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Research article

CXCL12-loaded-hydrogel (CLG): A new device for metastatic circulating tumor cells (CTCs) capturing and characterization

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ABSTRACT

Background: Circulating Tumor Cells (CTCs) represent a small, heterogeneous population that comprise the minority of cells able to develop metastasis. To trap and characterize CTCs with metastatic attitude, a CXCL12-loaded hyaluronic-gel (CLG) was developed. CXCR4+cells with invasive capability would infiltrate CLG.

Methods: Human colon, renal, lung and ovarian cancer cells (HT29, A498, H460 and OVCAR8 respectively) were seeded on 150 μl Empty Gels (EG) or 300 ng/ml CXCL12 loaded gel (CLG) and allowed to infiltrate for 16 h. Gels were then digested and fixed with 2 % FA-HAse for human cancer cell enumeration or digested with HAse and cancer cells recovered. CLG-recovered cells migrated toward CXCL12 and were tested for colonies/spheres formation. Moreover, CXCR4, E-Cadherin and Vimentin expression was assessed through flow cytometry and RT-PCR. The clinical trial "TRAP4MET" recruited 48 metastatic/advanced cancer patients (8 OC, 8 LC, 8 GBM, 8 EC, 8 RCC and 8 EC). 10 cc whole blood were devoted to PBMCs extraction (7 cc) and ScreenCell™ filters (3 cc) CTCs evaluation. Ficoll-isolated patient's PBMCs were seeded over CLG and allowed to infiltrate for 16 h; gels were digested and fixed with 2 % FA-HAse, cells stained and DAPI+/CD45-/pan-CK + cells enumerated as CTCs.

Results: Human cancer cells infiltrate CLG more efficiently than EG (CLG/EG ratio 1.25 for HT29/1.58 for A498/1.71 for H460 and 2.83 for OVCAR8). CLG-recovered HT29 cells display hybrid-mesenchymal features [low E-cadherin (40 %) and high vimentin (235 %) as compared to HT29], CXCR4 two-fold higher than HT29, efficiently migrate toward CXCL12 (two-fold higher

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than HT29) and developed higher number of colonies (171 ± 21 for HT29-CLG vs 131 ± 8 colonies for HT29)/larger spheres (spheroid area: 26561 ± 6142 μm^2 for HT29-CLG vs 20297 ± 7238 for HT29). In TRAP4MET clinical trial, CLG-CTCs were isolated in 8/8 patients with OC, 6/8 with LC, 6/8 with CRC, 8/8 with EC, 8/8 with RCC cancer and 5/8 with GBM. Interestingly, in OC, LC and GBM, CLG isolated higher number of CTCs as compared to the conventional ScreenCell™ (CLG/SC ratio = 1.88 for OC, 2.47 for LC and 11.89 for GBM). Bland and Altman blot analysis and Passing and Bablok regression analysis showed concordance between the methodological approaches but indicate that SC and CLG are not superimposable suggesting that the two systems select cells with different features.

Conclusion: CLG might represent a new and easy tool to isolate invasive CTCs in multiple cancers such as OC, LC and GBM at today orphan of reliable methods to consistently detect CTCs.

1. Introduction

Metastasis, responsible of >90 % of death for cancer [1,2], derives from a multistep, inefficient process in which primary tumor shapes distant organ microenvironment permissive to metastatic outgrowth [3,4]. In April 2024, the search of 'Circulating Tumour Cells' on ClinicalTrials.gov website returned 246 "active" studies, underling the interest in CTCs as biomarkers for personalized oncology. Although 1 × 10^6 CTCs per gram of tumor tissue are estimated to reach the circulation in a day [5], less than 0.01 %, endowed with hybrid epithelial-mesenchymal phenotype and invasive/stemness properties, successfully complete the metastatic cascade [6,7]. Nevertheless, specific features of metastatic capability are poorly characterized. In cancers such as renal, endometrium, colon, lung, and breast, CTCs are informative on prognosis and predictive of response [8–14]. A meta-analysis including 50 studies with 6712 breast cancer patients showed that CTCs decrease after therapy [15]. In Non Small Cell Lung Cancer (NSCLC) a meta-analysis including 22 studies with 1674 NSCLC patients showed that CTCs correlate with Progression Free Survival (PFS) and Overall Survival (OS) before and after treatment [16] and PD-L1 positive CTCs predicts tumoral PD-L1 [17]. Nevertheless, CTCs characterization is challenging due to heterogeneity, rarity (one CTC is neighbored by 1x10^6 WBC and 1x10^9 RBC per ml of blood) [6,7,18] and the absence of validated/standardized methods. EpCAM-positive CTCs selection with CellSearch is the only FDA approved technology in breast, prostate and colorectal cancer [19]. It relies on CTCs enrichment using ferrofluid nanoparticles coated with anti-EpCAM antibodies followed by immunofluorescence using anti-CK8, -CK18, -CK19 and -CD45 antibodies and DAPI nuclear staining. EpCAM is frequently expressed on the cell surface of many epithelial cancer cells and scarcely in blood cells, representing an ideal marker for CTCs assessment; nevertheless, EpCAM expression is dynamic and context dependent [20,21]. Thus, CellSearch holds intrinsic limitations such as the lack of tumor-specific antigens and low efficiency for EpCAM low-expressing tumors as CTCs might not express or loose EpCAM due to epithelial-mesenchymal transition (EMT), underestimating mesenchymal and EpCAM-low CTCs subpopulations [22,23]. EpCAM expression could correlate with patient's prognosis [24] and with organ tropism (EpCAM-negative CTCs associated with brain metastases; EpCAM-expressing CTC are associated with bone metastases [25–27]). Marker-independent techniques such as ScreenCell filters [28] and Parasortix [29] are based on CTCs physical proprieties, cell size or deformability respectively. Nevertheless, these methods cannot discriminate between CTCs and blood cells with similar physical proprieties. Microfluidic and nanotechnology approaches lack of automation, are expensive and in preclinical stage [18]. Thus, current methods display limitations [30] and do not take into account the heterogeneous, metastatic potential of CTCs being more focused on "searching for cancer cells" than "searching for metastatic cells" in the blood stream; this characteristic may lead to inaccurate results when using CTCs as a prognostic biomarker [30].

