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ORIGINAL ARTICLE

Aberrant Telomere Length in Circulating Cell-Free DNA as Possible Blood Biomarker with High Diagnostic Performance in Endometrial Cancer

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Abstract

To investigate the diagnostic performance of relative telomere length (RTL) in cell-free DNA (cfDNA) for endometrioid endometrial cancer (EC). We measured RTL in cfDNA of 40 EC patients (65 ± 12 years) and 31 healthy controls (HC) (63 ± 13 years), excluding in both groups other oncologic and severe non-oncologic diseases to limit confounders. Circulating cfDNA was extracted from serum using the QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany). After the quantitative real-time polymerase chain reaction, telomere repeat copy number to single-gene copy number ratio was calculated. RTL in cfDNA was found to be significantly lower in EC patients than in HC ($p < 0.0001$). The diagnostic performance of cfDNA RTL was estimated with receiver operating characteristics (ROC) curve analysis, which showed a diagnostic accuracy for EC of 0.87 (95% CI: 0.79–0.95, $p < 0.0001$). The cutoff cfDNA RTL value of 2.505 (T/S copy ratio) reported a sensitivity of 80.0% (95% CI: 64.35–90.95) and a specificity of 80.65% (95% CI: 62.53–92.55). Significant differences of RTL among EC stages or grades ($p = 0.85$ and $p = 0.89$, respectively) were not observed. Our results suggest that cfDNA RTL analysis may be a diagnostic tool for EC detection since the early stage, whilst its diagnostic performance seems unsatisfactory for cancer progression, staging, and grading. However, further studies are needed to confirm these preliminary findings. In particular, future investigations should focus on high-risk patients (such as those with atypical endometrial hyperplasia) that may benefit from this tool, because TL shortening is not specific for EC and is influenced by other oncologic and non-oncologic diseases.

Keywords Endometrial cancer · Endometrial hyperplasia · cfDNA · Telomere length · Diagnosis · Biomarkers

Introduction

Endometrial cancer (EC) represents the most common gynecologic malignancy in developed countries, with an age-standardized incidence of approximately 13.6 per 100,000 women [1]. The prognosis of this cancer is usually favorable in the early stage, with a 5-year overall survival rate approximating 80–90%, but it decreases to 57–46% in patients with advanced, high-grade tumors [2]. Although the many efforts to identify non-invasive diagnostic tests characterized by optimal sensitivity and specificity for early EC detection, many epigenetic or biochemical markers have not found a definitive place in routine diagnostics so far [3–7].

Telomeres are highly specialized regions of repetitive nucleotide sequences coupled with proteins and located at each end of a chromosome in eukaryotic cells [8]. In mammalians, the repetitive DNA sequence (TTAGGG)n is highly conserved, with an average length between 3 and 15 kb [9]. The most important telomere function is protecting the chromosome ends from fusion and degradation, thus deploying genomic stability [10]. Telomere length (TL) is typically preserved by

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telomerase, a ribonucleoprotein reverse transcriptase [11]. When telomeres shorten or undergo a process of uncapping by loss of proteins, the chromosome end is damaged, thus leading the way to the generation of DNA double-strand breaks and chromosomal rearrangements. The consequent activation of DNA repair events (DNA-damage response) and breakage-fusion-bridge cycles can then lead to amplification and overexpression of sub-telomeric oncogenes [12]. Impaired genomic stability, associated with the occurrence of both genetic and epigenetic alterations, can hence predispose to neoplastic transformation [13, 14]. Consistently, several studies showed that both telomere shortening and impaired telomerase activity was involved in the pathogenesis of a different type of cancers [15–21]. Many epidemiological studies have then corroborated these findings, by demonstrating that shorter TL is associated with increased cancer incidence as well as with both all-cause and cancer mortality [22–24].

Although TL is usually assessed in leukocytes, relative TL (RTL) can also be measured in serum cell-free DNA (cfDNA) [25]. Wu et al. described a significant reduction of repetitive telomere sequences in serum cfDNA of patients affected by breast cancer and suggested that TL could be considered a biomarker for early cancer detection [26]. Moreover, the longitudinal evaluation of cfDNA RTL has been recently proposed as a possible non-invasive risk predictive biomarker for hepatocellular carcinoma in patients with chronic Hepatitis B virus infection [27]. Considering this background, the present study aimed to investigate the diagnostic performance of RTL measured in serum cfDNA for endometrioid EC detection and staging.

Materials and Methods

Study Population

The EC group consisted of 40 consecutive patients with a new endometrioid EC diagnosis. We excluded patients affected by any other severe cardiovascular, autoimmune, metabolic or endocrine disease with concurrent pharmacological or non-pharmacological treatments, as well as smokers.

The patients were enrolled in the Obstetrics and Gynecology Clinics of the University Hospital of Verona (Italy). All patients, after diagnosis of endometrioid EC by endometrial biopsy, underwent standard preoperative evaluating procedures including gynecologic examination, transvaginal ultrasound, chest X-Ray, and abdomino pelvic magnetic resonance imaging and/or computed tomography [28]. Blood samples were collected before therapeutic procedures (i.e., surgery, chemotherapy or radiotherapy). All the patients underwent surgical treatment as appropriate. Histopathological analysis after surgery, that represents the standard of reference, confirmed a diagnosis of endometrioid EC in all the patients, and the cancer stage and grade were defined according to the International Federation of Gynecology and Obstetrics (FIGO) system criteria [29].

The control group consisted of 31 female healthy controls (HC), matched by age and body mass index (BMI) with the EC group. Also for controls, we excluded women affected by any other severe cardiovascular, autoimmune, metabolic or endocrine disease with concurrent pharmacological or non-pharmacological treatments, as well as smokers. All healthy controls underwent gynecologic evaluation and transvaginal ultrasound during the previous two years, in order to exclude any relevant gynecological comorbidity.

