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Total cardiolipin levels in gastric and colon cancer: evaluating the prognostic potential

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Abstract

Background Cardiolipin (CL) is a signature phospholipid of mitochondria that maintains the integrity of mitochondrial membrane and supports proper mitochondrial function. Alterations in CL level and composition can impair or, conversely, improve mitochondrial function and bioenergetics, both of which are critical for cancer metabolism. However, conflicting reports on CL levels across different cancer types and limited research using human patient samples limit our understanding of its diagnostic potential.

Methods This cross-sectional study explores CL concentrations in gastric and colon cancer tissues using a CL-specific fluorescent probe MitoCLue and compares them to adjacent healthy tissues.

Results In gastric cancer, CL levels showed no significant differences between tumor and healthy tissues, suggesting that metabolic shifts in gastric cancer do not affect total CL content. In contrast, colon cancer tissues exhibited a significant 33% increase in CL levels, indicating mitochondrial adaptation and/or increase in mitochondrial mass in colon cancer. No associations were found between CL levels and patient demographic factors; although a weak correlation with body mass index was noted.

Conclusion We successfully applied MitoCLue to quantitatively assess the total CL level in healthy and tumor tissues from patients with gastric or colon cancer. The distinct CL levels in gastric and colon cancer suggest that there are cancer-type specific mitochondrial adaptations, reflecting unique bioenergetic demands and metabolic reprogramming pathways. While a 33% increase in CL levels was observed in colon cancer tissues compared to healthy adjacent tissues, this modest variation may limit its utility as a standalone biomarker.

Keywords Colon cancer, Gastric cancer, Cardiolipin, Biomarker, Diagnostic

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Introduction

According to the World Health Organization, cancer remains one of the leading causes of death globally. The latest statistics indicate that nearly 20 million new cancer cases were reported in 2022 alone [1]. Colorectal cancer and gastric cancer are ranked among most common cancer types (3rd and 5th, respectively) with high lethality rates (2nd and 5th, respectively). The high incidence and mortality rates associated with gastric and colon cancers, especially in younger population (< 55 years old) highlight the urgent need for new prognostic biomarkers that could aid in the early detection, provide better prognostic value, and suggest personalized treatment strategies [2].

Cardiolipin (CL) is a unique phospholipid localized almost exclusively in the inner mitochondrial membrane (IMM), where it constitutes approximately 20% of the total IMM phospholipid content [3]. CL is essential for maintaining the structural integrity and function of mitochondria, playing a critical role in membrane morphology, stability, and dynamics [4]. Due to its molecules conical shape, CL helps sustain the IMM's intrinsic curvature, which is crucial for cristae formation and the stabilization of electron transfer system (ETS) supercomplexes — both necessary for efficient oxidative phosphorylation and ATP production [5–9]. Given its crucial functions, disruptions in CL content or metabolism can significantly impact mitochondrial function and contribute to a range of pathological conditions, including cardiovascular diseases, neurodegenerative disorders, aging and cancer [10, 11].

Alterations in CL content and composition are particularly interesting in cancer because they could have diagnostic and therapeutic implications. However, the immense diversity of cancer types means that there are myriads of different metabolic patterns that can influence CL levels in one direction or another. For example, previously we found that breast adenocarcinoma, T cell leukemia, colon carcinoma, and pancreatic carcinoma cells exhibit significantly higher levels of CL than rat cardiomyoblasts, which are considered to be rich in mitochondria [12]. Peyta et al. reported a similar trend – mitochondria of tumorigenic hepatic cells contain more CL compared to primary human hepatocytes [13]. Zhang et al. found that the human oncocytic thyroid tumors have a 2-fold higher CL level than non-oncocytic tumors and a 40-fold higher level than normal tissue [14]. In addition, they discovered greater diversity in the CL species in oncocytic tumors, with an increase in oxidized forms of CL.

In contrast, some studies report a decrease in total CL levels in specific types of cancer. Kiebish et al. discovered that in several mouse brain tumor models, mitochondria have a higher abundance of immature CL species and lower levels of mature CL species, but the total CL content was significantly decreased in most of the tumors. This reduction is naturally reflected in significantly lower activity of complexes I, I/III and II/III in brain tumors compared to healthy tissues and shifts cancer away from using any fuel other than glucose, promoting Warburg effect [15]. Furthermore, the findings of Peyta et al. do not follow those of Zhong et al., who observed that in patients with hepatocellular carcinoma (HCC), the total CL content in tumor tissue was lower compared to adjacent healthy tissue. Interestingly, they observed that in tumor tissues, the concentration of unsaturated CLs, and consequently their peroxidation products, gradually decreases as cancer progresses. This decrease and shift towards saturated CLs was found to enhance resistance to oxidative stress, reduce apoptotic susceptibility and enhance resistance to sorafenib in late-stage HCC [16].

These dichotomic discrepancies in the literature likely arise from fundamental metabolic differences between in vitro cellular systems, animal models, and actual human tumors. This hypothesis is supported by the fact that CL level can be influenced by various factors, including diet and cell medium supplementation, and several metabolites such as lactate [17] and fatty acids [18–20] have been shown to affect both composition and the level of CL.

Moreover, measuring CL concentration and identifying CL species present additional challenges. Quantitative analysis of CL is typically performed using high-performance liquid chromatography-electrospray ionization mass spectrometry (HPLC-ESI-MS), which requires lipid extraction from biological samples and subsequent lipid separation. However, the high diversity of CL species (saturated, unsaturated, oxidized, short and long fatty acid residues) creates significant variability in their physicochemical properties that will, obviously, result in the loss of at least some portion of the original CL content. It has been reported that matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) can be used directly on isolated mitochondrial fractions to measure CL concentration without the need of lipid extraction [21, 22]. However, the number of CL species detected and overall method sensitivity are heavily dependent on the matrix of choice [23]. Like ESI-MS, MALDI-MS suffers from 'ion suppression effect' caused by lipid-lipid interactions [24, 25]. Moreover, CL species do not ionize equally well, and the correlation between ionization intensity and molecular weight is not linear [26]. For instance, tetramyristoyl (14:0) CL, commonly used as a CL standard, ionizes much more efficiently than CLs with higher molecular weights, potentially leading to inaccurate results. On top of that, data analysis is complex and requires specialized software. Other MS techniques, such as desorption electrospray ionization mass spectrometry (DESI-MS) [14] or matrix-assisted laser desorption/

ionization mass spectrometry imaging (MALDI-MSI) [27], can also be used for semi-quantitative CL analysis directly in a tissue of interest.

