Italian Journal of
Gynæcology & Obstetrics
September 2017 - Vol. 29 - N. 3 - Quarterly - ISSN 2385 - 0868

# Direct quantification from serum samples of circulating cell-free DNA content: a complementary rapid and inexpensive tool for endometrial cancer management

Lucia Cicchillitti[1*], Giacomo Corrado[2*], Martina De Angeli[3], Emanuela Mancini[1], Ermelinda Baiocco[1],Lodovico Patrizi[3], Ashanti Zampa[1], Roberta Merola[4], Aline Martayan[4], Laura Conti[4], Giulia Piaggio[5**], Enrico Vizza[1**]

[1] Department of Experimental Clinical Oncology, Gynecologic Oncology Unit, IRCCS Regina Elena National Cancer Institute, Rome, Italy
[2] Department of Obstetrics and Gynecology, Gynecologic Oncology Unit, Catholic University of Sacred Heart, Rome, Italy
[3] Department of Biomedicine and Prevention, Obstetrics and Gynecology Unit, University of Rome "Tor Vergata", Rome, Italy
[4] Clinical Pathology, IRCCS Regina Elena National Cancer Institute, Rome, Italy
[5] Department of Research, Advanced Diagnostics and Technological Innovation, Area of Translational Research, IRCCS Regina Elena National Cancer Institute, Rome, Italy

[*] Contributed equally [**] Co-last authors

## ABSTRACT

**Introduction**: the aim of this study was to assess the potential role of cfDNA as a simple and not invasive tool for endometrial cancer (EC) diagnosis by comparing two rapid and inexpensive methodologies.

**Methods**: a cohort of 57 EC serum samples was analysed by both direct SYBR gold stain and qPCR-Alu. As control, we used 21 serum samples from healthy women. Concentrations were extrapolated from a standard curves. The DNA integrity index was calculated as the ratio of longer to total amount of DNA, quantified by qPCR-Alu247 and qPCR-Alu115. Receiver operating characteristic (ROC) analysis was performed to evaluate the discriminating capability between healthy and EC patients of both tests.

**Results**: cfDNA levels, measured with both SYBR gold stain and qPCR-Alu can differentiate between healthy women and EC patients. Augmented cfDNA content significantly increases in high EC grades.

*continued...*

*Corresponding Author: Dr. Giacomo Corrado*
*giacomo.corrado@alice.it*

*DOI: 10.14660/2385-0868-71*

## SOMMARIO

**Introduzione**: lo scopo di questo studio è quello di valutare il potenziale ruolo del DNA libero circolante (cfDNA) come strumento semplice e non invasivo per la diagnosi del cancro endometriale (EC) confrontando due metodologie rapide e poco costose.

**Metodi**: una coorte di 57 campioni di siero è stata analizzata attraverso saggio di colorazione diretta con SYBR gold che attraverso qPCR-Alu. Come controllo, abbiamo utilizzato 21 campioni di siero da donne sane. Le concentrazioni sono state extrapolate da curve standard. L'indice di integrità del DNA è stato calcolato come rapporto tra la più lunga e la quantità totale del DNA, quantificato da qPCR-Alu247 e qPCR-Alu115. L'analisi della caratteristica operativa del ricevitore (ROC) è stata effettuata per valutare la capacità discriminante tra pazienti sani e con EC con entrambi i test.

**Risultati**: i livelli di cfDNA, misurati con SYBR gold e qPCR-Alu, sono in grado di differenziare tra donne sane e pazienti con EC. Il livello di cfDNA aumenta notevolmente nei gradi elevati di EC. Valori più alti di indice di integrità del DNA si ottengono nei grado più elevati di EC, molto probabilmente associati al

*continua...*

*continue from Abstract...*
A higher DNA integrity index is observed in higher EC grade, very likely associated with the occurrence of tumor lymphovascular space invasion (LVSI).
**Discussion**: cfDNA content may help clinical management. SYBR gold assay may represent a sensitive tool for absolute quantification, whereas qPCR-Alu115 and DNA integrity index could be used as rapids complementary tools to stratify high grade EC with risk of metastasis.

**Key words**: uterine fibroids; uterine artery embolization; sexuality; quality of life.

*segue dal Sommario...*
verificarsi di invasione dello spazio linfovascolare tumorale (LVSI).
**Discussione**: il contenuto di cfDNA può aiutare la gestione clinica. Il test attraverso colorazione con SYBR gold può rappresentare uno test sensibile per la quantificazione assoluta del cfDNA, mentre valori ottenuti da qPCR-Alu115 e l'indice di integrità del DNA potrebbero essere utilizzati come rapide metodologie complementari per stratificare EC di alto grado con rischio di metastasi.