Telomere Length qRT-PCR

Circulating serum cfDNA was isolated from 200 μL of serum using QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The RTL was assessed using the protocol described by Cawthon, with minor modification [30]. This method used quantitative real-time polymerase chain reaction (qRT-PCR) to measure the ratio between telomere repeat copy number (T) and single-gene copy number (S) in each sample. Briefly, the PCRs (2 μL of each DNA) were carried out in using 10 μL volume and 250 μM of each dNTP (GE Healthcare, Little Chalfont, UK), 1× HotStart Buffer (Qiagen, Hilden, Germany), 2.5 mM MgCl2, 1.5 units Hot-Start polymerase (Qiagen, Hilden, Germany), 2 μM SYTO 9 (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and 1 × ROX reference dye (Thermo Fisher Scientific Waltham, Massachusetts, USA). The primers for telomeres and single-copy gene 36B4 (encodes acidic ribosomal phosphoprotein PO) were added to final concentrations of 0.9 μM and 0.3 μM, respectively.

The primer sequences were: TeFo (5'-CGGTTTGT TTGGGTTTGGGTTTGGGTTTGGGTT-3′), TeRe (5'-GGCTTGCCTTACCCTTACCCTTACCC TTACCCTTACCCT-3′), 36B4Fo (5′- CAGCAAGT GGGAAGGTGTAATCC-3′) and 36B4Re (5'-CCCATTCT ATCATCAACGGGTACAA-3′.)

The enzyme was activated at 95 °C for 10 min, followed by 30 cycles of 95 °C for 5 s, 58 °C for 10 s, and 72 °C for 40 s for the 36B4 reaction, or 20 cycles of 95 °C for 5 s, 54 °C for 45 s, and 72 °C for 45 s for the telomere reaction. The inter-run calibration reaction in each qRT-PCR run contained a standard curve, ranging from 100 ng to 6.25 ng of K562 (Promega, Madison, WI) was included. The T/S ratio was calculated as $2 - [Ct (telomeres)/Ct (36B4)] = 2 - \Delta Ct$. To assess the relative T/S ratio of the sample, the T/S ratio was normalized to ΔCt of standard K562. The final formula for the relative T/S ratio was $2 - (\Delta CtSample - \Delta CtStandard)$. The slope of the standard curve generated for the 36B4 and telomere reaction was −3.45 and − 3.21, respectively.

Statistical Analysis

Descriptive statistics were reported according to data distribution as mean ± standard deviation (SD), or median and interquartile range (IQR) for continuous variables; the categorical variables were reported as absolute number and percentage (%). Fisher's exact test, parametric (t-test, ANOVA) and non-parametric (Mann–Whitney, Kruskal-Wallis) tests were used to compare baseline characteristics and RTL, as appropriate. The RTL values were reported as median and IQR. A comparison of RTL values between two groups was performed with the Mann-Whitney test. The diagnostic performance of cfDNA RTL was estimated using receiver operator characteristic (ROC) curve analysis. The Yuden's J statistic was used in conjunction with ROC analysis to identify the maximum value of Youden's J index identifying the optimal cut-off of cfDNA RTL that maximizes the sensitivity and specificity. The 95% Confidence intervals (CIs) have been reported. The statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego California USA), and the level of statistical significance was set at $p < 0.05$.

Ethics and Methodological Standards

The design, analysis, interpretation of data, drafting and revisions conform the Helsinki Declaration, the Committee on Publication Ethics (COPE) guidelines (http://publicationethics.org/), and the STARD (Standards for Reporting Diagnostic accuracy studies) statements, available through the EQUATOR (enhancing the quality and transparency of health research) network (www.equator-network.org). The study was approved by the independent Institutional Review Board of the University of Verona. Each patient enrolled in the study signed informed consent for all the procedures, to allow blood sample collection and analysis, data collection and analysis for research purposes. The study was not advertised, and no remuneration was offered to the patients to enter or continue the study. An independent data safety and monitoring committee evaluated the results.

Results

We did not find significant differences between EC and HC groups for age ($p = 0.51$), BMI ($p = 0.68$), and ethnicity ($p = 0.88$). Demographics characteristics of the HC group and demographic characteristics and definitive EC stage of the EC group are reported in Table 1.

The values of cfDNA RTL were found to be lower in the EC group, with median 1.58 (IQR: 0.89–2.31), than in the HC group, with median 3.18 (IQR: 2.60–5.13) ($p < 0.0001$; Fig. 1). The area under the ROC curve reported a diagnostic accuracy for EC detection, distinguishing EC patients from HC, of 0.87 (95% CI: 0.79–0.95, $p < 0.0001$) (Fig. 2). The maximal Youden's J index was 0.61 at the cutoff value of 2.505 (T/S copy ratio) for cfDNA RTL, and the optimal sensitivity and specificity were 80.0% (95% CI: 64.35–90.95) and 80.65% (95% CI: 62.53–92.55), respectively. When the analysis of diagnostic performance was limited to EC patients with I stage EC, the diagnostic accuracy was slightly increased to 0.89 (95% CI: 0.81–0.97, $p < 0.0001$) (Fig. 2). No significant differences in cfDNA RTL values were found comparing different EC stages and grades. The EC group was analyzed comparing lymph node involvement (IIIC1 and IIIC2 stage, median 1.60 and IQR 0.88–2.78) versus no lymph node involvement (I and II stage, median 1.56 and IQR 0.89–2.31), and we did not find a significant difference ($p = 0.85$) (Fig. 3). Similarly, no statistically significant difference in cfDNA RTL values was found comparing low grades (1–2, median 1.58 and IQR 0.85–2.34) versus high grade (3, median 1.43 and IQR 0.91–2.42) endometrioid EC ($p = 0.89$) (Fig. 4).

Table 1 Demographics characteristics of endometrial cancer (EC) and healthy control (HC) groups, and definitive endometrioid EC stage and grade in EC group

Variable	EC group ($n = 40$)	HC group ($n = 31$)
Age, years (±SD)	65 (±12)	63 (±13)
BMI, (±SD)	31.3 (±9.18)	30.4 (±8.7)
FIGO stage, n (%)		
Ia	19 (47.5)	
Ib	10 (25.0)	
II	6 (15.0)	
IIIa	0	
IIIb	0	
IIIc1	3 (7.5)	
IIIc2	1 (2.5)	
IV	1 (2.5)	
Histological grade, n (%)		
1	9 (22.5)	
2	21 (52.5)	
3	10 (25.0)	

SD = Standard deviation; EC = Endometrial cancer; HC = Healthy control; BMI = Body mass index

Discussion

In the area of oncological diagnostics, genetic (i.e. mutations, polymorphisms) and epigenetic (i.e. microRNAs, DNA methylation, histones modifications) biomarkers have been investigated in cancer patients, with the aim to develop highly sensitive and specific non-invasive biomarker [31].