Another approach to measure total CL concentration involves the use of specific fluorescent probes. We have previously developed an advanced CL-specific probe, MitoCLue (CAS No.: 2648425-06-1, 3,6-di(azetidin-1-yl)-10-(3-(trimethylsilyl)propyl)acridin-10-ium iodide), which can be used for CL quantification in isolated mitochondrial fractions without the need for expensive equipment or specialized software [12]. This method is simple, relatively fast, and offers a practical alternative for the total CL analysis.

Given that the level of CL depends on the origin of the sample, it is crucial to focus on patient-derived tumors to better understand the role of CL in human cancer progression and its potential as a biomarker. In this study, we measured CL levels in malignant neoplasms from the stomach and colon using MitoCLue and compared them to adjacent healthy tissues. Additionally, we analyzed various clinical parameters such as sex, age, body mass index, and smoking status, all of which may influence CL levels.

Methods

Tissue collection

This study was conducted as a retrospective cross-sectional observational analysis, using tumor and adjacent healthy tissues collected from patients diagnosed with gastric and colon cancer. Retrospectively collected samples were used from the Biobank run by the Institute of Clinical and Preventive Medicine, University of Latvia, and Riga East University hospital. Stringent sample collection protocol has been followed while embedding samples to the biobank. Tissue samples were obtained immediately after the surgical sample has been removed at surgery. Cancerous tissue as well as normal tissue without the evidence of malignant infiltration were resected from the surgically removed material and evaluated by gross evaluation. Approximately 100 mg of each tissue was provided for the measurements. Samples were snap-frozen in liquid nitrogen. Thereafter, samples were stored in a -80° C freezer until selected for the analysis. A formal sample size calculation was not performed due to the retrospective nature of the study and the limited availability of high-quality paired tissue samples from the biobank. We analyzed the maximal number of eligible samples available to ensure robust and meaningful comparisons within the constraints of this dataset.

Isolation of mitochondrial fractions

The biopsy material from healthy or tumor tissue was homogenized on ice in 1:10 (w/v) medium containing 180 mM KCl, 10 mM Tris/HCl and 1 mM EGTA (pH 7.7 at 4 °C) using a Teflon glass homogenizer. The homogenate was centrifuged at 750 × *g* for 5 min at 4 °C, after that supernatant was separated and centrifuged at 6800 × *g* for 10 min at 4 °C. The obtained mitochondrial pellet was resuspended and washed twice (10 min at 6800 × *g*) with buffer containing 10 mM KCl and 10 mM Tris/HCl (pH 6.7 at 4 °C). The resulting mitochondrial pellets were frozen at − 80 °C and stored until analysis. Prior to analysis, the mitochondrial pellets were diluted with 300 µL of 20 mM HEPES buffer (pH 6.8).

Determination of protein concentration

Protein concentration was measured using the Lowry method [28]. Briefly, the mitochondrial solution was diluted with 0.1% Triton X-100 and 20 µL of the diluted sample or standard (bovine serum albumin, BSA) was added into wells of a 96-well plate. To each well, 200 µL of freshly prepared Lowry reagent (a mixture of Lowry A and B solutions in a 50:1 ratio) were added, and the plate was incubated at room temperature for 10 min. Then, 20 µL of Folin-Ciocalteu reagent was added, followed by mixing for 30 s. The plate was incubated for an additional 30 min at room temperature. Absorbance was measured at 650 nm using Hidex Sense microplate reader (Hidex, Finland). Protein concentration was calculated based on a standard curve generated with known concentrations of BSA, after blank subtraction.

Citrate synthase activity assay

The activity of citrate synthase (CS) was measured using the method of Srere [29], with slight modifications. Briefly, 10 µL of the diluted mitochondrial solution was added to 90 µL of the assay medium, which contained 100 mM Tris (pH 8.1), 0.25% Triton X-100, 5 mM EDTA, 0.1 mM DTNB (5,5'-Dithiobis(2-nitrobenzoic acid), Ellman reagent), 0.25 mM acetyl-CoA, and 0.5 mM oxaloacetic acid. The assay was performed in duplicate using 96-well plates, and the reaction was monitored with a Hidex Sense microplate reader (Hidex, Finland) by measuring absorbance at 412 nm at 25 °C, with readings taken every 20 s for 10 min. A standard curve with known concentrations of CoA was generated in parallel and used to calculate the concentration of CoA–thionitrobenzoic acid (CoA-TNB) formed in the reaction. The rate of CoA-TNB formation was determined from the rate of absorbance change. Specific activity (SA) of CS is expressed in U/mg protein, where 1 U of CS catalyzes the formation of 1 µmol of citrate per minute.

Preparation of CL standard

Cardiolipin (CL) containing liposomes for quantitative analysis of CL were prepared by the thin film method. Briefly, the desired volume of stock solutions of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, 25 mg/ml

solution in $CHCl_3$, Avanti Polar Lipids) and bovine heart CL (5 mg/ml solution in EtOH, Sigma-Aldrich) were completely evaporated under reduced pressure, and the resulting lipid film was resuspended in HEPES buffer (20 mM, pH 6.8) to obtain 100:300 μM CL/DOPC solution. The obtained large multilamellar liposomes were sonicated in a bath-type sonicator (Cole Parmer Ultrasonic Cleaner 8891CPX, USA) at room temperature for 30 min followed by extrusion (LiposoFast-Basic, Avestin) through a 100 nm polycarbonate filter (Whatman® Nuclepore™). The quality of the resulting small unilamellar vesicles (size, polydispersity index) was tested by a dynamic light scattering technique (Zetasizer Nano ZSP, Malvern Panalytical Ltd., UK). All liposome samples were freshly prepared prior to each set of experiments. Liposomes containing CL analytical standard consisting of unnatural 24:1(3)-14:1, 14:1(3)-15:1, 15:0(3)-16:1, 22:1(3)-14:1 CL`s (Cardiolipin Mix I, Avanti Polar Lipids) were prepared in a similar manner.