**Parole chiave**: endometrial cancer, circulating cell-free DNA, SYBR gold assay, qPCR-Alu, DNA integrity index, lymphovascular space invasion, cancer grade, cancer stage, rOC curve, cut-off.

## INTRODUCTION

Worldwide, endometrial cancer (EC) is the second most common gynecological occurring in reproductive and postmenopausal women, and the sixth most common cancer overall among women [1]. About 75% of women have a cancer confined to the uterus (stage I) and have generally a good prognosis since early diagnosis, however the prognosis for recurrent or metastatic EC remains poor. Identification of patients with poor prognosis represents a particular therapeutic challenge.

Circulating cell-free DNA (cfDNA) provides rapid and noninvasive "liquid biopsy" tool, which gives important complementary information on diagnosis and therapeutic management in cancer patients [2]. CfDNA is a double-stranded molecule, highly fragmented, including nuclear and mitochondrial DNA that is released in the blood stream through several physiological and pathological processes, such as apoptosis, necrosis, autophagy, secretion, and necroptosis [3-6]. The presence of cfDNA within the plasma was first reported in the blood of healthy individuals [7]. Because of technological limitations, only several years later Stroun at al provided the first study supporting that cfDNA does indeed contain tumor DNA in cancer patients [8]. The higher levels of cfDNA are found in serum or plasma of cancer patients than in healthy controls [9,10]. Elevated release of cfDNA occurs in several other disorders, such as infectious and autoimmune diseases, stroke, infarction and trauma [11], thus more specific and accurate methodologies are needed to discriminate the source of cfDNA. CfDNA harbours cancer specific somatic and genetic alterations, such as gene mutations, methylation and microsatellite instability [6,12,13] that discriminate cancer cfDNA from normal cfDNA and assure it as a specific biomarker that provides personalized information to detect residual disease or monitor tumor progression during therapy. However, the technical complexity and high cost associated with analysis of cancer associated genetic and somatic alterations in cfDNA represent a limitation in its practical application in routine patient management.

CfDNA level rapidly increases in blood stream during tumor development mainly by an excessive DNA release by apoptotic and necrotic cells, and the variability of its levels, ranging from 3% to 93% of the total cfDNA in cancer patients [14], associates with tumor burden, stage, vascularity, cellular turnover, and response to therapy with highest levels in advanced and metastatic disease [15,16]. In healthy individuals, the cfDNA in circulating blood is released by apoptosis, whereas in cancer patients the source is throught both apoptosis as well as by necrosis [17]. The major portion of cfDNA in cancer patients is of apoptotic origin, i.e. shorter fragments with 180-200 base pairs or multiples of this unit in length. Increased levels of longer fragments of cfDNA in blood has been shown to be a predictive marker for the presence of malignant tumor DNA [17-19]. Basing on these evidences, currently the DNA integrity, calculated as the ratio of longer to shorter DNA fragments , has been assessed for its diagnostic and prognostic potential in cancer patients.

Alu-quantitative real-time PCR (qPCR-Alu) is the most common method used to detect DNA integrity [20]. The quantification of cfDNA by the qPCR of Alu repeats uses sets of Alu primers that

amplifies shorter (truncated by apoptosis) and longer DNA fragments, with a detection limit of approximately 0.01 ng [21]. Alu sequences are the most abundant short interspersed elements accounting for approximately 10% of the human genome. The molecular weight of each Alu element is 300 bp. [22]. The most commonly used primers for qPCR-Alu are Alu115 and Alu247 [23]. Annealing sites of Alu115 are within the Alu247 sequence, thus results from qPCR-Alu115 represent the total amount of cfDNA. Alu247 primers amplifies only longer DNA fragments. DNA integrity is generally measured as a ratio of longer to shorter DNA fragments, or as a ratio of longer to total amount of DNA quantified by qPCR-Alu247 and qPCR-Alu115,

In our study, we used two rapid and not-expensive methodologies, the SYBR gold staining and the qPCR-Alu, to evaluate the utility of cfDNA content and degree of fragmentation as prognostic tool to help clinical management in EC. Both methods are based on the direct analysis on serum, without preceding DNA purification, thus overcoming artifacts associated with DNA isolation, such as the prevailing short-comings of DNA extraction methods. The SYBR gold assay is based on use of the fluorochrome SYBR gold stain and is a simple, inexpensive and accurate test for total cfDNA quantification [24]. Several studies have already shown the specificity and clinical relevance of this fluorometric assay [25-29].