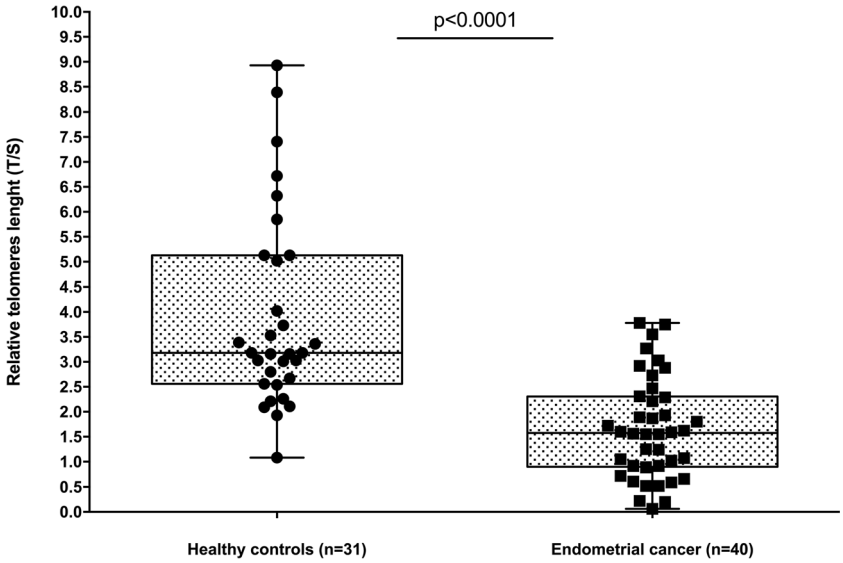

Fig. 1 Dot plot with box and whiskers graph showing the relative telomere length (RTL) values in patients with endometrial cancer (EC) and healthy controls (HC). The center line denotes the median value (EC = 1.58; HC = 3.18), the box contains the 25th (EC = 0.89; HC = 2.60) to 75th (EC = 2.31; HC = 5.13) percentiles of dataset. The black whiskers mark the minimum and maximum values. The dots show the individual data points. T = Telomere repeat copy number; S = Single-gene copy number

The analysis of cfDNA recently became of exceptional interest for the diagnosis of many human disorders, and its potential clinical utility has been demonstrated for early detection of cardiovascular, autoimmune, and neoplastic diseases, as well as for prenatal screening [32–35]. In patients affected by malignant diseases, free-nucleic acids released from neoplastic cells circulate in the bloodstream as tumor-derived cfDNA, in addition to normal cell-derived cfDNA, and represent a potential source of cancer genetic material allowing identification of tumor-associated modifications [36–39]. CfDNA may provide a noninvasive "liquid biopsy", which could give important information about the diagnosis, possible targets of therapy, and drug resistance mechanisms in patients with cancers [40].

Various strategies have been investigated with the aim to use circulating cfDNA as a potential cancer biomarker. The measurement of absolute cfDNA level reported in cancer patients was generally higher than healthy controls, but the increased level is reported varying widely, from 0.01% to more than 90%. The variability of cfDNA levels in cancer patients, that could be explained by different tumor stage, cellular turnover, vascularity, and response to therapy, limits the use of absolute cfDNA level as cancer biomarker [41]. Another cfDNA analysis requires information on specific genetic or epigenetic modifications present in the tumor lesion, making them unsuitable for monitoring cancer progression. A similar but alternative cfDNA evaluation uses a panel of frequently mutated genes in a specific type of cancer; nevertheless, the detection performance still remains undefined [42–44]. Moreover, further studies investigated and demonstrated a potential link between circulating cell-free mitochondrial DNA (cfmtDNA) levels and cancers [45, 46].

Differently to some of the techniques described above, TL estimated by RTL in serum cfDNA proposed by Wu et al. [26] may be considered a possible biomarker for early cancer detection without the need for any prior information. These authors hypothesized that the cfDNA released by tumor cells and circulating in the bloodstream is characterized by shorter telomeres and may allow identifying telomeric abnormality related to breast cancer in the serum of affected patients. They reported an aberrant reduction of telomere repetitive sequences in circulating cfDNA of breast cancer patients and then suggested that cfDNA RTL may be considered a promising biomarker for early cancer detection. On that basis, they

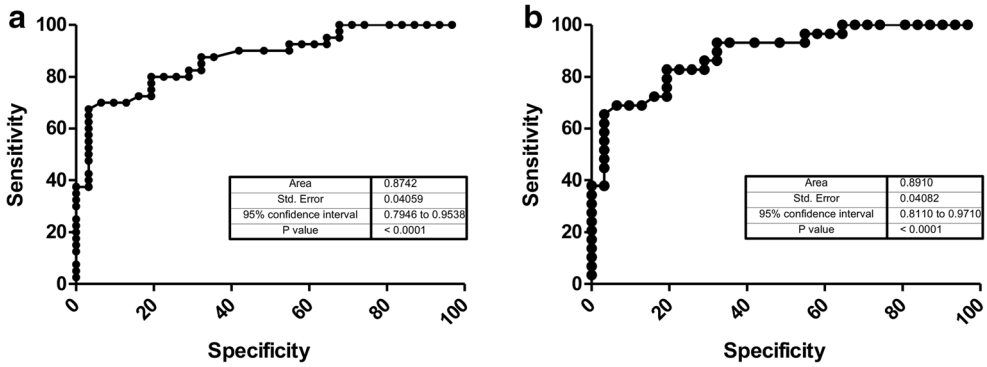

Fig. 2 Receiver operating characteristics (ROC) curve analysis using relative telomere length (RTL) to distinguish total endometrial cancer patients from healthy controls (A) and stage I endometrial cancer patients from healthy controls (B)