CXCR4 is a 48 kDa seven-span transmembrane domains G-protein-coupled chemokine receptor (GPCRs) that binds the chemokine CXCL12. CXCR4 is frequently overexpressed in solid tumors where affects proliferation, survival, metastatic dissemination and microenvironment composition [31–34]. In preclinical models of ovarian, lung, renal and colorectal cancer, CXCR4-expressing tumor cells are invasive, highly chemo-resistant and possess stem-like proprieties [31,35]. We previously demonstrated that cisplatin selects a cancer-initiating cell subset CD133+CXCR4+EpCAM-able to induce primary tumor recurrence and metastatic dissemination in lung cancer [36–38]. Mechanistically, cisplatin promotes the expansion and recruitment of CCR2+CXCR4+Ly6C^{high} inflammatory monocytes and the production of CXCL12 that attract circulating CD133+CXCR4+ metastasis initiating cells to the lung [36]. A new, simple device based on the commercially available Belotero Hyaluronic gel dermal filler, loaded with the chemokine CXCL12 (CXCL12-loaded hydrogel, CLG) reproduced a pseudo niche attracting immune and circulating cancer cells CXCR4+ [39]. We hypothesized that this device might represent a suitable and cheap approach to trap and characterize subset of CTCs endowed with metastatic capacity. Thus, we aim to evaluate the efficacy of CLG in isolating patients derived CTCs in advanced cancers. To this aim, forty-eight patients with ovarian (OC), endometrial (EC), lung (LC), glioblastoma (GBM), renal (RCC) and colorectal cancer (CRC) with advanced disease were enrolled in TRAP4MET clinical trial and evaluated for CTCs at the diagnosis.

2. Results

2.1. Human cancer cells efficiently infiltrate CLG

To test CLG efficiency in recovering cancer cells, human colon, renal, lung and ovarian cancer cells (HT29, A498, H460 and OVCAR8 respectively) infiltrate empty (EG) or CLG gels for 16 h. Gels were digested, cells fixed and DAPI-stained for enumeration or

recovering for plastic growing (Fig. 1A). Gels infiltration assay showed that human cancer cells infiltrate CLG more efficiently than EG (CLG/EG fold 1.25 for HT29/1.58 for A498/1.71 for H460 and 2.83 for OVCAR8) (Fig. 1B).

2.2. HT29-CLG cells express high CXCR4, migrate efficiently toward CXCL12 and display mesenchymal features

Only HT29 cells, with highest CXCR4 expression (21.5 %, not shown), were successfully gel-recovered and in vitro grown (HT29-EG are HT29 recovered from EG while HT29-CLG are HT29 cells recovered from CLG). Fig. 2A–B shows that HT29-CLG cells express 2-fold higher CXCR4 and more efficiently (2-fold) migrate toward BSA and CXCL12 [40] as compared to HT29. Compared to parental, HT29-CLG cells developed higher number of colonies (171 ± 21 for HT29-CLG vs 131 ± 8 for HT29 colonies, Fig. 2C) and better growth in hanging drops (total spheroid area: 26561 ± 6142 for HT29-CLG vs 20297 ± 7238 for HT29 colonies; spheroid index: 0.9671 ± 0.022 for HT29-CLG vs 0.9199 ± 0.074 for HT29 colonies, Fig. 2D). HT29-CLG cells expressed lower E-cadherin (40 %) and higher vimentin (235 %) mRNA as compared to parental cells (Fig. 2E). Moreover, HT29-CLG cells were resistant to 5-Fluoracil and display less surface EpCAM (Fig. S1 A-B) as compared to the parental cell lines. These data suggest that CLG selects cancer cell subpopulation with more aggressive and hybrid-mesenchymal features.