Our study indicates the utility of quantification of cfDNA level by SYBR gold and qPCR-Alu and degree of fragmentation by qPCR-Alu247 value /qPCR-Alu115 value in association with the clinicopathological characteristics of pre-surgical EC patients as complementary tools in clinical diagnosis.

## MATERIAL AND METHODS

**Patient cohort**: All healthy volunteers and EC patients were recruited at the Regina Elena National Cancer Institute. We collected serum samples from 21 healthy volunteers and 57 EC patients. According with the histologic grade, we analyzed samples from 12 G1, 28 G2 and 17 G3 ECs. **Table I** depicts clinical-pathological characteristics of patients enrolled in this study. The blood of cancer patients was obtained before surgery and before the beginning of any treatment. Information about patients was obtained by reviewing their medical charts.

**Table 1.**
*Clinico-pathological features of our cohort of 57 EC patients.*

| Endometrial cancer (grade) | G1 | G2 | G3 |
|---|---|---|---|
| No of cases | 12 | 28 | 17 |
| Median Age (years) | 57 (range 48-71) | 57 (range 30-73) | 61 (range 52-78) |
| <65 years old (%) | 58,3 | 76,6 | 64,7 |
| >65 years old (%) | 41,7 | 23,4 | 35,3 |
| BMI <30 (%) | 58,3 | 53,3 | 70,5 |
| BMI >30 (%) | 41,7 | 46,7 | 29,5 |
| Figo Stage (%) | | | |
| I | 100 | 89,6 | 41,2 |
| II | 0 | 6,8 | 23,5 |
| III | 0 | 3,4 | 35,3 |
| IV | 0 | 0 | 0 |
| Lymph node metastases (%) | 0 | 0 | 17,6 |

**Serum blood samples storage**: Whole blood samples were collected in Vacutainer tubes without anticoagulant. After collection of the whole blood, the blood was leaved at room temperature to allow to clot. The blood serum will be separated by centrifuging at 1,000–2,000 x g for 10 minutes in a refrigerated centrifuge and stored at –20°C or lower.

**Measurement of cfDNA levels**: SYBR gold stain was performed as described by Goldeshtein et al. [24]. SYBR Gold Nucleic Acid Gel Stain (Invitrogen) was diluted first at 1:1000 in dimethyl sulphoxide (DMSO) and then at 1:8 in phosphate-buffered saline (PBS). Ten microliters of DNA solutions or sera were applied to a black 96-well plates. Forty microliters of diluted SYBR Gold were added to each well (final dilution 1:10,000) and fluorescence measured with a 96well fluorometer at an emission wavelength of 535 nm and an excitation wavelength of 485 nm. Serum samples were diluted in PBS fivefold (20%). Assay was performed in triplicate. Standards were prepared with commercial Salmon sperm DNA.

For qPCR-Alu assay, serum preparation was performed as described by Umetani et al. [19]. Briefly, serum proteins which might hinder the qPCR results by binding to template DNA or DNA polymerase were deactivated by mixing 20 µL of each serum sample with 20 µL of a preparation buffer that contained 2.5% of tween 20, 50 mmol/L Tris, and 1 mmol/L EDTA. This mixture was digested with proteinase K (20 µg) solution for 50 min (Promega) at 56°C, followed by 5 min of heat deactivation and insolubilization for 10 min at 95°C. After subsequent centrifugation of 10,000×g for 5 min, 0.2 µL of supernatant was used as a template for each quantitative real-time polymerase chain reaction (qRT-PCR) using SYBR Green Master Mix (Applied Biosystems, CA, USA)

followed by evaluation of the average of CT values from triplicate reactions from Real Time PCR software. qPCR reaction.

The absolute equivalent amount ofDNA in each sample was determined by a standard curve with serial dilutions (15 ng-0.015 pg) of gently prepared genomic DNA obtained from peripheral blood leukocytes of healthy donor volunteers. A negative control (without template) was performed in each plate. All qPCR assays were performed in a blinded fashion without knowledge of specimen identity. Mean values were calculated from triplicate reactions. The sequences of the ALU115 primers were forward: 5-CCTGAGGTCAGGAGTTCGAG-3 and reverse: 5-CCCGAGTAGCTGGGATTACA-3; ALU247 primers were forward: 5-GTGGCTCACGCCTGTAATC-3 and reverse: 5-CAGGCTG GAGTGCAGTGG-3. DNA integrity index was calculated as qPCR-Alu247 value / qPCR-Alu115 value of each sample.