Determination of cardiolipin concentration

The concentration of cardiolipin (CL) was measured using a previously described method [12], adapted to a 96-well plate format and performed in triplicate. Briefly, 20 μL of diluted mitochondrial solution was added to 80 μL of assay medium containing 20 mM HEPES (pH 6.8) and 20 μM MitoCLue. The plate was then incubated for 15 min at 25 °C in a plate shaker. Fluorescence intensity was measured using a CLARIOstar Plus microplate reader (BMG Labtech, Germany) with excitation and emission wavelengths of 497 nm and 529 nm, respectively. Prior to determining CL concentration in the mitochondrial fractions, a CL standard titration (CL-DOPC liposomes as described above, CL in 0–10 μM range) was performed in triplicate to generate a calibration curve. The CL concentration in each sample well was calculated by interpolation from the calibration curve, and the total mitochondrial CL concentration was calculated using Eq. 1:

$$C_{CL} = \frac{n \times D \times 1000}{V \times P}; nmol/mg\ prot \quad (1)$$

Where:

n is the amount of CL in the sample well, nmol;

D is the sample dilution factor;

V is the sample volume added into the reaction well, μL;

P is the mitochondrial protein concentration of the sample, mg/ml.

CL concentration in respect to CS activity was calculated using Eq. 2:

$$/\, C_{CL} = \frac{C_{CL}}{SA_{CS}}; nmol/U\ CS \quad (2)$$

Where:

C_{CL} is the concentration of CL in nmol/mg prot;

SA_{CS} is the specific activity of the CS in U/ mg prot.

Statistical analysis

Statistical analysis was performed using R Studio (version 4.4.1) with the *ggbetweenstats* package for both data analysis and visualization. To determine the appropriate statistical methods, the normality of the data was first assessed using the Shapiro-Wilk test. Since the data did not follow a normal distribution, non-parametric tests were employed throughout the analysis.

For paired comparisons of CL levels between healthy and tumor tissues within the same patient group, the Wilcoxon signed-rank test was used. For unpaired comparisons between independent groups, the Mann-Whitney U test was applied. Results are reported with *p*-values and 95% confidence intervals where applicable, and a significance threshold of $p < 0.05$ was considered for all tests.

In addition, Spearman's rank correlation coefficient (Spearman's ρ) was used to assess potential correlations between CL levels and patient age or BMI. Correlation coefficients were interpreted as follows: strong correlation ($\rho \geq 0.7$), moderate correlation ($0.7 > \rho \geq 0.5$), weak correlation ($0.5 > \rho \geq 0.3$), and no correlation ($\rho \leq 0.3$). All correlations are reported with corresponding *p*-values, with statistical significance set at $p < 0.05$.

Results

Demographic and clinical data

The study included frozen tumor and adjacent healthy tissue material from two groups of patients: patients with colon cancer ($n = 41$) and with gastric cancer ($n = 40$). The descriptive statistics for both groups are summarized in the Table 1. In the colon cancer group, the patients were almost equally distributed by sex, 51% being male ($n = 21$) and 49% female ($n = 20$), but the gastric cancer group had a higher proportion of females, with 70% female ($n = 28$) and only 30% ($n = 12$) male patients. The patients in both groups were predominantly elderly, with a median age of 72 years and 68 years for colon and gastric cancer patients, respectively. The median body mass index (BMI) was similar across groups, at 27.9 kg/m² for colon cancer patients and 25.9 kg/m² for gastric cancer patients. In terms of BMI categories, 41% of colon cancer patients were classified as overweight (BMI 25–30), and 22% as obese (BMI > 30). Similarly, 38% of gastric cancer patients were overweight, while 18% were obese. Around 60% ($n = 24$) of the patients were current or ex-smokers in the colon cancer group, while in the gastric cancer group the percentage of current or ex-smokers was reverse. No

Table 1 Descriptive statistics of patient groups

	Colon cancer	Gastric cancer
	N **= 41**	***N*** **= 40**
Sex		
Male	21 (51%)	12 (30%)
Female	20 (49%)	28 (70%)
Age (years)		
Mean (SD)	69 (10)	67 (12)
Median [Min, Max]	72 [34, 81]	68 [31, 86]
BMI, kg/m²		
Mean (SD)	27.2 (4.7)	26.4 (5.8)
Median [Min, Max]	27.9 [18.0, 40.5]	25.9 [18.8, 53.4]
BMI category		
Underweight (< 18.5 kg/m²)	1 (2.4%)	0 (0%)
Normal (18.5–24.9 kg/m²)	14 (34%)	18 (45%)
Overweight (25–30 kg/m²))	17 (41%)	15 (38%)
Obese (> 30 kg/m²))	9 (22%)	7 (18%)
Smoking status		
Never smoked	24 (59%)	16 (40%)
Current or ex-smoker	17 (41%)	24 (60%)
Stage		
I	-	4 (10%)
II	-	12 (30%)
III	-	20 (50%)
IV	-	2 (5%)
No Data	-	2 (5%)
T Stage		
T1	-	3 (7.5%)
T2	-	6 (15%)
T3	-	8 (20%)
T4	-	23 (57.5%)
N Stage		
N0	-	11 (27.5%)
N1	-	10 (25%)
N2	-	8 (20%)
N3	-	11 (27.5%)
M Stage		
M1	-	38 (95%)
M2	-	2 (5%)

data on the stage of cancer was available for the colon cancer cohort, whereas most gastric cancer patients (50%) were diagnosed with stage III without metastases (95%). None of the patient in both groups received chemotherapy or radiotherapy prior to surgery.

CL standard comparison

Quantitative analysis of CL was performed using CL-containing liposomes as a standard. For the preparation of the standard, we evaluated two commercially available CL solutions with concentrations predetermined by the manufacturer: CL from bovine heart, which contained approx. 90% 18:2(4), 5% 18:1(4), and 5% CL with an unknown fatty acid distribution, and analytical CL Mix I, containing equal amounts of 14:1(3)-15:1, 15:0(3)-16:1, 22:1(3)-14:1 and 24:1(3)-14:1 CLs. MitoCLue bound to CLs in a 2:1 ratio using both standard preparations, and the titration slopes were nearly identical. Therefore, unlike mass spectrometry-based techniques, MitoCLue provides a more accurate measurement of total CL concentration, as it binds equally well to various CL species regardless of the length of the fatty acid chain or degree of unsaturation (Fig. 1). Furthermore, the less expensive CL from bovine heart can be used successfully as a titration standard, as it produces comparable results.