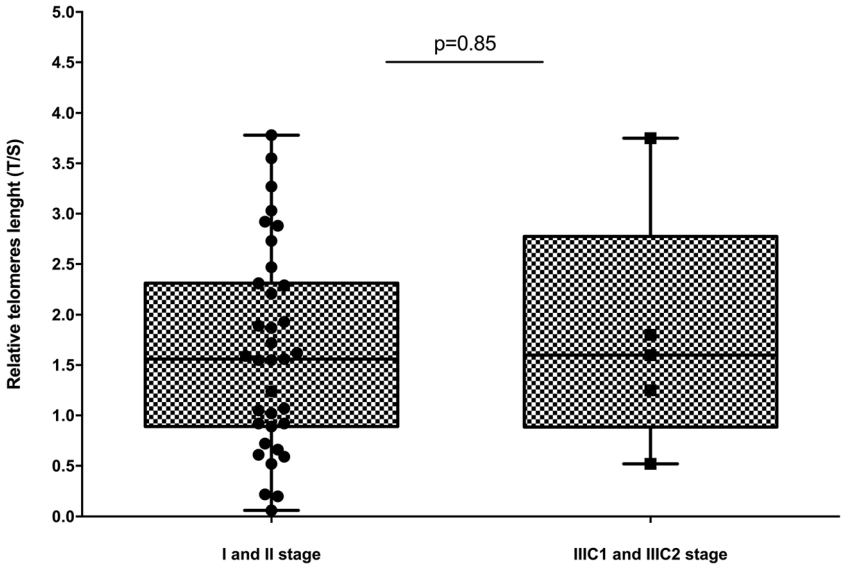

Fig. 3 Dot plot with box and whiskers graph showing the relative telomere length (RTL) values in patients with I and II stage endometrial cancer (EC) and patients with IIIC1 and IIIC2 stage EC. The center line denotes the median value (I and II stage = 1.56; IIIC1 and IIIC2 stage = 1.60), the box contains the 25th (I and II stage = 0.89; IIIC1 and IIIC2 stage = 0.88) to 75th (I and II stage = 2.31; IIIC1 and IIIC2 stage = 2.78) percentiles of dataset. The black whiskers mark the minimum and maximum values. The dots show the individual data points. T = Telomere repeat copy number; S = Single-gene copy number

suggested that this biomarker should be investigated in other types of cancers as a diagnostic tool, if cfDNA RTL shortening will be confirmed as a constant characteristic of carcinogenesis processes [26]. To the best of our knowledge, TL evaluated by RTL in serum cfDNA has never been previously assessed in patients with EC.

Main Results

We have estimated TL by RTL in serum cfDNA using qRT-PCR after the extraction of cfDNA with QIAamp DNA Blood Mini Kit. Our results show that cfDNA RTL was significantly lower in the EC group compared to the HC group, reporting a high diagnostic accuracy of 0.87 (95% CI: 0.79–0.95, $p < 0.0001$) for endometrioid EC. Therefore, results contribute to the accumulating evidence suggesting that telomere shortening, genomic instability, and increased cell death are present in endometrioid EC. Indeed, susceptibility to DNA damage and impaired repair function are present in EC, such as loss of mismatch repair proteins, ARID1A mutation, and microsatellite instability [47, 48]. Although the physiology and biology of cfDNA are not completely understood, results may strengthen the hypothesis that the RTL in cfDNA of the EC group is due to the presence of cancer-derived cfDNA with shortened telomeres in the serum of affected patients. Moreover, although TL is expected to be shortened with the cancer progression, we found that RTL of cfDNA among different cancer stages based on lymph node involvement (I-II vs. IIIC1/IIIC2; $p = 0.85$) or grades (1–2 vs. 3; $p = 0.89$) was not significantly different, and high diagnostic performance was confirmed by our results even in I stage EC (95% CI: 0.81–0.97, $p < 0.0001$). This result may strengthen the

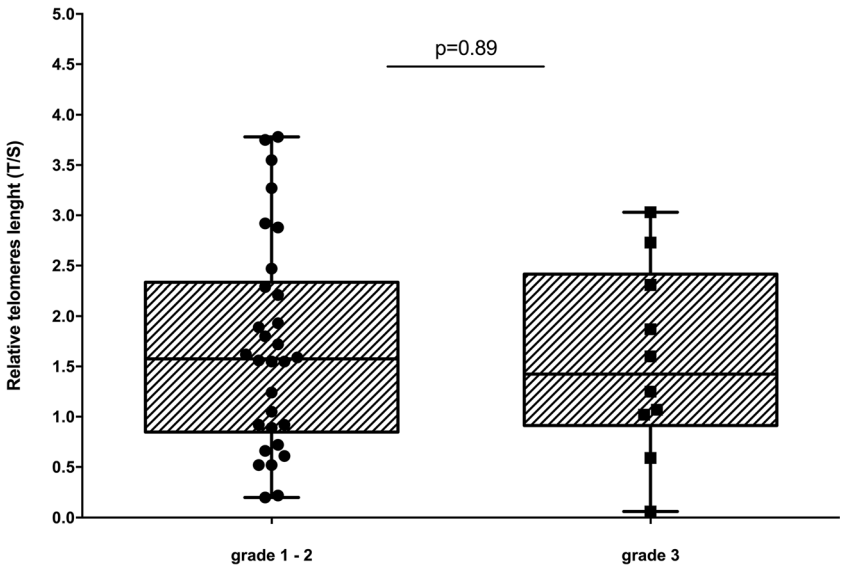

Fig. 4 Dot plot with box and whiskers graph showing the relative telomere length (RTL) values in patients with grade 1 and 2 endometrial cancer (EC) and patients with grade 3 endometrial cancer (EC). The center line denotes the median value (grade 1 and 2 = 1.58; grade 3 = 1.43), the box contains the 25th (grade 1 and 2 = 0.85; grade 3 = 0.91) to 75th (grade 1 and 2 = 2.34; grade 3 = 2.42) percentiles of dataset. The black whiskers mark the minimum and maximum values. The dots show the individual data points. T = Telomere repeat copy number; S = Single-gene copy number

hypothesis that the telomere crisis is present even in the early-stage tumors and stress the potential utility of cfDNA RTL as a non-invasive biomarker in early-stage EC. On that basis, RTL in cfDNA may be further investigated as a non-invasive biomarker that may allow early detection of EC for all the stages of the disease.