**Statistical analysis**

Data were reported as mean and standard deviation. In all experiments, comparisons of results between two groups were based on Student's t-test and one-way analysis of variance (ANOVA). P≤0.05 was deemed to be significant variation. The predictive capability (i.e., diagnostic performance) of cfDNA was investigated by means of the area under the ROC (Receiver-Operating Characteristics) curve (AUC). Cut-offs were extrapolated from the curve.

## RESULTS

Comparison between SYBR gold staining and qPCR-Alu115 assay for cfDNA measurement in EC.

Clinicopathological features of EC patients enrolled in this study are depicted in Table 1. Serum samples from 21 healthy patients were used as control. We analysed serum samples from 12 G1, 28 G2, and 17 G3 EC patients. We prepared a standard curve using several dilutions of commercial Salmon Sperm DNA to extrapolate DNA concentrations. The fit of the standard curve (R2) was higher than 0,98. We performed the SYBR gold assay using 10 µL of serum and 40 µL of diluted SYBR gold. Fluorescence was measured with a 96 well fluorometer at an emission wavelength of 535 nm and an excitation wavelength of 485 nm. For qPCR-Alu, we digested 20 µL of serum with proteinase K and used 0.2 µL for the assay. The absolute equivalent amount of DNA in each sample was determined by a standard curve with serial dilutions (15 ng-0.015 pg) of genomic DNA extracted from peripheral blood leukocytes using a specific set of primers for Alu115 amplification. The fit of the standard curve (R2) was higher than 0,99. A significant increase in cfDNA content in G1 and G2 EC patients compared to G1 EC and control group was observed using both assays (**Fig. 1A and 1B**). CfDNA levels in G2 and G3 EC serum samples were very similar. Analysis performed by SYBR gold assay allowed us to detect a significant increase also in G1 EC compared to control samples. This alteration of cfDNA total amount was not related to EC stage, since not significant differences were detected using both assays (**Fig. 1C and 1D**). Our results indicate that cfDNA levels increase in EC and is associated with EC grading. Moreover, SYBR gold assay seems to have a stronger sensibility in discriminating low grade EC from healthy serum samples (**Fig. 1A and 1B**).

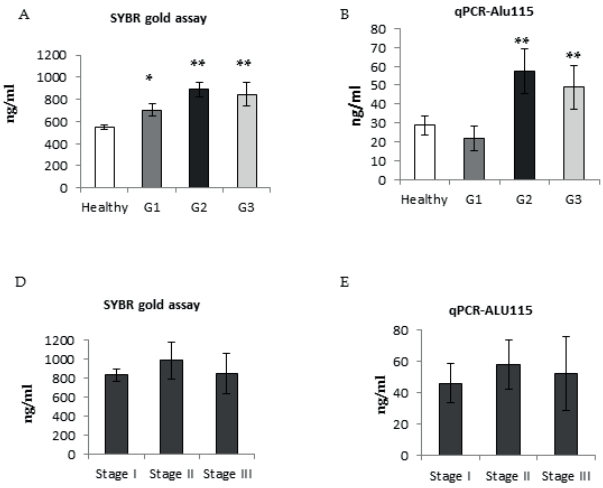


**Figure 1.**
*Comparison between cfDNA level in healthy volunteers and EC patients. Measurement of mean of cfDNA content with SYBR gold assay (1A) and qPCR-Alu115 analysis (1B). Evaluation of cfDNA content in different EC stages with with SYBR gold assay (1C) and qPCR-Alu115 analysis (1D). Statistical significance: : *P≤0.05, **P≤0.01. The error bars indicate the standard error.*

Evaluation of the discriminating capability between healthy and EC patients of SYBR gold and qPCR-Alu115 assays.

To evaluate the predictive capability (i.e. diagnostic significance) of cfDNA measurements by SYBR gold and qPCR-Alu115 assays in EC serum samples, we performed the receiver operating characteristic (ROC) analysis. The ROC curves obtained by plot at different cut-offs (**Fig. 2A and 2B**) showed a moderate predictive accuracy for both tests (**Tab. 2**).