CL levels in healthy and tumor tissues

The analysis of CL levels began with the isolation of mitochondrial fractions from tissue samples by homogenization and differential centrifugation. This step is crucial to prevent nonspecific interactions between MitoCLue and other cellular components. Once the mitochondrial fractions are isolated, it is necessary to measure mitochondrial content to accurately relate and normalize the CL levels. Various mitochondrial markers are commonly used for this purpose, including mitochondrial protein content, citrate synthase (CS) activity, activity of mitochondrial respiratory complexes, mitochondrial DNA (mtDNA) content, or CL levels themselves. The measurement of respiratory complex activity is time-consuming and would have created a bottleneck in the overall analysis, while mtDNA is considered the least reliable marker for estimating mitochondrial content [30]. Therefore, we analyzed CL levels between healthy and tumor tissues in patients with gastric or colon cancer, using two normalization approaches: CL concentration relative to protein content (nmol/mg prot.) and to CS activity (nmol/U CS).

We compared CL concentration in healthy and tumor tissue from the same patients, therefore Wilcoxon signed-rank test was used as the data did not follow normal distribution. In gastric cancer patients, the median CL concentration in healthy tissue was 121.1 nmol/mg prot., compared to 110.0 nmol/mg prot. in tumor tissue (Fig. 2A). Although this 9.1% decrease in CL levels approached statistical significance ($V = 519.0$, $p = 0.07$, 95% CI = [-0.01, 0.60]), these changes are not substantial enough to be considered a reliable clinical marker.

When normalized to CS activity, the median CL concentration was 809.5 nmol/U CS in healthy tissues and 752.9 nmol/U CS in tumor tissues (Fig. 2B), with no statistically significant difference ($V = 442.0$, $p = 0.67$, 95% CI = [-0.27, 0.41]). Also, no statistically significant variation in CL levels was observed across the different cancer stages using both metrics (ESI). These results suggest that CL levels do not differ between normal and malignant tissues in gastric cancer. Although numerous studies have reported that gastric cancer cells and tissues tend to shift toward glycolysis [31], particularly under hypoxic conditions, and exhibit reduced expression of specific

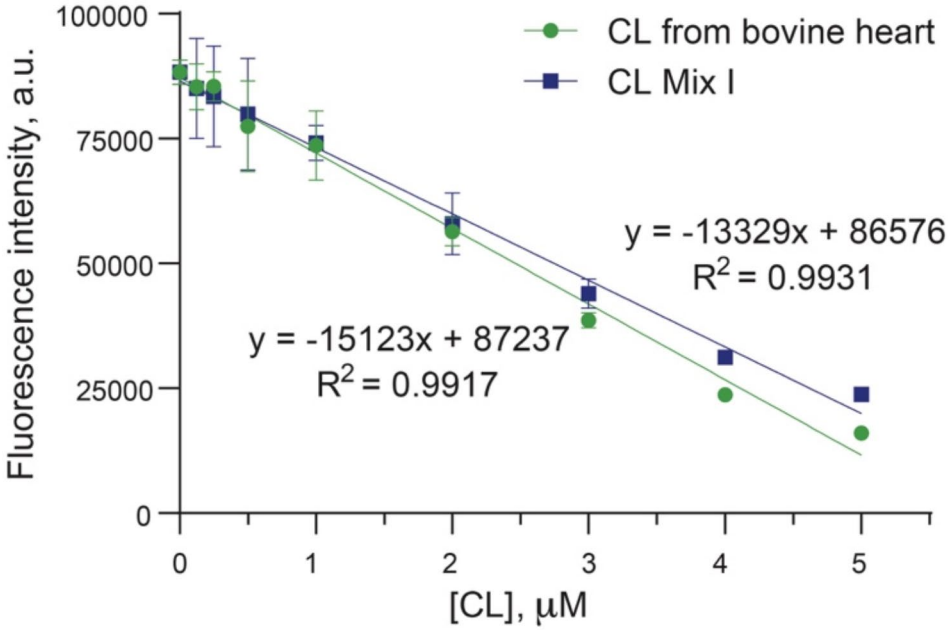

Fig. 1 Titration of CL-containing liposomes with MitoCLue, using different CL standards. The CL from bovine heart (green line) contained ≥ 90% 18:2(4) CL, the CL Mix I (blue line) contained 14:1(3)-15:1, 15:0(3)-16:1, 22:1(3)-14:1, 24:1(3)-14:1 CL in equal amounts

mitochondrial proteins (e.g. mitofusin-2, Mfn2) [32] and enzymes (e.g. aconitase, ACO2) expression [33], CL does not appear to play a significant role in the metabolic reprogramming of gastric cancer.

In samples from patients with colon cancer, no statistically significant difference in CL concentration was observed (V = 386.0, $p = 0.57$, 95% CI = [-0.43, 0.24]) between healthy (97.3 nmol/mg prot.) and tumor tissues (102.0 nmol/mg prot.), when CL concentrations were normalized to protein content (Fig. 2C). However, when normalized to CS activity (Fig. 2D), a 33% increase in median CL level was found in tumor tissues (684.7 nmol/U CS) compared to healthy tissues (514.8 nmol/U CS), which was statistically significant (V = 232.0, $p = 0.01$, 95% CI = [-0.69, -0.15]). Using this metric, CL concentration was found to be higher in tumor tissues than adjacent colon tissues in 29 of 41 (70.7%) patients.