Strength, Limitations and Possible Applications

For the proper interpretation of study results and their possible applications, different points need to be discussed. Regarding the limitations of our study, they could be considered the low number of women included, the use of QIAamp DNA Blood Mini kit, and some limitations of the techniques used. QIAamp DNA Blood Mini Kit reported in the healthy group compared to cancer group a slightly but significantly lower total yielded cfDNA [26], although this element is overwhelmed by the fact that absolute cfDNA level was not investigated in our study. Furthermore, qRT-PCR showed that the inter-laboratory coefficient of variation can be as high as 20% [49], although similarly to QIAamp DNA Blood Mini Kit limit RTL is a proportion and may overwhelm the limits of absolute quantification. Finally, the low number of patients limits the evaluation of cfDNA RTL differences among EC stages and grades. This is of paramount importance to evaluate the utility of this tool for prognosis and staging, and further studies with larger groups are needed to confirm no differences among cancer grades, stages and particularly lymph node involvement state.

On the other hand, the strict inclusion and exclusion criteria represent a strong point of the study and a robust background for the data analysis we performed. The inclusion/exclusion criteria were designed to exclude in both groups any other severe disease, with the aim to avoid any potential known and unknown confounder able to influence cfDNA level and TL. Moreover, the EC group and HC group were matched for age and BMI, since age and BMI influenced both cfDNA level and TL [50–53]. About confounders, a strong point of cfDNA RTL measurement is that RTL is a proportion, allowing to reduce the influence of confounders that may alter the absolute level of cfDNA and not the TL.

Regardless strength and limitations of our study, further studies are mandatory to confirm the diagnostic performance, the clinical utility, the cost-effectiveness, and the potential target population of this non-invasive biomarker as a diagnostic tool for EC. The influence of age, BMI, other clinical and demographic factors such as metabolic regulation, diet, hormonal balance, and smoke on the cfDNA RTL in EC patients need further assessment [54–59]. Indeed, reduced TL has been reported associated to aging [60], cardiovascular diseases [61], diabetes [62], schizophrenia [63], depression [64], decline cognitive function [65], cancer [66], smoke [67], diet and BMI [68], and overall mortality [60].

Therefore, although reduced RTL in cfDNA was investigated only in breast cancer [26] and in hepatocellular carcinoma for patients with chronic Hepatitis B virus infection [27], the reduced cfDNA RTL cannot be considered potentially specific for EC, and factors such as age, BMI, non-oncologic and oncologic diseases represent possible confounders. Indeed, an isolated reduced RTL in cfDNA in the general population would provide difficult interpretation. On that basis, cfDNA RTL cannot be used as population screening for the EC detection but may represent a diagnostic tool for EC in high-risk patients, such as those with endometrial hyperplasia and specifically in patients with atypical complex hyperplasia. In these patients, the ability of cfDNA RTL to differentiate endometrial hyperplasia from EC may allow better tailoring of treatment and follow-up, particularly in young patients who would benefit from a fertility-sparing approach. This would be in line with what was already proposed for hepatocellular carcinoma in patients with chronic Hepatitis B virus infection [27] and in breast cancer [26], where cfDNA RTL can be a possible biomarker for the detection of early-stage disease and allow prompt treatment.

Conclusion

The results of our pilot study suggest that measuring RTL in circulating serum cfDNA may be a potentially valuable, non-invasive, simple and relatively inexpensive diagnostic tool for early detection of EC, whilst its diagnostic performance seems almost unsatisfactory for detecting cancer progression, grading and staging. The high performance of cfDNA RTL even in early-stage EC could be useful to allow early and prompt treatment and avoid the disease progression. Nevertheless, because reduced cfDNA RTL is not specific for EC and is influenced by several confounding factors, it cannot be used as population screening for the EC detection; conversely, it may represent a potential non-invasive tool for early identification of EC in the high-risk population, such as those with atypical endometrial hyperplasia or genetic predisposition. If cfDNA RTL will be able to differentiate atypical endometrial hyperplasia from EC, it may allow better tailoring of treatment and follow-up. However, larger studies may be necessary to confirm our preliminary findings and broaden the use of RTL in clinical practice. Moreover, prospective and longitudinal investigations will also be necessary to identify and confirm high-risk patients who may benefit from this tool, considering that reduced RTL is not specific to EC and its use as population screening is not feasible.

Author Contribution G Lippi, M Franchi, F Ghezzi, M Benati and M Montagnana: study conceptualization, and protocol planning. M Benati, E Danese, M Mazzon, E Paviati and M Montagnana: laboratory analysis, data extraction, and statistical analysis. S Garzon, AS Laganà, J Casarin,

S Giudici and R Raffaelli: clinical evaluation, follow-ups, data collection, and statistical analysis. G Lippi, F Ghezzi, M Franchi and M Montagnana: project administration, methodology validation, and supervision. S Garzon, AS Laganà, M Benati and M Montagnana: Manuscript writing/editing. G Lippi, F Ghezzi, M Franchi: Manuscript revision and final approval. All the authors conform the International Committee of Medical Journal Editors (ICMJE) criteria for authorship, contributed to the intellectual content of the study and gave approval for the final version of the article.

Compliance with Ethical Standards

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Conflict of Interest The authors have no proprietary, financial, professional or other personal interest of any nature in any product, service or company. The authors alone are responsible for the content and writing of the paper.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