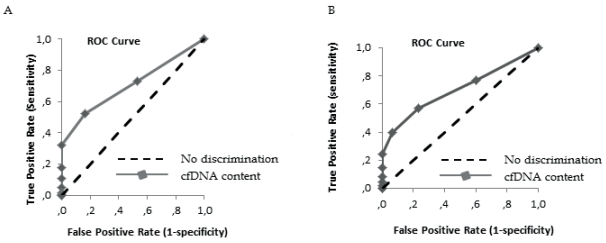


**Figure 2.**
*Receiver operative characteristics (ROC) curves for cfDNA levels measured with SYBR gold stain (2A) and qPCR-Alu115 (2B)*

We identified two cut-offs that best discriminated cfDNA content between healthy women and EC patients: one cut-off, equal to 800 ng/ml, for the SYBR gold staining with 57% sensitivity and 77% specificity, and another, equal to 20 ng/m,l for qPCR-Alu115 assay with 52% sensitivity and 84% specificity (**Tab. 2**).

**Table 2.**
*Receiver operative characteristics (ROC) analyses. Receiver operative characteristics (ROC) results and optimal cut-offs values for cfDNA evaluated by SYBR gold stain and qPCR-Alu115 analysis. AUC: area under the ROC curve. CI: 95% confidence interval .*

| | SYBR Gold Assay | qPCR assay-Alu115 |
|---|---|---|
| **AUC (CI)** | 0,704 (0.632-0.777) | 0,701 (0.612-0.789) |
| **SE** | 0.037 | 0.045 |
| **Cut-off** | 800 ng/ml | 20 ng/ml |
| **Sensitivity** | 57% | 52% |
| **Specificity** | 77% | 84% |

We used these two cut-offs to cluster samples and evaluate the percentage of serum samples with cfDNA content higher than the cut-off values in G1, G2 and G3 EC samples. The number of patients with high levels of cfDNA was higher high grade EC, and this increase was larger for qPCR-Alu115 analysis compared to SYR gold staining. Results are shown in **Table 3**.

**Table 3.**
*Cluster analysis of EC patients with high levels of cfDNA. Percentage of serum samples from G1,G2 and G3 EC patients with cfDNA content higher than optimal cuf-offs extrapolated by ROC analysis measured by SYBR gold assay (800 ng/ml) and qPCR-115 quantification (20 ng/ml).*

| | cfDNA concentration by SYBR Gold assay >800 ng/ml | cfDNA concentration by qPCR-Alu115 assay >20ng/ml |
|---|---|---|
| **G1** | 33.3% | 33.3% |
| **G2** | 46.4% | 60.7% |
| **G3** | 53.0% | 70.6% |

DNA integrity index discriminates aggressive EC.

We also assessed the contribution of the degree of fragmentation in discriminating cfDNA from EC and from healthy sera, measuring the DNA integrity index as qPCR-Alu247 value / qPCR-Alu115value of each sample. We prepared the standard curve with genomic DNA from leukocytes by qPCR with specific primers for Alu247 amplification.

The fit of the standard curve (R2) was higher than 0,99. From the curve, we extrapolated Alu247 concentration in all samples. Then, we calculated DNA integrity as a ratio of qPCR-Alu247 to qPCR-Alu115. We performed ROC analysis by plot at different cut-offs of DNA integrity values. The ROC curve obtained showed a low predictive accuracy ($0.5 < AUC \leq 0.7$) in discriminating healthy volunteers and EC patients. However, analysis of the average of values obtained among different EC grades showed that the DNA integrity index was significantly higher in G3 ($p<0,01$) compared to G1 and G2 EC samples (**Fig. 3A**), whereas not significant alterations were observed among the different stages (**Fig. 3B**). This result indicates a trend towards lower level of DNA fragmentation in higher EC grade, not dependent on stage. We then investigated the possible involvement of lymphovascular space invasion (LVSI) in cfDNA release and integrity. We divided G2-G3 EC serum samples from patients with LVSI (LVSI+) and without LVSI (LVSI-). Measurement of cfDNA, evaluated by SYBR gold assay (**Fig. 3C**) and qPCR-Alu115 was very similar in LVSI+ and LVSI- samples, whereas a significant modulation was observed only for cfDNA fragmentation levels, as indicated by the higher DNA integrity index in LVSI+ serum samples (**Fig. 3E**).