This finding is in a good agreement with reports, indicating that mitochondrial respiration is significantly more active in colon cancer compared to adjacent healthy tissues [34], accompanied by higher mitochondrial DNA expression [35]. Moreover, mitochondrial metabolism has been shown to promote and regulate tumorigenesis via the Wnt signaling pathway [36], contributing to the progression and aggressiveness of colon cancer [37]. Therefore, CL appears to support this type of metabolic reprogramming, in contrast to gastric cancer. Remarkably, we found no differences between groups when CL concentrations were normalized to mitochondrial protein content. This result may be attributed to the possible presence of non-mitochondrial proteins that were not completely separated during centrifugation. Further purification of the mitochondrial fractions was not considered due to the small size of the tissue samples, as this would have resulted in a lower isolation yield. In this scenario, CS activity appears to be a more precise and specific marker for normalization. On the other hand, CS activity may vary under different pathological conditions, including cancer; [38, 39] however, this variability is also observed in other mitochondrial mass markers, such as mitochondrial protein concentration, mtDNA copy number, and the activity of mitochondrial respiratory complexes. It also has been shown that surgical intervention can damage mitochondria and suppress enzymatic activity [40], therefore, paired tissue comparisons within the same patients were done to mitigate these confounding effects.

Influence of other clinical parameters on CL levels

Using the available patient data, we explored whether gender, age, body mass index (BMI) and smoking status influence CL concentration in healthy (left panel) and tumor (right panel) tissue of gastric cancer patients. The differences between the groups and the correlations with these parameters, where CL concentrations were normalized to CS activity, are shown in Fig. 3. Similar results were observed when CL concentrations were normalized to total mitochondrial protein content and are shown in the Supplementary Material.

No significant differences in CL levels were observed between males and females (Fig. 4A, B), and no correlation with age was identified in either healthy or

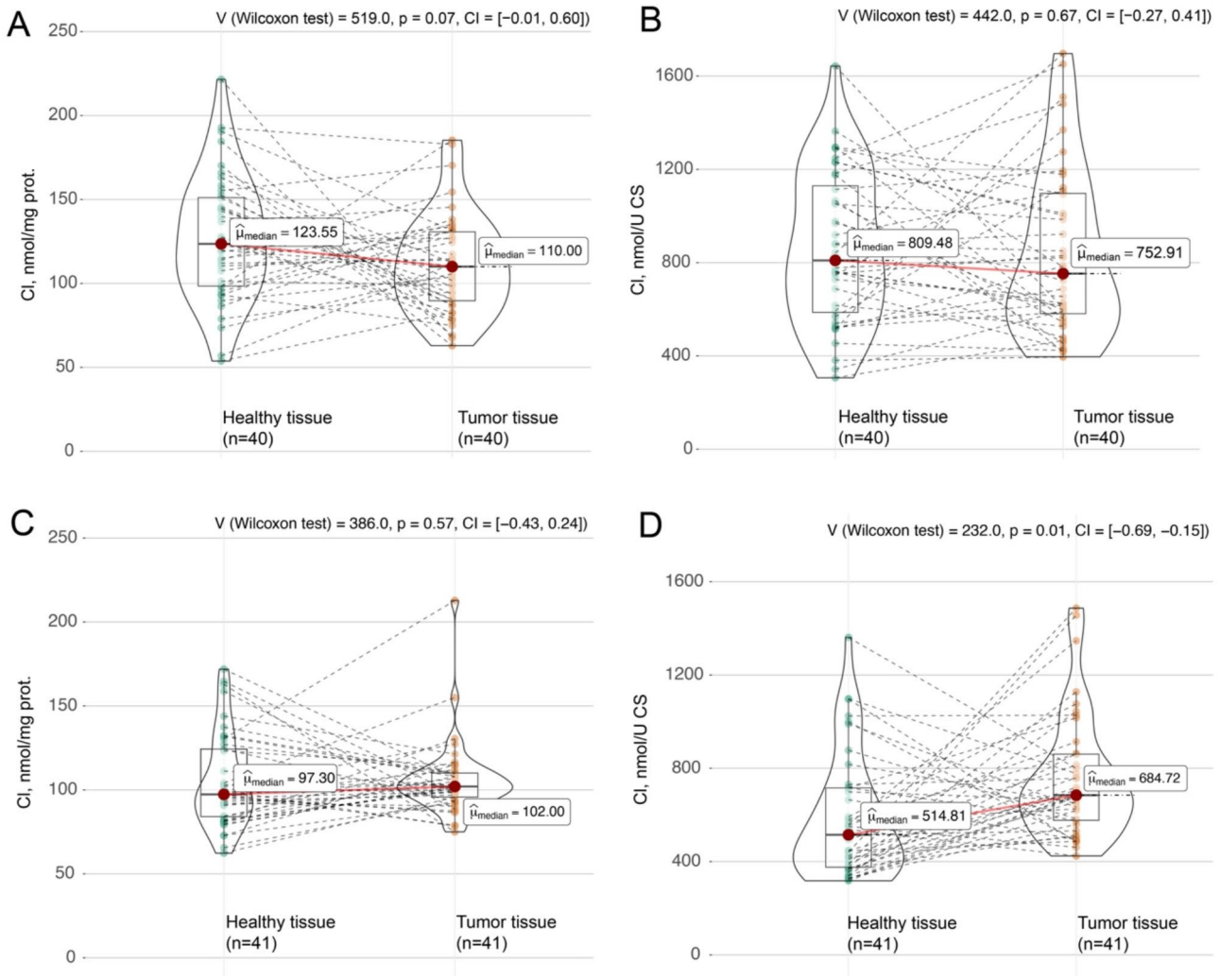

Fig. 2 CL concentrations in normal and tumor tissue from patients: (**A**) with gastric cancer normalized to mitochondrial protein content; (**B**) with gastric cancer normalized to CS activity.; (**C**) with colon cancer normalized to mitochondrial protein content; (**D**) with colon cancer related to CS activity. The data was analyzed using Wilcoxon signed-rank test, and the difference was considered significant when $p < 0.05$

tumor tissues (Fig. 4C, D). A weak positive correlation (Spearman`s $\rho = 0.35$, $p = 0.0282$) was found between BMI and CL concentration in healthy tissues (Fig. 4E), but not in malignant tissues (Fig. 4F). The weak association between BMI and CL levels in healthy tissues suggests a potential influence of BMI on mitochondrial lipid composition under normal physiological conditions. Previously, it has been shown that high fat diet can significantly influence both CL levels and composition [41]. However, we did not have any information about dietary habits of the patients, therefore further research with strict dietary control is necessary to better understand and validate these correlations. In addition, no clear association was established between CL levels and smoking status in either type of tissue. Overall, these results suggest that CL levels in gastric cancer patients are not significantly affected by sex, age, smoking status, while BMI may have a minor impact.