2.3. TRAP4MET clinical trial aims at isolating CLG-CTCs

To test CLG efficiency in recovering human cancer cells from human blood, HT29 or H460 cells were spiked in healthy donor (HD) blood, the mixture Ficoll-paqued and allowed to infiltrate CLG. As shown in Fig. S2A, HT29 and H460 cells (identified as DAPI+/pan-CK+/CD45-cells) were successfully recovered from HD blood with a CTCs count of 4 and 2.6 cancer cells/cc respectively (HT29 recovery rate of 14 %; H460 recovery rate 9 %). Thus, CLG enables the enrichment and enumeration only of cancer cells displaying infiltrating features present in blood. To evaluate the efficacy of CLG in enumerating and characterizing patients derived CTCs, 48 patients with advanced solid cancers were enrolled in the TRAP4MET trial (Table 1); specifically, eight patients with ovarian (OC), endometrial (EC), colorectal (CRC), renal (RCC), lung (LC) cancer and glioblastoma (GBM) were enrolled. CTCs were searched at the diagnosis of advanced/metastatic disease comparing CLG versus ScreenCell filters, a commercial size-based enrichment method CE-labelled assessed in several malignancies [41–46]. For CLG-CTCs, PBMC were isolated and 12-16 x 10^6 seeded on CLG in 8-well chamber slide. Cells were allowed to infiltrate the gels for 16 h and CTCs were counted as DAPI + nuclei, pan-CK+ and $CD45^-$ (Fig. 3A–B). CTCs were also evaluated with the ScreenCell and identified as hematoxylin/pan-CK + cells. As reported in Table 2 and Fig. 4A, CTCs were successfully isolated with both methods (Fig. 4, Table 2 and Table S1), as 363 total CTCs (ranging from 0 to 80, in 3 cc blood) were isolated with ScreenCell and 661 with CLG (ranging from 0 to 133, in 7 cc blood). CLG-CTCs were isolated in 8/8