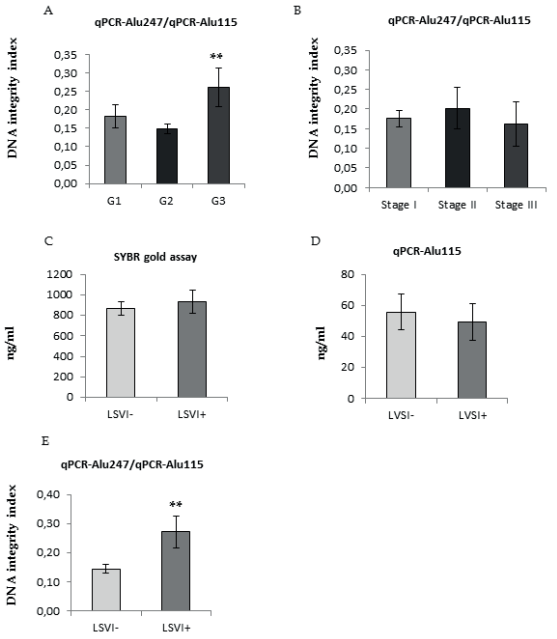


**Figure 3.**
*DNA integrity indexes in different EC grades (3A) and stages (3B). Mean levels of cfDNA measured by SYBR gold assay (3C), qPCR-Alu115 assay (3D), and evaluation of DNA integrity index (3E) in tumors without and with lymphovascular space invasion (LVSI- and LVSI+, respectively). Statistical significance: : *P≤0.05, **P≤0.01. The error bars indicate the standard error.*

## DISCUSSION

EC is the most common type of uterine cancer. The exact cause of EC is unknown. The main risk factors of EC are family history of EC, personal history of certain gynecological diseases, alcohol consumption, and metabolic disorders characteristic of metabolic syndrome. At diagnosis, about 75% of women have a cancer confined to the uterus (stage I) and the prognosis is good, however the prognosis for recurrent or metastatic EC remains poor, thus more sensitive methods and complementary tools to help clinical diagnosis and improve the stratification of EC patients are needed.

In our study, we calculated the amount and the degree of fragmentation of cfDNA in EC serum samples in order to assess its potential role as a simple and inexpensive not invasive tool for EC detection and diagnosis. This study included a cohort of 57 EC serum samples from pre-surgical patients. CfDNA concentration in blood serum was evaluated by SYBR gold assay and by qPCR of Alu sequences, which are 300 base pair (bp) interspersed repeat elements in the human genome, having a copy number of approximately 1 million copies per genome[1]. We used serum since it is believed to be better source of cfDNA than plasma [31].

We observed a significant increase of cfDNA content in high grade EC with both methods, thus indicating the relevance and specificity of cfDNA quantification in EC blood sera. Interestingly, by qPCR-Alu115 assay we could not detect differences between G1 EC and healthy blood sera, whereas a significant modulation was observed using the SYBR gold assay. It is possible that this inability to discriminate between G1 EC and healthy serum samples through qPCR-Alu115 assay is due to the lack of amplification of very small DNA fragments that very likely compromise the yield, whereas the direct SYBR gold assay detects fragments of nucleic acids as small as 12– 18 base pairs.

CfDNA can arise from cancer cells but also from cells in the tumor microenvironment, immune cells or other body organs. Under normal physiologic circumstances, apoptotic and necrotic debris are cleared by infiltrating phagocytes. This mechanism is very likely hampered within the tumoral mass, thus resulting in accumulation of cellular debris and its secretion, includen DNA, into the circulation [32]. CfDNA is also actively released in circulation during tumor growth, thus further increasing its content [33]. We also hypothesises that in G1 EC the clearance capacity of phagocytic is still efficient resulting in a strong DNA nuclease activity and DNA degradation. This mechanism may explain the lack of discriminating capability of the qPCR-Alu115 method thus resulting in the lack of detection of differences between healthy and G1 EC samples. For these reasons, we suggest that SYBR Gold stain may represent a better tool in terms of absolute cfDNA quantification since it does not require prior processing of samples and further amplification steps.

An important criterion for further therapy in cancer is represented by LVSI status. LVSI is characterised by lymphatic and blood vessel invasion involved in tumor metastasis by enhancing dissemination of viable cancer cells and release of DNA from malignant tumor into the blood. Moreover, several studies suggested that LVSI status represents an important prognostic factor for relapse of disease and poor survival in patients with several types of cancer [34-39], included EC [40].

The integrity of cfDNA is elevated in many kinds of cancer, and this biomarker shows great promise for diagnosing cancer and other diseases because of its high sensitivity [20]. Interestingly, we observed a significant increase of DNA integrity in high grade EC serum samples. Moreover, cluster analysis of tumors with LVSI and without LVSI strongly indicate that DNA integrity index may be a sensitive tool for discriminating more aggressive and metastatic EC.

Altogether, our data indicate that assessment of cfDNA content may help clinical management. In particular, we suggest that SYBR gold assay may represent a sensitive tool for EC detection, in particular for low grade EC, whereas both qPCR-Alu115 and DNA integrity index could be a rapid tool to stratify high grade EC with risk of metastasis. Future efforts need to better address and validate the clinical utility of cfDNA analysis in order to harmonize the techniques involved in its quantification and also to include well-powered sample size in study.

## CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

## REFERENCES


1) Weiderpass E, Labrèche F. **Malignant tumors of the female reproductive system**. Saf Health Work. 2012;3(3):166-80.

2) Schwarzenbach H, Hoon DS, Pantel K. **Cell-free nucleic acids as biomarkers in cancer patients**. Nat Rev Cancer. 2011;11(6):426-37.

3) Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD, 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):1659-65.

4)Wang W, Kong P, Ma G, Li L, Zhu J, Xia T, Xie H, Zhou W, Wang S. **Characterization of the release and biological significance of cell-free DNA from breast cancer cell lines**. Oncotarget. 2017;8(26):43180-19.

5) Marzese DM, Hirose H, Hoon DS. **Diagnostic and prognostic value of circulating tumor-related DNA in cancer patients**. Expert Rev Mol Diagn. 2013;13(8):827-44.

6) M. Demers, D.D. Wagner. **Neutrophil extracellular traps: A new link to cancer-associated thrombosis and potential implications for tumor progression**. OncoImmunology 2013; 2(2):e22946.

7) Mandel P, Metais P. **Les acides nucleiques du plasma sanguine chez I'homme**. C R Seances Soc Biol Fil. 1948;142:241Y24.3.

8) Stroun M, Anker P, Maurice P, Lyautey J, Lederrey C, Beljanski M. **Neoplastic characteristics of the DNA found in the plasma of cancer patients**. Oncology 1989;46:318–22.

9) Stötzer OJ, Lehner J, Fersching-Gierlich D, Nagel D, Holdenrieder S. **Diagnostic relevance of plasma DNA and DNA integrity for breast cancer**. Tumour Biol J Int Soc Oncodevelopmental Biol Med. 2014;35:1183–91.

10) Sunami E, Vu AT, Nguyen SL, Giuliano AE, Hoon DS. **Quantification of LINE1 in circulating DNA as a molecular biomarker of breast cancer**. Ann N Y Acad Sci. 2008;1137:171–74.

11) Holdenrieder S, Stieber P. **Clinical use of circulating nucleosomes**. Crit Rev Clin Lab Sci. 2009; 46:1-24.

12) Jung K, Fleischhacker M, Rabien A. **Cell-free DNA in the blood as a solid tumor biomarker–a critical appraisal of the literature**. Clin Chim Acta Int J Clin Chem. 2010;411:1611–24.

13) Galanopoulos M, Tsoukalas N, Papanikolaou IS, Tolia M, Gazouli M, Mantzaris GJ. **Abnormal DNA methylation as a cell-free circulating DNA biomarker for colorectal cancer detection: A review of literature**. World J Gastrointest Oncol. 2017;9(4):142-152.

14) Chu D, Park BH. **Liquid biopsy: unlocking the potentials of cell-free DNA**. Virchows Arch. 2017 May 2. doi: 10.1007/s00428-017-2137-8.

15) Schwarzenbach H, Hoon DS, Pantel K. **Cell-free nucleic acids as biomarkers in cancer patients**. Nat Rev Cancer 2011; 11: 426–37.

16) Holdenrieder S, Stieber P, Bodenmuller H, Busch M, Fertig G, Furst H, Schalhorn A, Schmeller N, Untch M, Seidel D. **Nucleosomes in serum of patients with benign and malignant diseases**. Int J Cancer 2001; 95: 114–20.

17) Jahr S, Hentze H, Englisch S, Hardt D, Fackelmeyer FD, Hesch RD, 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;61:1659–65

18) Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, Thornton K, et al. **Circulating mutant DNA to assess tumor dynamics**. Nat Med. 2008;14:985–90.

19) Umetani N, Kim J, Hiramatsu S, Reber HA, Hines OJ, Bilchik AJ, et al. **Increased integrity of free circulating DNA in sera of Patients with colorectal or periampullary cancer: direct quantitative PCR for ALU repeats**. Clin Chem. 2006;52:1062–69.

20) Yu J, Gu G, Ju S. **Recent advances in clinical applications of circulating cell-free DNA integrity**. Lab Med. 2014 Winter;45(1):6-11.

21) Walker JA, Kilroy GE, Xing J, Shewale J, Sinha SK, Batzer MA. **Human DNA quantitation using Alu element-based polymerase chain reaction**. Anal Biochem. 2003;315:122-28

22) Lander ES, Linton LM, Birren B, et al. **Initial sequencing and analysis of the human genome**. Nature. 2001;409:860-921

23) Umetani N, Kim J, Hiramatsu S, et al. **Increased integrity of free circulating DNA in sera of patients with colorectal or periampullary cancer: direct quantitative PCR for ALU repeats**. Clin Chem. 2006;52:1062-69.