A similar analysis (Fig. 3, left panel – healthy tissue, right panel – tumor tissue) was done to examine potential associations between CL level and clinical parameters of colon cancer patients. Consistent with our findings in gastric cancer, sex (Fig. 3A, B), age (Fig. 3C, D), BMI (Fig. 3E, F) or smoking status (Fig. 3G, H) had no impact on total CL in healthy or malignant tissues from patients with colon cancer.

Discussion

The results of this study provide the first insights into CL levels in human tissue samples from patients suffering from gastric and colon cancer, highlighting both consistencies and discrepancies with prior research. This study adheres to the STROBE guidelines for cross-sectional observational studies, ensuring transparency and completeness in reporting.

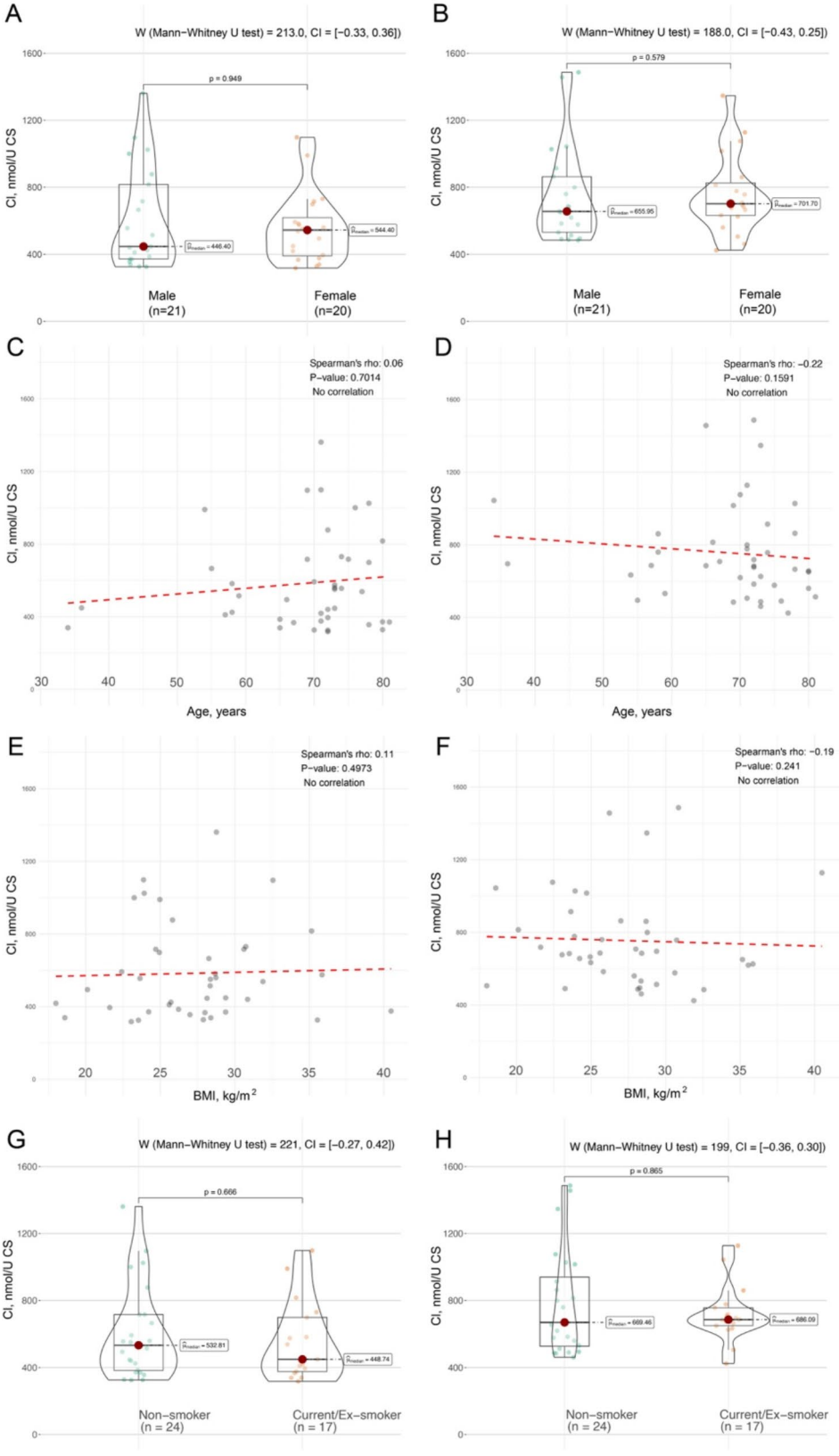

Fig. 3 (See legend on next page.)

(See figure on previous page.)

Fig. 3 Associations of total CL level with clinical parameters of colon cancer patients. The left panel in each pair (**A**, **C**, **E**, **G**) represents healthy tissue, while the right panel (**B**, **D**, **F**, **H**) represents tumor tissue. (**A**, **B**) CL levels by gender; (**C**, **D**) Correlation between CL levels and age; (**E**, **F**) Correlation between CL levels and BMI; (**G**, **H**) CL levels by smoking status. CL levels were normalized to CS activity. Differences between groups were assessed using the Mann-Whitney U test, and the difference was considered significant when $p < 0.05$. Correlations were evaluated using Spearman's correlation and considered significant at $p < 0.05$. CS – citrate synthase, BMI – body mass index

Several studies have highlighted metabolic reprogramming in gastric cancer cells, particularly involving various alterations in mitochondrial functions [42–45]. This observation led us to the hypothesis that considerable changes in mitochondrial mass, potentially reflected in CL levels, might occur in gastric tumors. However, previous studies in gastric tumors demonstrated that mtDNA copy number (mtCN), that somewhat reflects total mitochondrial mass, was very variable and did not correlate with overall survival of gastric cancer patients [46]. Similar results were reported by Lee et al., who found that while 64.2% of GC tissues exhibited higher mtCN than adjacent healthy tissue, there was no association with any clinicopathological characteristics [47]. Recent meta-analysis revealed that high heterogeneity in mtCN exists in tumor versus the adjacent counterpart tissues, and authors did not find any significant differences in mtCN in patients with gastric cancer [48]. Therefore, there is no agreement on mitochondrial mass influence in gastric cancer highlighting a need for additional markers of mitochondrial mass, such as total CL content.