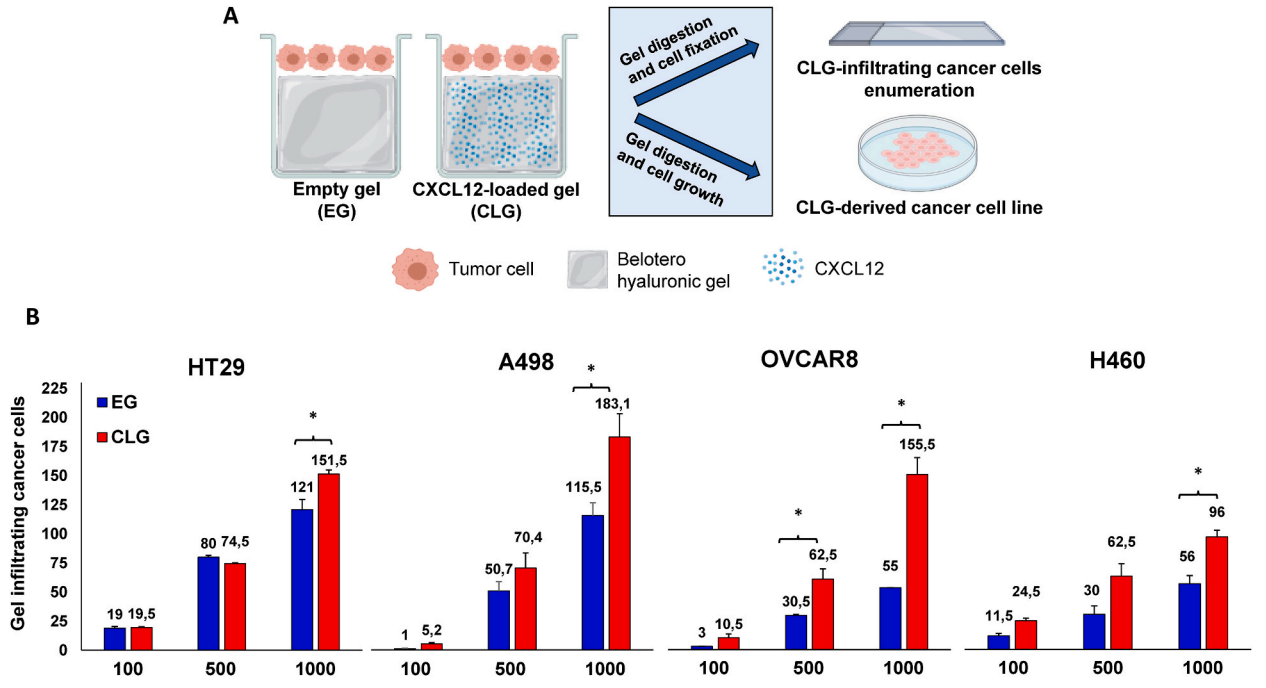

Fig. 1. Human cancer cells efficiently infiltrate CLG. (A) Schematic representation of Gel infiltration assay. (B) Human colon, renal, lung, and ovarian cells (HT29, A498, H460 and OVCAR8) were seeded on empty (EG) or CXCL12-loaded Hydrogel (CLG) on 8-well chamber slide in Serum Free (SF) media and allowed to infiltrate 16 h. Cells were fixed with 2%FA + Hyaluronidase (HAase) for 6 h and stained with DAPI for enumeration at fluorescent microscope. B 100, 500 or 1000 A498, HT29, H460 and OVCAR8 were allowed to infiltrate EG or CLG. Data in bar graphs represent mean ± SEM of at least two independent experiments. A two-tailed *t*-test was used to assess significance. *P < 0·05, **P < 0·01 ***P < 0·001.

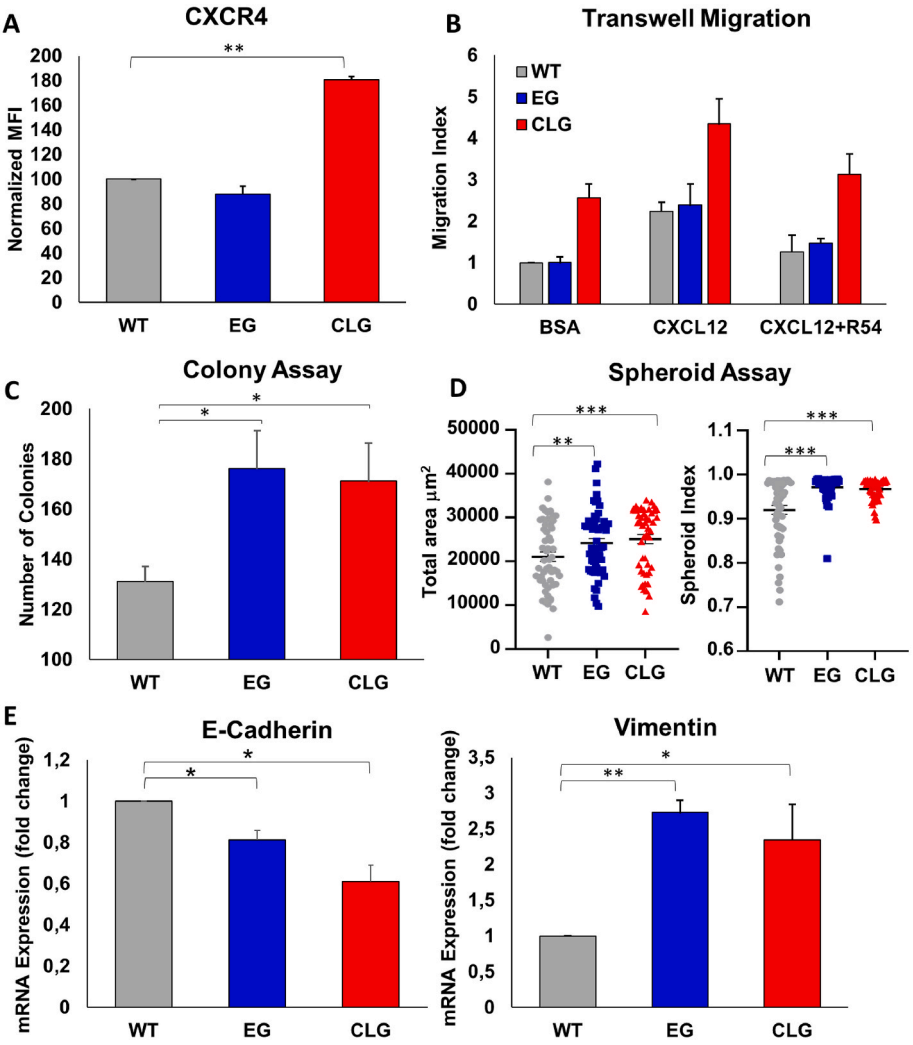

Fig. 2. HT29-CLG cells express high CXCR4, migrate efficiently toward CXCL12 and show mesenchymal features. (A) **HT29**-EG- (HT29-EG) and CLG- (HT29-CLG) HT29-derived immortalized cells CXCR4 expression; (B) CXCL12-induced migration; (C) colony assay; (D) spheroid formation capability and (E) E-cadherin and Vimentin RNA expression. Data in bar graphs represent mean ± SEM of at least two independent experiments. A two-tailed *t*-test was used to assess significance. *P < 0·05, **P < 0·01 ***P < 0·001.

