulating DNA quantitative analysis for cancer screening. *Adv Sci*. 2020;7(18):2000486.
43 Rosen AW, Gögenur M, Paulsen IW, et al. Perioperative changes in cell-free DNA for patients undergoing surgery for colon cancer. *BMC Gastroenterol*. 2022;22(1):168.
44 Wei Z, Shah N, Deng C, Xiao X, Zhong T, Li X. Circulating DNA addresses cancer monitoring in non small cell lung cancer patients for detection and capturing the dynamic changes of the disease. *SpringerPlus*. 2016;5(1):531.
45 Evrard C, Ingrand P, Rochelle T, et al. Circulating tumor DNA in unresectable pancreatic cancer is a strong predictor of first-line treatment efficacy: the KRASCIPANC prospective study. *Dig Liver Dis*. 2023;55(11):1562–1572.
46 Pastor B, Abraham JD, Pisareva E, et al. Association of neutrophil extracellular traps with the production of circulating DNA in patients with colorectal cancer. *iScience*. 2022;25(2):103826.
47 Margraf S, Lögters T, Reipen J, Altrichter J, Scholz M, Windolf J. Neutrophil-derived circulating free DNA (cf-DNA/NETs): a potential prognostic marker for posttraumatic development of inflammatory second hit and sepsis. *Shock*. 2008;30(4):352–358.
48 Jahr S, Hentze H, Englisch S, et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Res*. 2001;61(4):1659–1665.
49 Loyfer N, Magenheim J, Peretz A, et al. A DNA methylation atlas of normal human cell types. *Nature*. 2023;613(7943):355–364.
50 Lehmann-Werman R, Neiman D, Zemmour H, et al. Identification of tissue-specific cell death using methylation patterns of circulating DNA. *Proc Natl Acad Sci U S A*. 2016;113(13):E1826–E1834.
51 Mattox AK, Douville C, Wang Y, et al. The origin of highly elevated cell-free DNA in healthy individuals and patients with pancreatic, colorectal, lung, or ovarian cancer. *Cancer Discov*. 2023;13(10):2166–2179.
52 Katsman E, Orlanski S, Martignano F, et al. Detecting cell-of-origin and cancer-specific methylation features of cell-free DNA from Nanopore sequencing. *Genome Biol*. 2022;23(1):158.
53 Műzes G, Bohusné Barta B, Szabó O, Horgas V, Sipos F. Cell-free DNA in the pathogenesis and therapy of non-infectious inflammations and tumors. *Biomedicines*. 2022;10(11):2853.
54 Delgado-Rizo V, Martínez-Guzmán MA, Iñiguez-Gutierrez L, García-Orozco A, Alvarado-Navarro A, Fafutis-Morris M. Neutrophil extracellular traps and its implications in inflammation: an overview. *Front Immunol*. 2017;8:81.
55 Zucoloto AZ, Jenne CN. Platelet-neutrophil interplay: insights into neutrophil extracellular trap (NET)-Driven coagulation in infection. *Front Cardiovasc Med*. 2019;6:85.
56 Pretorius E, Thierry A, Sánchez C, et al. Circulating microclots are structurally associated with Neutrophil Extracellular Traps and their amounts are strongly elevated in long COVID patients. *Res Sq*. 2024. https://doi.org/10.21203/rs.3.rs-4666650/v1 [Preprint].
57 Wigerblad G, Kaplan MJ. Neutrophil extracellular traps in systemic autoimmune and autoinflammatory diseases. *Nat Rev Immunol*. 2023;23(5):274–288.
58 Thierry AR, Salmon D. Inflammation-, immunothrombosis,- and autoimmune-feedback loops may lead to persistent neutrophil self-stimulation in long COVID. *J Med Virol*. 2024;96(8):e29887.
59 Osumi H, Shinozaki E, Yamaguchi K, Zembutsu H. Early change in circulating tumor DNA as a potential predictor of response to chemotherapy in patients with metastatic colorectal cancer. *Sci Rep*. 2019;9(1):17358.
60 Wei T, Zhang Q, Li X, et al. Monitoring tumor burden in response to FOLFIRINOX chemotherapy via profiling circulating cell-free DNA in pancreatic cancer. *Mol Cancer Ther*. 2019;18(1):196–203.
61 Demaria M, O'Leary MN, Chang J, et al. Cellular senescence promotes adverse effects of chemotherapy and cancer relapse. *Cancer Discov*. 2017;7(2):165–176.
62 Tissot C, Toffart AC, Villar S, et al. Circulating free DNA concentration is an independent prognostic biomarker in lung cancer. *Eur Respir J*. 2015;46(6):1773–1780.
63 Cervantes A, Adam R, Roselló S, et al. Metastatic colorectal cancer: ESMO clinical practice guideline for diagnosis, treatment and follow-up. *Ann Oncol*. 2023;34(1):10–32.
64 Moik F, Ay C, Pabinger I. Risk prediction for cancer-associated thrombosis in ambulatory patients with cancer: past, present and future. *Thromb Res*. 2020;191:S3–S11.
65 Khorana AA, Mackman N, Falanga A, et al. Cancer-associated venous thromboembolism. *Nat Rev Dis Primers*. 2022;8(1):11.
66 Hisada Y, Geddings JE, Ay C, Mackman N. Venous thrombosis and cancer: from mouse models to clinical trials. *J Thromb Haemostasis*. 2015;13(8):1372–1382.
67 Malka D, Girard N, Smadja DM, et al. Prévention et prise en charge des thromboses associées au cancer: questions pratiques à propos de l'anticoagulation. *Bull Cancer*. 2023;110(2):212–224.
68 Hisada Y, Garratt KB, Maqsood A, et al. Plasminogen activator inhibitor 1 and venous thrombosis in pancreatic cancer. *Blood Adv*. 2021;5(2):487–495.
69 Hao YJ, Chang LW, Yang CY, et al. The rare circulating tumor microemboli as a biomarker contributes to predicting early colorectal cancer recurrences after medical treatment. *Transl Res*. 2024;263:1–14.
70 Moik F, Colling M, Mahé I, Jara-Palomares L, Pabinger I, Ay C. Extended anticoagulation treatment for cancer-associated thrombosis—rates of recurrence and bleeding beyond 6 months: a systematic review. *J Thromb Haemostasis*. 2022;20(3):619–634.
71 Mauracher L-M, Posch F, Martinod K, et al. Citrullinated histone H3, a biomarker of neutrophil extracellular trap formation, predicts the risk of venous thromboembolism in cancer patients. *J Thromb Haemostasis*. 2018;16(3):508–518.
72 Modestino L, Cristinziano L, Trocchia M, et al. Melanoma-derived soluble mediators modulate neutrophil biological properties and the release of neutrophil extracellular traps. *Cancer Immunol Immunother*. 2023;72(10):3363–3376.
73 Thierry AR, Pisareva E. A new paradigm of the origins of circulating DNA in patients with cancer. *Cancer Discov*. 2023;13(10):2122–2124.
74 Henriksen TV, Reinert T, Christensen E, et al. The effect of surgical trauma on circulating free DNA levels in cancer patients—implications for studies of circulating tumor DNA. *Mol Oncol*. 2020;14(8):1670–1679.
75 Urbini M, Marisi G, Azzali I, et al. Dynamic monitoring of circulating tumor DNA in patients with metastatic colorectal cancer. *JCO Precision Oncology*. 2023;7:e2200694.
76 Campani C, Imbeaud S, Ziol M, et al. Dynamic evolution of circulating tumor DNA in patients with hepatocellular carcinoma across tumor stages and treatments. *Dig Liver Dis*. 2023;55:S213–S214.