References

1. Colombo N, Creutzberg C, Amant F, Bosse T, González-Martín A, Ledermann J, Marth C, Nout R, Querleu D, Mirza MR, Sessa C, ESMO-ESGO-ESTRO Endometrial Consensus Conference Working Group (2016) ESMO-ESGO-ESTRO consensus conference on endometrial cancer: diagnosis, treatment and follow-up. Int J Gynecol Cancer 26:2–30. https://doi.org/10.1097/IGC.0000000000000609
2. Yeramian A, Moreno-Bueno G, Dolcet X, Catasus L, Abal M, Colas E, Reventos J, Palacios J, Prat J, Matias-Guiu X (2013) Endometrial carcinoma: molecular alterations involved in tumor development and progression. Oncogene 32:403–413. https://doi.org/10.1038/onc.2012.76
3. Montagnana M, Lippi G, Ruzzenente O, Bresciani V, Danese E, Scevarolli S, Salvagno GL, Giudici S, Franchi M, Guidi GC (2009) The utility of serum human epididymis protein 4 (HE4) in patients with a pelvic mass. J Clin Lab Anal 23:331–335. https://doi.org/10.1002/jcla.20340
4. Montagnana M, Benati M, Danese E, Giudici S, Perfranceschi M, Ruzzenenete O, Salvagno GL, Bassi A, Gelati M, Paviati E, Guidi GC, Franchi M, Lippi G (2017) Aberrant MicroRNA expression in patients with endometrial cancer. Int J Gynecol Cancer 27:459–466. https://doi.org/10.1097/IGC.0000000000000913
5. Benati M, Montagnana M, Danese E, Paviati E, Giudici S, Franchi M, Lippi G (2017) Evaluation of mir-203 expression levels and DNA promoter methylation status in serum of patients with endometrial cancer. Clin Lab 63:1675–1681. https://doi.org/10.7754/Clin.Lab.2017.170421
6. Benati M, Montagnana M, Danese E, Paviati E, Giudici S, Ruzzenente O, Franchi M, Lippi G (2018) The clinical significance of DJ-1 and HE4 in patients with endometrial cancer. J Clin Lab Anal 32:e22223. https://doi.org/10.1002/jcla.22223
7. Casarin J, Bogani G, Serati M, Pinelli C, Laganà AS, Garzon S, Raspagliesi F, Ghezzi F (2019) Presence of glandular cells at the preoperative cervical cytology and local recurrence in endometrial cancer. Int J Gynecol Pathol. https://doi.org/10.1097/PGP.0000000000000642
8. Blackburn EH (1991) Structure and function of telomeres. Nature 350:569–573. https://doi.org/10.1038/350569a0
9. Moyzis RK, Buckingham JM, Cram LS, Dani M, Deaven LL, Jones MD, Meyne J, Ratliff RL, Wu JR (1988) A highly conserved repetitive DNA sequence, (TTAGGG) n, present at the telomeres of human chromosomes. Proc Natl Acad Sci U S A 85:6622–6626. https://doi.org/10.1073/pnas.85.18.6622
10. McClintock B (1941) The stability of broken ends of chromosomes in Zea Mays. Genetics 26:234–282
11. Blasco MA (2005) Telomeres and human disease: ageing, cancer and beyond. Nat Rev Genet 6:611–622. https://doi.org/10.1038/nrg1656
12. Lo AW, Sabatier L, Fouladi B, Pottier G, Ricoul M, Mumane JP (2002) DNA amplification by breakage/fusion/bridge cycles initiated by spontaneous telomere loss in a human cancer cell line. Neoplasia 4:531–538. https://doi.org/10.1038/sj.neo.7900267
13. Maser RS (2002) Connecting chromosomes, crisis, and cancer. Science 297:565–569. https://doi.org/10.1126/science.297.5581.565
14. Bunting SF, Nussenzweig A (2013) End-joining, translocations and cancer. Nat Rev Cancer 13:443–454. https://doi.org/10.1038/nrc3537
15. Counter CM, Hirte HW, Bacchetti S, Harley CB (1994) Telomerase activity in human ovarian carcinoma. Proc Natl Acad Sci U S A 91:2900–2904. https://doi.org/10.1073/pnas.91.8.2900
16. Hastie ND, Dempster M, Dunlop MG, Thompson AM, Green DK, Allshire RC (1990) Telomere reduction in human colorectal carcinoma and with ageing. Nature 346:866–868. https://doi.org/10.1038/346866a0
17. Sieglová Z, Žilovcová S, Čermák J, Říhová H, Březinová D, Dvořáková R, Marková M, Maaloufová J, Sajdová J, Březinová J, Zemanová Z, Michalová K (2004) Dynamics of telomere erosion and its association with genome instability in myelodysplastic syndromes (MDS) and acute myelogenous leukemia arising from MDS: a marker of disease prognosis? Leuk Res 28:1013–1021. https://doi.org/10.1016/j.leukres.2003.11.020
18. McGrath M, Wong JY, Michaud D, Hunter DJ, De Vivo I (2007) Telomere length, cigarette smoking, and bladder cancer risk in men and women. Cancer Epidemiol Biomark Prev 16:815–819. https://doi.org/10.1158/1055-9965.EPI-06-0961
19. Shao L, Wood CG, Zhang D, Tannir NM, Matin S, Dinney CP, Wu X (2007) Telomere dysfunction in peripheral lymphocytes as a potential predisposition factor for renal cancer. J Urol 178:1492–1496. https://doi.org/10.1016/j.juro.2007.05.112
20. Wu X, Amos CI, Zhu Y, Zhao H, Grossman BH, Shay JW, Luo S, Hong WK, Spitz MR (2003) Telomere dysfunction: a potential cancer predisposition factor. J Natl Cancer Inst 95:1211–1218. https://doi.org/10.1093/jnci/djg011
21. Jang JS, Choi YY, Lee WK, Choi JE, Cha SI, Kim YJ, Kim CH, Kam S, Jung TH, Park JY (2008) Telomere length and the risk of lung cancer. Cancer Sci 99:1385–1389. https://doi.org/10.1111/j.1349-7006.2008.00831.x
22. Willeit P, Willeit J, Mayr A, Weger S, Oberhollenzer F, Brandstätter A, Kronenberg F, Kiechl S (2010) Telomere length and risk of incident cancer and cancer mortality. JAMA 304:69–75. https://doi.org/10.1001/jama.2010.897
23. Weischer M, Nordestgaard BG, Cawthon RM, Freiberg JJ, Tybjærg-Hansen A, Bojesen SE (2013) Short telomere length, cancer survival, and cancer risk in 47102 individuals. J Natl Cancer Inst 105:459–468. https://doi.org/10.1093/jnci/djt016
24. Rode L, Nordestgaard BG, Bojesen SE (2015) Peripheral blood leukocyte telomere length and mortality among 64 637 individuals