patients with OC, 6/8 with LC, 6/8 with CRC, 5/8 with GBM, 8/8 with EC and 8/8 with RCC (Table 2). Bland and Altman blot analysis suggested concordance between CLG and ScreenCells method (Fig. 4B). Passing and Bablok regression analysis indicate that the methods are not superimposable (Slope A 0.28, 95 % CI 0 to 0.83; Intercept B 1.20, 95 % CI 0.54 to 2.57; Spearman's coefficient = 0.415, deviation from linearity p < 0.01) (Table 3 and Fig. 4B) suggesting that the two methods could discriminate cell populations with different features. Interestingly, in lung cancer a significantly higher CTCs number was retrieved with CLG (Fig. 4C). In lung, glioblastoma and ovarian cancer patients, although not significant, there is higher number of CTCs in the CLG as compared to SceenCell in paired analysis (Fig. 4D). Thus, CLG identifies higher number of CTCs as compared to ScreenCell in ovarian, lung cancer and glioblastoma (CLG/SC ratio = 1.88 for OC, 2.47 for LC and 11.89 for GBM) and may enrich CTCs with specific biological features.

3. Discussion

In the present manuscript, a hyaluronic acid-based gel (dermal filler)-CXCL12 loaded (CLG) was considered for CTCs enumeration and characterization. *In vitro* studies demonstrated that CLG-recovered cancer cells displayed hybrid-mesenchymal features and enhanced CXCL12-dependent migration. Cancer cells migration/invasion capacities are critical for the metastatic cascade (eg. intravasation, extravasation and colonization of distant sites) and are frequently gained through the acquisition of mesenchymal phenotype. Thus, CXCL12-dependent migration may identify CTCs with metastatic attitude with a possible prognostic/predictive meaning [5,47]. TRAP4MET trial demonstrated that CLG is able to isolate CTCs from blood of ovarian, endometrial, lung, renal, colorectal cancer and glioblastoma patients as efficiently (renal) or even better (lung, ovarian, glioblastoma) than the size dependent

Table 1
Patient characteristics. ADC = Adenocarcinoma, HG Serous = High grade serous ovarian cancer; NOS= Not otherwise specified non-small cell lung cancer.

	Colon	Endometrial	Glioblastoma		Lung		Ovarian		Renal	
	N (%)	N (%)	N (%)		N (%)		N (%)		N (%)	
Age (median years)										
<62	4 (50)	1 (12.5)	5 (62.5)		2 (25)		2 (25)		8 (100)	
≥62	4 (50)	7 (87.5)	3 (37.5)		6 (75)		6 (75)			
Gender										
Male	4 (50)		4 (50)		5 (62.5)				6 (75)	
Female	4 (50)	8 (100)	4 (50)		3 (37.5)		8 (100)		2 (25)	
Stage										
Locally Advanced		6 (75)	6 (75)				5 (62.5)			
Metastatic	8 (100)	2 (25)			8 (100)		3 (37.5)		8 (100)	
Missing			2 (25)							
Histological variant										
ADC	7 (87.5)	Endometrial 5 (62.5)	IDH-WT	5 (62.5)	ADC	7 (87.5)	Serous	5 (62.5)	Clear Cell	4 (50)
Mucinous	1 (12.5)	Mucinous 1 (12.5)	Astrocytoma	1 (12.5)	NOS	1 (12.5)	HG Serous	2 (25)	Mixed	1 (12.5)
		Serous 1 (12.5)	Missing	2 (25)			Missing	1 (12.5)	Missing	3 (37.5)
		Clear Cell 1 (12.5)								