24) Goldshtein H, Michael J, Hausmann JM, Douvdevani A. **A rapid direct fluorescent assay for cell-free DNA quantification in biological fluids**. Ann Clin Biochem. 2009;46(Pt 6):488-9

25) Letendre JA, Goggs R. **Measurement of plasma cell-free DNA concentrations in dogs with sepsis, trauma, and neoplasia**. J.Vet Emerg Crit Care (San Antonio). 2017; 27(3): 307-14.

26) Lazarev I, Leibovitch L, Czeiger D, Sion-Vardi N, Geffen DB, Douvdevani A, et al. **Cell-free DNA blood levels in colorectal cancer patients do not correlate with mismatch repair-proficiency**. In Vivo. 2014; 28(3): 349-54.

27) Agassi R, Czeiger D, Shaked G, Avriel A, Sheynin J, Lavrenkov K, et al. **Measurement of circulating cell-free DNA levels by a simple fluorescent test in patients with breast cancer**. Am. J. Clin Pathol. 2015; 143(1): 18-24.

28) Czeiger D, Shaked G, Sebbag G, Vakhrushev A, Flomboym A, Lior Y, et al. **Elevated Cell-Free DNA Measured by a Simple Assay Is Associated With Increased Rate of Colorectal Cancer Relapse**. Am J Clin Pathol. 2016; 145: 852-57.

29) Czeiger D, Shaked G, Eini H, Vered I, Belochitski O, Avriel A, et al. **Measurement of circulating cell-free DNA levels by a new simple fluorescent test in patients with primary colorectal cancer**. Am J Clin Pathol. 2011; 135(2):264-70.

30) Deininger P. **Alu elements: know the SINEs**. Genome Biol. 2011;12:236.

31) Umetani N, Hiramatsu S, Hoon DS. **Higher amount of free circulating DNA in serum than in plasma is**

**not mainly caused by contaminated extraneous DNA during separation**. Ann N Y Acad Sci. 2006;1075:299-307.
32) Diaz LA, Bardelli A. **Liquid biopsies: genotyping circulating tumor DNA**. J Clin Oncol Off J Am Soc Clin Oncol. 2014;32:579–86.
33) Jahr S, Hentze H, Englisch S, Hardt D, Fackelmeyer FD, Hesch RD, 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;61:1659–65.
34) O'Hanlan KA, Kargas S, Schreiber M, Burrs D, Mallipeddi P, Longacre T, et al. **Ovarian carcinoma metastases to gastrointestinal tract appear to spread like colon carcinoma: implications for surgical resection**. Gynecol Oncol. 1995;59:200–20.
35) Paladini D, Cross P, Lopes A, Monaghan JM. **Prognostic significance of lymph node variables in squamous cell carcinoma of the vulva**. Cancer. 1994;74:2491–96.
36) Roman LD, Felix JC, Muderspach LI, Varkey T, Burnett AF, Qian D, et al. **Influence of quantity of lymph-vascular space invasion on the risk of nodal metastases in women with early-stage squamous cancer of the cervix**. Gynecol Oncol. 1998;68:220–25.
37) Schorge JO, Molpus KL, Koelliker D, Nikrui N, Goodman A, Fuller AF., **Jr Stage IB and IIA cervical cancer with negative lymph nodes: the role of adjuvant radiotherapy after radical hysterectomy**. Gynecol Oncol. 1997;66:31–5.
38) Talbot IC, Ritchie S, Leighton MH, Hughes AO, Bussey HJ, Morson BC. **Spread of rectal cancer within veins. Histologic features and clinical significance**. Am J Surg. 1981;141:15–17.
39) Katz A, Strom EA, Buchholz TA, Theriault R, Singletary SE, McNeese MD. **The influence of pathologic tumor characteristics on locoregional recurrence rates following mastectomy**. Int J Radiat Oncol Biol Phys. 2001;50:735–42.
40) Mariani A, Webb MJ, Keeney GL, Aletti G, Podratz KC. **Predictors of lymphatic failure in endometrial cancer**. Gynecol Oncol. 2002;84:437–442.
41) Mariani A, Webb MJ, Keeney GL, Aletti G, Podratz KC. **Endometrial cancer: predictors of peritoneal failure**. Gynecol Oncol. 2003;89:236–242.