from the general population. J Natl Cancer Inst 107:1–8. https://doi.org/10.1093/jnci/djv074
25. Wan S, Hann HW, Myers RE, Fu X, Hann RS, Kim SH, Tang H, Xing J, Yang H (2012) Telomere length in circulating serum DNA as a novel non-invasive biomarker for cirrhosis: a nested case-control analysis. Liver Int 32:1233–1241. https://doi.org/10.1111/j.1478-3231.2012.02801.x
26. Wu X, Tanaka H (2015) Aberrant reduction of telomere repetitive sequences in plasma cell-free DNA for early breast cancer detection. Oncotarget 6:29795–29807. https://doi.org/10.18632/oncotarget.5083
27. Wan S, Hann HW, Ye Z, Hann RS, Lai Y, Wang C, Li L, Myers RE, Li B, Xing J, Yang H (2017) Prospective and longitudinal evaluations of telomere length of circulating DNA as a risk predictor of hepatocellular carcinoma in HBV patients. Carcinogenesis 38:439–446. https://doi.org/10.1093/carcin/bgx021
28. Franchi M, Garzon S, Zorzato PC, Laganà AS, Casarin J, Locantore L, Raffaelli R, Ghezzi F (2019) PET-CT scan in the preoperative workup of early stage intermediate- and high-risk endometrial cancer. Minim Invasive Ther Allied Technol. https://doi.org/10.1080/13645706.2019.1624576
29. Creasman W (2009) Revised FIGO staging for carcinoma of the endometrium. Int J Gynecol Obstet 105:109–109. https://doi.org/10.1016/j.ijgo.2009.02.010
30. Cawthon RM (2002) Telomere measurement by quantitative PCR. Nucleic Acids Res 30:e47–e51
31. Montagnana M, Lippi G (2017) Cancer diagnostics: current concepts and future perspectives. Ann Transl Med 5:268–271. https://doi.org/10.21037/atm.2017.06.20
32. Cerne D, Bajalo JL (2014) Cell-free nucleic acids as a non-invasive route for investigating atherosclerosis. Curr Pharm Des 20:5004–5009. https://doi.org/10.2174/1381612819666131206110317
33. Siravegna G, Marsoni S, Siena S, Bardelli A (2017) Integrating liquid biopsies into the management of cancer. Nat Rev Clin Oncol 14:531–548. https://doi.org/10.1038/nrclinonc.2017.14
34. Swarup V, Rajeswari MR (2007) Circulating (cell-free) nucleic acids - a promising, non-invasive tool for early detection of several human diseases. FEBS Lett 581:795–799. https://doi.org/10.1016/j.febslet.2007.01.051
35. Danese E, Montagnana M, Fava C, Guidi G (2014) Circulating nucleic acids and hemostasis: biological basis behind their relationship and technical issues in assessment. Semin Thromb Hemost 40:766–773 https://doi.org/10.1055/s-0034-1387923
36. Schwarzenbach H, Müller V, Stahmann N, Pantel K (2004) Detection and characterization of circulating microsatellite-DNA in blood of patients with breast cancer. Ann N Y Acad Sci 1022:25–32. https://doi.org/10.1196/annals.1318.005
37. Schwarzenbach H, Eichelser C, Kropidlowski J, Janni W, Rack B, Pantel K (2012) Loss of heterozygosity at tumor suppressor genes detectable on fractionated circulating cell-free tumor DNA as indicator of breast cancer progression. Clin Cancer Res 18:5719–5730. https://doi.org/10.1158/1078-0432.CCR-12-0142
38. Phallen J, Sausen M, Adleff V, Leal A, Hruban C, White J, Anagnostou V, Fiksel J, Cristiano S, Papp E, Speir S, Reinert T, Orntoft MW, Woodward BD, Murphy D, Parpart-Li S, Riley D, Nesselbush M, Sengamalay N, Georgiadis A, Li QK, Madsen MR, Mortensen FV, Huiskens J, Punt C, van Grieken N, Fijneman R, Meijer G, Husain H, Scharpf RB, Diaz Jr LA, Jones S, Angiuoli S, Ørntoft T, Nielsen HJ, Andersen CL, Velculescu VE (2017) Direct detection of early-stage cancers using circulating tumor DNA. Sci Transl med 9:eaan2415. https://doi.org/10.1126/scitranslmed.aan2415
39. Medford AJ, Gillani RN, Park BH (2018) Detection of cancer DNA in early stage and metastatic breast cancer patients. Methods Mol Biol 1768:209–227. https://doi.org/10.1007/978-1-4939-7778-9_13
40. Schwarzenbach H, Hoon DS, Pantel K (2011) Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer 11:426–437. https://doi.org/10.1038/nrc3066
41. Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD, Knippers R (2001) DNA fragments in the blood plasma of cancer patients: Quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res 61:1659–1665
42. Heitzer E, Ulz P, Geigl JB (2015) Circulating tumor DNA as a liquid biopsy for cancer. Clin Chem 61:112–123. https://doi.org/10.1373/clinchem.2014.222679
43. Perakis S, Auer M, Belic J, Heitzer E (2017) Advances in circulating tumor DNA analysis. Adv Clin Chem 80:73–153. https://doi.org/10.1016/bs.acc.2016.11.005
44. Ulz P, Heitzer E, Geigl JB, Speicher MR (2017) Patient monitoring through liquid biopsies using circulating tumor DNA. Int J Cancer 141:887–896. https://doi.org/10.1002/ijc.30759
45. Marzese DM, Hirose H, Hoon DS (2013) Diagnostic and prognostic value of circulating tumor-related DNA in cancer patients. Expert Rev Mol Diagn 13:827–844. https://doi.org/10.1586/14737159.2013.845088
46. Chan KC, Jiang P, Zheng YW, Liao GJ, Sun H, Wong J, Siu SS, Chan WC, Chan SL, Chan AT, Lai PB, Chiu RW, Lo YM (2013) Cancer genome scanning in plasma: detection of tumor-associated copy number aberrations, single-nucleotide variants, and Tumoral heterogeneity by massively parallel sequencing. Clin Chem 59:211–224. https://doi.org/10.1373/clinchem.2012.196014
47. The Cancer Genome Atlas Research Network (2013) Integrated genomic characterization of endometrial carcinoma. Nature 497:67–73 https://doi.org/10.1038/nature12113
48. MacKay HJ, Levine DA, Bae-Jump VL, Bell DW, McAlpine JN, Santin A, Fleming GF, Mutch DG, Nephew KP, Wentzensen N, Goodfellow PJ, Dorigo O, Nijman HW, Broaddus R, Kohn EC (2017) Moving forward with actionable therapeutic targets and opportunities in endometrial cancer: NCI clinical trials planning meeting report on identifying key genes and molecular pathways for targeted endometrial cancer trials. Oncotarget 8:84579–84594 https://doi.org/10.18632/oncotarget.19961
49. Martin-Ruiz CM, Baird D, Roger L, Boukamp P, Krunic D, Cawthon R, Dokter MM, van der Harst P, Bekaert S, de Meyer T, Roos G, Svenson U, Codd V, Samani NJ, McGlynn L, Shiels PG, Pooley KA, Dunning AM, Cooper R, Wong A, Kingston A, von Zglinicki T (2015) Reproducibility of telomere length assessment: an international collaborative study. Int J Epidemiol 44:1673–1683. https://doi.org/10.1093/ije/dyu191
50. Cicchillitti L, Corrado G, De Angeli M, Mancini E, Baiocco E, Patrizi L, Zampa A, Merola R, Martayan A, Conti L, Piaggio G, Vizza E (2017) Circulating cell-free DNA content as blood based biomarker in endometrial cancer. Oncotarget 8:115230–115243. https://doi.org/10.18632/oncotarget.23247
51. Vizza E, Corrado G, De Angeli M, Carosi M, Mancini E, Baiocco E, Chiofalo B, Patrizi L, Zampa A, Piaggio G, Cicchillitti L (2018) Serum DNA integrity index as a potential molecular biomarker in endometrial cancer. J Exp Clin Cancer Res 37:16–24. https://doi.org/10.1186/s13046-018-0688-4
52. Harley CB (1991) Telomere loss: mitotic clock or genetic time bomb? Mutat Res 256:271–282. https://doi.org/10.1016/0921-8734(91)90018-7
53. Rizvi S, Raza ST, Mahdi F (2014) Telomere length variations in aging and age-related diseases. Curr Aging Sci 7:161–167 https://doi.org/10.2174/1874609808666150122153151
54. Goya RG, Cónsole GM, Spinelli OM, Carino MH, Riccillo F, Corrons FJ (2003) Glucocorticoid-induced apoptosis in lymphoid organs is associated with a delayed increase in circulating deoxyribonucleic acid. Apoptosis 8:171–177. https://doi.org/10.1023/A:1022922726418