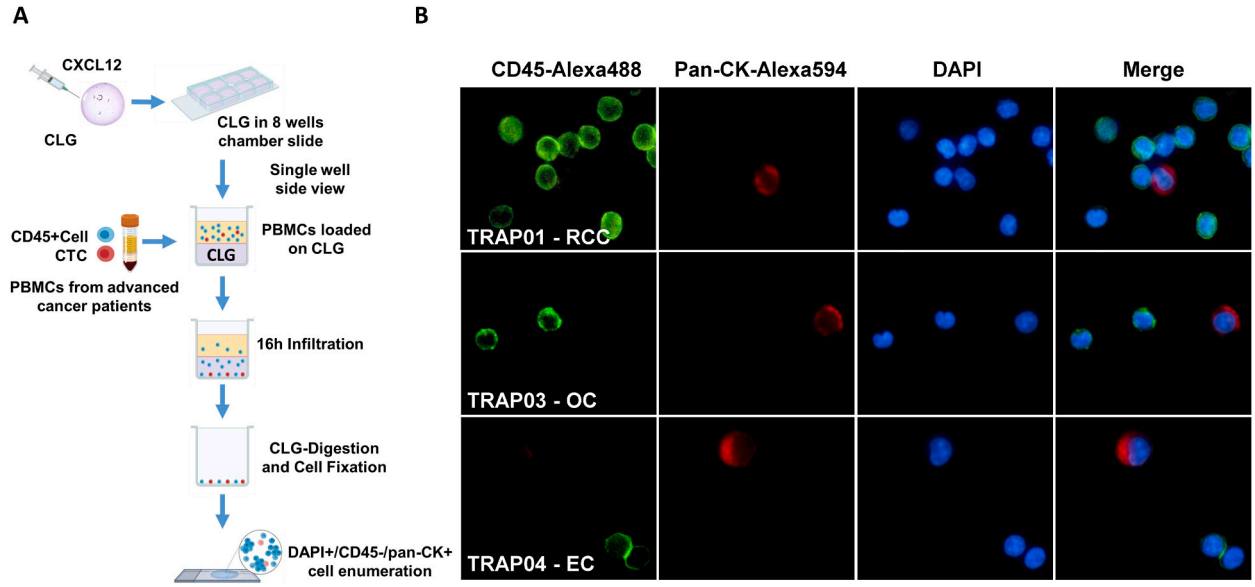

Fig. 3. CLG efficiently recovered CTCs from cancer patient's blood (TRAP4MET clinical trial). (A) Schematic representation of CTCs isolation using CLG. (B) CTCs isolated from patients from the TRAP4MET clinical trial using CLG. 7 cc patient's blood were Ficoll-paqued and isolated cells suspended in Serum Free media and placed on CLG in 8-well chamber slide. Cells were allowed to migrate 16h and then fixed in 2 % FA-HAase and stained with DAPI, Alexa488-anti human CD45 and Alexa594-*anti*-human panCK. As negative control, HD blood samples were searched for DAPI+/pan-CK+/CD45-cells (DAPI+/pan-CK+/CD45-mean count/cc of 0,33 ± 0,32).

Table 2
Isolated CTCs-TRAP4MET clinical trial patients. CTCs were isolated using Screen Cell and CXCL12-loaded hydrogel (CLG) from 48 patients. CTCs counts as number of CTCs/cc of blood ±standard deviation. CRC = colorectal cancer, EC = endometrial cancer, GBM = glioblastoma, LC= Lung cancer, OC= Ovarian Cancer, RCC= Renal cell carcinoma.

Disease	CTCs/cc SC	CTCs/cc CLG	CLG/SC ratio
CRC	5,46 ± 9,04	1,41 ± 1,19	0,26
EC	4,25 ± 8,48	0,91 ± 0,80	0,21
GBM	0,17 ± 0,31	1,98 ± 2,87	11,89
LC	0,54 ± 0,67	1,34 ± 0,86	2,47
OC	1,00 ± 1,13	1,88 ± 1,62	1,88
RCC	3,50 ± 5,49	4,29 ± 6,12	1,22
TOT	**2,49 ± 5,64**	**1,97 ± 2,97**	