To our surprise, we did not observe a significant alteration in total CL level between healthy and tumor tissues in gastric cancer. Thus, despite reported metabolic shifts in gastric cancer, total CL content, and, therefore, mitochondrial mass is not affected. This finding supports previous results of mtCN studies and challenges Sotgia's hypothesis regarding increased mitochondrial mass in gastric cancer, but do not exclude increased expression of particular mitochondrial proteins. Recently, it has been shown that *Helicobacter pylori* infection can influence mitochondrial metabolism and potentially impact clinical outcomes in gastric cancer [49]. However, in this study we did not examine whether *H. pylori* infection impacts CL levels, as detailed information about the infection status in the studied cohort was limited. Future research investigating the relationship between *H. pylori* infection and CL levels in gastric cancer could provide insights into how *H. pylori* alters mitochondrial lipid composition and its potential implications for disease progression and treatment response.

In colon cancer, mitochondrial mass reported as mtCN has been shown to be more than twice as high in tumors as in healthy tissue, as reported by Wang et al. [47]. Additionally, patients with higher mtCN had a worse prognosis after surgery compared to those with lower mtCN in their tumor tissues. Our results are consistent with these reports, revealing a 33% increase in CL level in colon tumor tissues and indicating alterations in CL metabolism and general mitochondrial function in colon cancer. This observation aligns well with prior research by Zichri et al. [50] and Chun et al. [51], who demonstrated that increased CL concentration optimizes efficient electron transfer, promotes ATP production and minimizes ROS production in human colon cancer cells (HCT116). Specifically, Chun et al. found that the level of CL in colon cancer cells is influenced by the expression of long-chain-fatty-acid—CoA ligase 5 (ASCL5), which, in turn, is induced by hypoxia-inducible factors HIF-1α and HIF-2α, as well as oncogenic *KRAS* mutation. These molecular mechanisms may explain the increase in CL levels observed in our study and point to mitochondrial adaptation in colon tumors.

Thus, the total CL level remains unaffected by the metabolic shift in gastric cancer, which represents a classic "glycolytic" type of cancer [52]. In contrast, colon cancer, which relies more heavily on oxidative phosphorylation, appears to involve CL in the modulation of mitochondrial metabolism, as its level is increased [37]. This observation suggests that treatments targeting CL [53, 54], mitochondrial metabolism directly [55] or indirectly via HIF-1α modulation [56, 57] or utilizing mitochondria-specific drug delivery systems [58] may hold greater efficacy for colon cancer patients. However, the question remains: can CL serve as a reliable biomarker in this context?

A biomarker is defined as a molecule used to monitor disease progression or predict an individual's risk of experiencing specific clinical events [59]. Given that CL is a ubiquitous lipid found in all healthy cells that have mitochondria, it lacks the specificity needed to become a predictive biomarker, however, significant increase in the concentration might serve as a prognostic marker. While we observed a statistically significant increase in total CL levels in colon cancer, the magnitude of this increase is modest, limiting CL's potential clinical value as a prognostic biomarker for routine practice. Currently, molecular biomarkers for colon cancer include mutations in genes such as *EGFR*, *KRAS*, *BRAF*, and *HER2* that have established clinical significance. However, some of these biomarkers, such as *KRAS* and *HER2*, have shown somewhat controversial predictive effects, while others, like *BRAF* and *EGFR*, lack guiding significance in the treatment of colorectal cancer. Thus, it remains necessary to continue to identify new biomarkers for more precise diagnostics and effective therapies [60].