55. Pérez LM, Amaral MA, Mundstock E, Barbé-Tuana FM, Guma FTCR, Jones MH, Machado DC, Sarria EE, Marques e Marques M, Preto LT, Epifanio M, Meinem Garbin JG, Mattiello R (2017) Effects of diet on telomere length: systematic review and meta-analysis. Public Health Genomics 20:286–292. https://doi.org/10.1159/000486586
56. Cherepanova AV, Tamkovich SN, Bryzgunova OE, Vlassov VV, Laktionov PP (2008) Deoxyribonuclease activity and circulating DNA concentration in blood plasma of patients with prostate tumors. Ann N Y Acad Sci 1137:218–221. https://doi.org/10.1196/annals.1448.016
57. Astuti Y, Wardhana A, Watkins J, Wulaningsih W (2017) Cigarette smoking and telomere length: a systematic review of 84 studies and meta-analysis. Environ Res 158:480–489. https://doi.org/10.1016/j.envres.2017.06.038
58. Haghiac M, Vora NL, Basu S, Johnson KL, Presley L, Bianchi DW, de Mouzon SH (2012) Increased death of adipose cells, a path to release cell free DNA into systemic circulation of obese women. Obesity (Silver Spring) Md 20:2213–2219. https://doi.org/10.1038/oby.2012.138
59. Needham BL, Adler N, Gregorich S, Rehkopf D, Lin J, Blackburn EH, Epel ES (2013) socioeconomic status, health behavior, and leukocyte telomere length in the National Health and nutrition examination survey, 1999–2002. Soc Sci Med 85:1–8. https://doi.org/10.1016/j.socscimed.2013.02.023
60. Lu W, Zhang Y, Liu D, Songyang Z, Wan M (2013) Telomeres-structure, function, and regulation. Exp Cell Res 319:133–141 https://doi.org/10.1016/j.yexcr.2012.09.005
61. Haycock PC, Heydon EE, Kaptoge S, Butterworth AS, Thompson A, Willeit P (2014) Leucocyte telomere length and risk of cardiovascular disease: systematic review and meta-analysis. BMJ 349:g4227. https://doi.org/10.1136/bmj.g4227
62. Pavanello S, Angelici L, Hoxha M, Cantone L, Campisi M, Tirelli AS, Vigna L, Pesatori AC, Bollati V (2018) Sterol 27-hydroxylase polymorphism significantly associates with shorter telomere, higher cardiovascular and Type-2 diabetes risk in obese subjects. Front Endocrinol 9:309. https://doi.org/10.3389/fendo.2018.00309
63. Russo P, Prinzi G, Proietti S, Lamonaca P, Frustaci A, Boccia S, Amore R, Lorenzi M, onder G, Marzetti E, Valdiglesias V, Guadagni F, Valente MG, Cascio GL, Fraietta S, Ducci G, Bonassi S (2018) Shorter telomere length in schizophrenia: evidence from a real-world population and meta-analysis of most recent literature. Schizophr Res 202:37–45. https://doi.org/10.1016/j.schres.2018.07.015
64. Needham BL, Mezuk B, Bareis N, Lin J, Blackburn EH, Epel ES (2015) Depression, anxiety and telomere length in young adults: evidence from the National Health and nutrition examination survey. Mol Psychiatry 20:520–528. https://doi.org/10.1038/mp.2014.89
65. Yaffe K, Lindquist K, Kluse M, Cawthon R, Harris T, Hsueh WC, Simonsick EM, Kuller L, Li R, Ayonayon HN, Rubin SM, Cummings SR (2011) Telomere length and cognitive function in community-dwelling elders: findings from the health ABC study. Neurobiol Aging 32:2055–2060. https://doi.org/10.1016/j.neurobiolaging.2009.12.006
66. Sun B, Wang Y, Kota K, Shi Y, Motlak S, Makambi K, Loffredo CA, Shields PG, Yang Q, Harris CC, Zheng YL (2015) Telomere length variation: a potential new telomere biomarker for lung cancer risk. Lung Cancer 88:297–303. https://doi.org/10.1016/j.lungcan.2015.03.011
67. Latifovic L, Peacock SD, Massey TE, King WD (2016) The influence of alcohol consumption, cigarette smoking, and physical activity on leukocyte telomere length. Cancer Epidemiol Biomarkers Prev 25:374–380. https://doi.org/10.1158/1055-9965.EPI-14-1364
68. Shammas MA (2011) Telomeres, lifestyle, cancer, and aging. Curr Opin Clin Nutr Metab Care 14:28–34. https://doi.org/10.1097/MCO.0b013e32834121b1