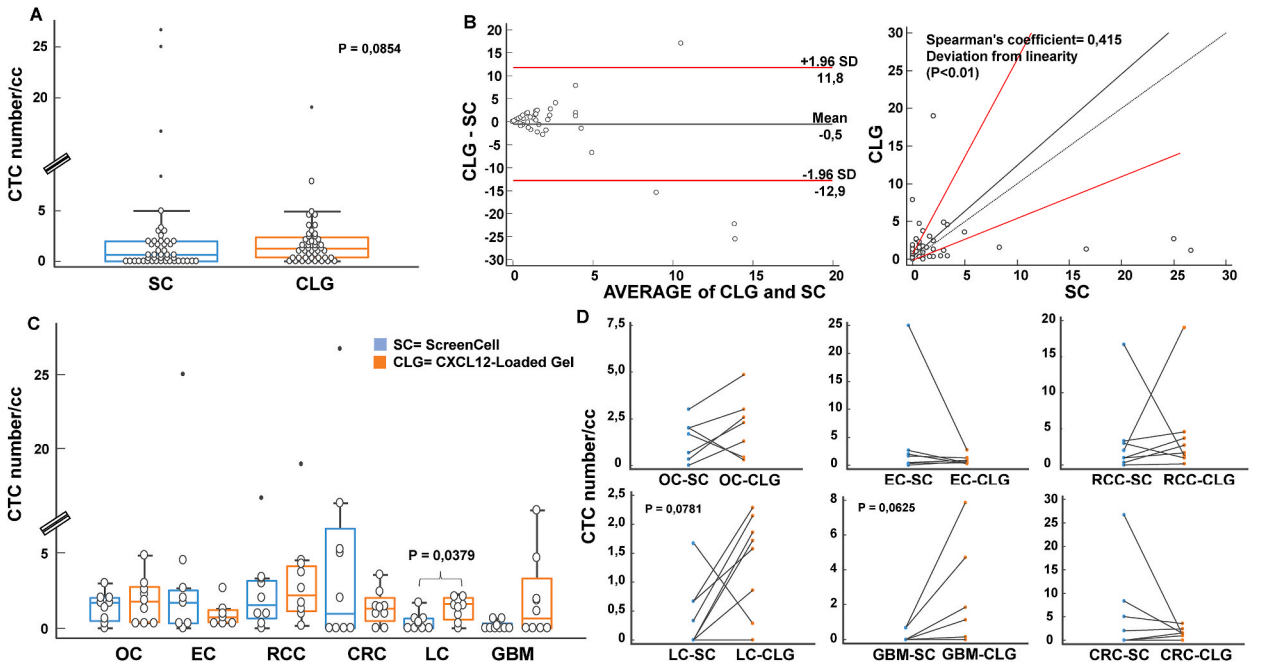

Fig. 4. CLG isolated CTCs with higher efficiency than Screen Cell in OC, LC and GBM patients. Ten milliliters of blood were collected, 7 cc used for CLG-CTCs and 3 cc for Screen Cell™ CTCs isolation. (A) Total CTCs isolated with CLG vs SC; (B) Correlation analysis (Passing-Bablok regression and Bland-Altman plot); (C) Mann-Whitney test for independent samples analysis; (D) Wilcoxon test for paired samples analysis.

Table 3
Passing and Bablok regression analysis. Intecept A and Slope B with their 95 % confidence interval (CI).

Passing and Bablok Regression Equation: y = 0,28 + 1,20 x						
Intercept A	95 % CI	Fixed/constant bias	Slope B	95 % CI	Proportional bias	Cusum test for linearity
0,28	0 to 0,83	YES	1,20	0,54 to 2,57	NO	Significant deviation from linearity

ScreenCell. Herein, isolated CTCs infiltrate the hydrogel and sense CXCL12. CLG-CTCs were identified as epithelial (CD45-/pan-CK+/DAPI+) within the majority of immune (CD45+/pan-CK-/DAPI+) cells. The ongoing molecular characterization will better define the biological features of CLG-CTCs as also immune/mesenchymal cancer associated cells express pan-CK [48–50]. It was previously reported that pseudo niche might trap CTCs. In murine model a subcutaneous hyaluronic acid-erythropoietin/CXCL12 cancer trap attracted circulating prostate cancer cells reducing metastasis [51] and in murine breast cancer intraperitoneal poly-caprolactone microspheres-CXCL12 increased immune cell recruitment [52]. We previously demonstrated that CLG attracted CXCR4+ CTCs in "in vivo" melanoma model [39]; herein we showed for the first time that CLG is able to isolate and enumerate human migrating/infiltrating CTCs in patients affected by advanced/metastatic solid cancer (lung, colon, ovarian, endometrium, lung and glioblastoma). CLG is feasible and sensitive in solid cancers (lung, ovarian and glioblastoma) orphans of clinically validated and regulatory-cleared method for CTCs. In glioblastoma, although rare, extracranial metastasis are reported as tumor representative and relevant for prognosis [53]. GBM-CTCs acquired mesenchymal/stem markers, are resistant to genotoxic treatments, able to repopulate locally and contribute to new tumor formation [54,55]. Herein we found that CLG identified CTCs in 5/8 GBM patients with a CTCs count 10-fold higher than ScreenCell suggesting a potential use in glioblastoma CTCs detection. In ovarian cancer, spread is mainly peritoneal; nevertheless, CTCs were relevant for prognosis [56–58]. EpCAM-based approach was limited in OC [59] while size based, density gradients and microfluidic devices were reported [60]. Nevertheless, multiplicity of devices and lack of external validation limited their use [61]. OC-CTCs were reported to overexpress CXCR4, MUC1, CK19, CD24, CD44, and TIMP1 and may have hybrid epithelial/mesenchymal phenotype [60,62–64]. In the manuscript, CLG identified CTCs in 8/8 OC patients with a CTCs count of 1,88 ± 1,62 CTCs/cc, which is almost 2-fold higher than ScreenCell. In lung cancer, CTCs are prognostic [65,66] and isolated through size, density gradients, EpCAM-immunomagnetic and microfluidic devices. EpCAM-negative CTCs were significantly higher than EpCAM-positive CTCs in stage IV patients and correlate with shorter OS and therapy resistance [67–69]. Moreover, CTCs from NSCLC patients co-express keratin and vimentin [70] suggesting cellular transition toward the mesenchymal phenotype. In TRAP4MET, CLG-CTCs cells were isolated in 6/8 LC patient's with a mean count of 1,34 ± 0,86 CTCs/cc which is 2.47 fold higher compared to ScreenCell (p = 0.0379) suggesting that the evaluation through CLG may improve CTCs isolation. To this aim, a new Clinical Trial (TRAP4MET-1) is actually enrolling 60 OC and 60 NSCLC patients to evaluate the prognostic/predictive role of CLG-CTCs with

particular interest for PARPis sensitivity or immunotherapy eligibility and to elucidate their molecular features.