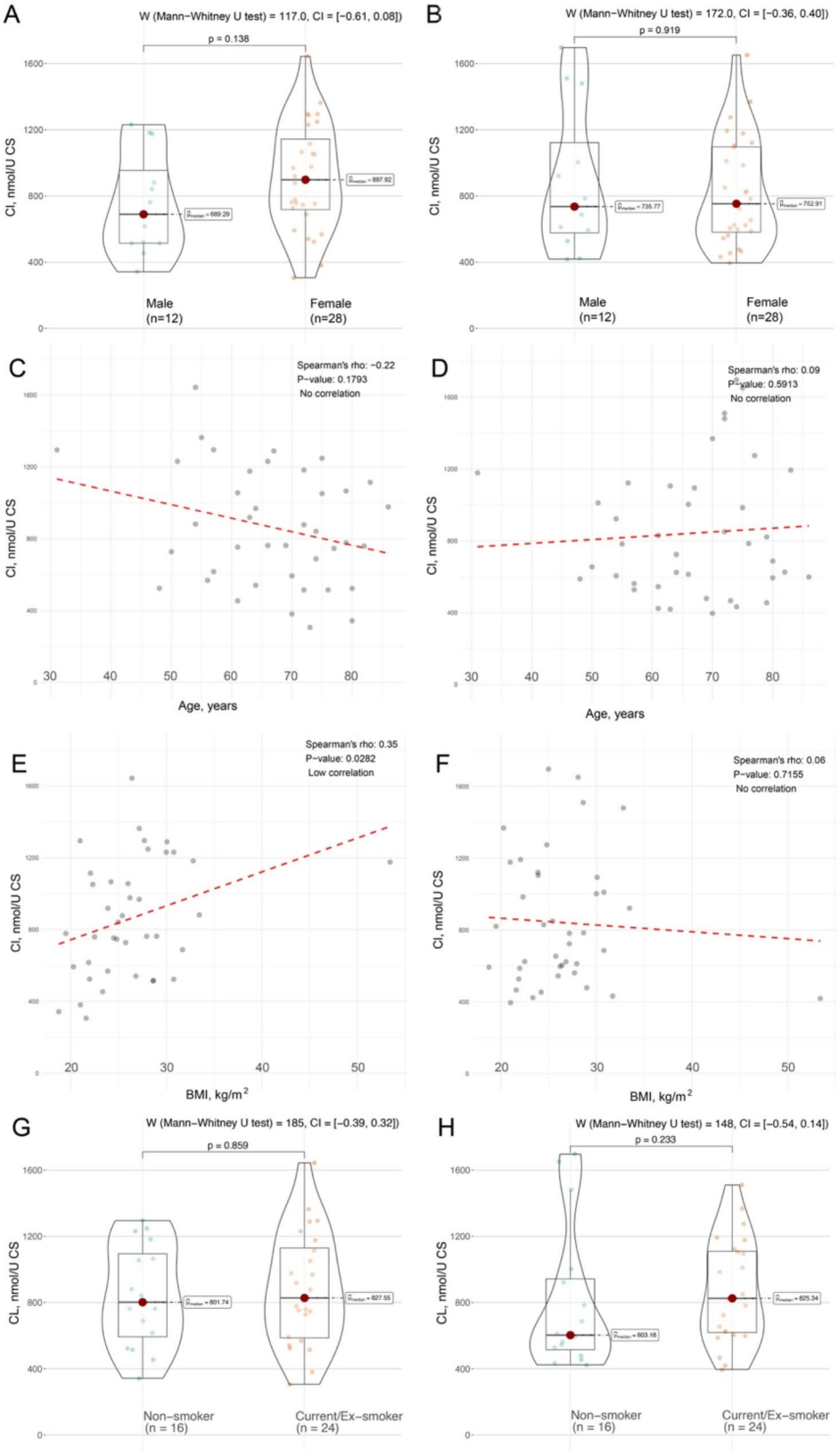

Fig. 4 (See legend on next page.)

(See figure on previous page.)

Fig. 4 Associations of total CL level with clinical parameters of gastric cancer patients. The left panel in each pair (**A**, **C**, **E**, **G**) represents healthy tissue, while the right panel (**B**, **D**, **F**, **H**) represents tumor tissue. (**A**, **B**) CL levels by gender; (**C**, **D**) Correlation between CL levels and age; (**E**, **F**) Correlation between CL levels and BMI; (**G**, **H**) CL levels by smoking status. CL levels were normalized to CS activity. Differences between groups were assessed using the Mann-Whitney U test, and the difference was considered significant when $p < 0.05$. Correlations were evaluated using Spearman's correlation and considered significant at $p < 0.05$. CS – citrate synthase, BMI – body mass index

We also found no correlation between CL concentrations and other clinical parameters such as sex, age or smoking status. Although a weak positive correlation was found between BMI and CL levels in healthy gastric tissue, this link was absent in malignant tissue, suggesting that further investigation is needed to verify this association.

Previous studies have shown that cardiolipin (CL) levels and acyl chain composition undergo significant changes not only in genetic mitochondrial disorders such as Barth syndrome but also in various diseases, including type 2 diabetes mellitus, Parkinson's disease, and ischemic heart disease [61, 62]. Elevated expression of certain genes, such as *ALCAT1*, can also modulate CL levels in particular tissues [63]. However, it remains unclear whether these alterations in CL composition have systemic effects or if comorbidities influence CL levels in tumors. Furthermore, certain medications, such as doxorubicin [64] and etomoxir [65], are known to decrease and increase CL levels, respectively, which should also be considered. As our study did not assess these parameters, further investigations are needed to address these gaps and to explore their potential implications for oncological diseases.

In this study, MitoCLue proved itself to be a robust tool for the determination of the total CL concentration, but several limitations should be acknowledged. First, while MitoCLue enables the measurement of total CL in mitochondrial fractions, nonspecific binding may lead to minor overestimations in total CL content. Second, it does not allow to distinguish between CL species, nor reveal specific CL pattern, such as degree of unsaturation or oxidized CLs. However, similar limitations are shared by other methodologies, such as different MS techniques, because they cannot cover the full scope of CL species and lack standards that would give correct results. Future studies might benefit from a more complex approach combining several existing techniques: fluorescent probes for total CL measurement, HPLC-ESI-MS or quantitative MALDI-MS for the profiling of CL species and DESI-MS or MALDI-MSI for the determination of the localization in the tissue sample. Also, larger-scale multicenter prospective studies are necessary to ensure reproducibility and generalizability of these results. Although our findings are limited by sample size in this study, they provide valuable insight into CL level variations in gastric and colon cancer, improving our understanding of differences in mitochondrial dynamics and bioenergetic function of mitochondria in distinct types of cancer.

Conclusions

In this study, we successfully applied MitoCLue, a CL-specific fluorescent probe, to quantitatively assess the total CL level in healthy and tumor tissues from patients with gastric or colon cancer. We recommend using CS activity as a more specific marker of mitochondrial mass when normalizing CL levels in crude mitochondrial preparations. The results of this study reveal that there are no alterations in total CL levels between normal and tumor tissues in gastric cancer patients. Although a 33% increase in CL levels was observed in colon tumor tissues compared to healthy adjacent tissues, this is a much more modest increase than several-fold changes that has been reported in cell culture studies by our and other groups. Thus, we emphasize the need for more research focused on human tissue samples. While CL may play a role in colon cancer progression, total CL measurement alone cannot serve as a reliable biomarker in this context. Additionally, we found that sex, age, smoking status, and BMI had minimal or no impact on CL levels in both gastric and colon cancer patients. Further studies are needed to validate preclinical observations of CL level alterations in other cancer types and explore their potential clinical implications.

Supplementary Information

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Supplementary Material 1

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None.

Author contributions
Conceptual research was conducted by PA and PD. The research design was primarily undertaken by PA, PD, and ML. Data analysis and interpretation were performed by PD. Patient tissue samples provided by AP, AF, ML. Manuscript drafting involved PD, ML, and PA. All authors conducted a thorough review of the final manuscript and subsequently provided their formal approval.

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Data availability
Access to the data analysed in this research is available upon request.

Declarations

Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Medical and Biomedical Research Ethics Committee of the Riga East University Hospital Support Foundation, (Registration No. 5000 8147021), protocol 12-A/23, March 9, 2023) and the Central Medical Ethics Committee of Latvia (Latvian Cabinet of Ministers Order No. 267), protocol 1/19-06-27, 27 June 2019. Signed consent was obtained from all individuals involved in the study at the time of recruitment to the biobank.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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