3.1. Limitation of the study

The TRAP4MET trial recognizes four main limitations: 1. patient's/number of tumor analyzed and, thus, 2. lack of prognostic/predictive meaning; 3. comparison between very different approaches (CLG identifies CTCs through invasion of hydrogel/migration toward CXCL12 while Screen Cell separates CTCs based on size); 4. lack of CLG-CTCs molecular characterization. As OC and LC were the best performing, the ongoing new clinical trial (TRAP4MET-1) will enroll 60 OC and 60 NSCLC patients to evaluate the CLG isolated CTCs prognostic/predictive value and to attempt their molecular characterization.

4. Conclusions

CLG, CXCL12-loaded gel, allows peripheral blood CTCs identification and enumeration. The CXCL12 allowed to capture end enumerate the CTCs possibly more prone to generate metastasis mainly in patients with ovarian, lung and glioblastoma. In conclusion, CLG is an easy and feasible device to isolate, enumerate and characterize human metastatic CTCs through a CTCs functional isolation.

5. Materials and methods

5.1. Cell lines

Human colon cancer cells HT29, human ovarian cancer cells OVCAR 8 and human lung cancer cells H460 were cultured in RPMI (Invitrogen, San Diego, CA, USA). Human Renal cancer cells A498 were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco BRL, Grand Island, NY). Cell lines derive from "NCI 60 cancer cell line collection" obtained directly from the National Cancer Institute's Developmental Therapeutics program (NCI-DTP) [71]. Medium was supplemented with 10 % fetal bovine serum (FBS), penicillin (100 μg/mL), streptomycin (100 μg/mL) (Invitrogen, Carlsbad, CA). Cells were maintained in 5 % CO_2 at 37 °C and test proved mycoplasma-free. For cytotoxicity assay, 2000 cells/well were seeded in triplicate into 96-well plates and 24 h later cells were treated with 5-Fluoruracil (5-FU). Cells were incubated at 37 °C with 5 % CO2 for 72 h, followed by SRB assay. The optical density was determined at 540 nm by a microplate reader. IC_{50} is the concentration at which growth was inhibited by 50 %.

5.2. Cell migration

For migration assay 24-well inserts (Corning, Corning, NY) with poly-carbonate membranes (8 μm pore size) were used. Membranes were coated with collagen (human collagen type I/III) and fibronectin (20 μg/mL). HT-29, HT29-EG and HT29-CLG cells (1 × 105 cells/well) were seeded into the upper chambers in RPMI medium with 0.5 % BSA alone or in the presence of the CXCR4 antagonist R54 [40]; 100 ng/mL CXCL12 was added in the lower chambers. After 16 h in a humidified incubator, non-migrating cells were removed from the upper chamber using a cotton swab, and the cells that had migrated to the lower surface of the membrane insert were fixed in 4 % (w/v) paraformaldehyde in PBS and stained with DAPI (1:25.000, sc-3598 Santa Cruz for 15min). Cells were imaged fluorescent microscope (Carl Zeiss, Axio Scope.A1) and counted (number of cells in 10 randomly chosen fields). Migration was defined as migration index (number of cells migrating toward CXCL12/number of cells migrating toward BSA).

5.3. Colony formation assay

70–80 % grown human colon cancer cells (HT29, HT29-EG and HT29-CLG) were detached with trypsin and vital cells counted (Trypan blue exclusion). 500 cells were seeded into single well of 6-MW in 2 ml and allowed to grow for one week then cells were fixed and stained using crystal violet and washed in PBS. Colonies of at least 50 cells counted.

5.4. Three-dimensional spheroids growth

HT29, HT29-EG and HT29-CLG spheroid were obtained through hanging drops. 50 μL media containing 500 cells ($1x10^4$ cells/ml) was seeded into inverted Petri dish lid and at least 20 drops per dish were plated to allow grown for 72 h. Images of individual spheroids were captured via optical inverted microscope (Axiovert10 Carl Zeiss, Germany, using 10x objective) on day 3. Images were analyzed with ImageJ (v. 1.53).

5.5. EG/CLG infiltration-recovery assay

The commercially available Hyaluronic Acid based gel Belotero Intense® was purchased from Merz Pharma. CXCL12 (300 ng/ml, R&D System) was dropped onto the sterile gel, gently mixed and immediately used. 150 μl Empty Gels (EG) or CLG were placed in a single well of an 8-well chamber slide. For gel infiltration assay 100-500-1000 A498 (renal cancer), HT29 (colon cancer), OVCAR8 (ovarian cancer) or H460 (lung cancer) cells were seeded over the gels in 150 μl Serum Free (SF) media and allowed to infiltrate 16 h. Cells were then fixed with 400 μl 2%FA + Hyaluronidase (HAase) for 6 h, washed three times in PBS and stained with DAPI (1:25.000, sc-3598 Santa Cruz) for enumeration at fluorescent microscope (Carl Zeiss Axio Scope.A1). For gel recovery, 200 cells HT29, A498,

OVCAR8 or H460 were suspended in 150 μl SF media, seeded on 150 μl EG or CLG and allowed to migrate ON. Cells were recovered by gel digestion with HAase and grown in complete media for 6 days.

Human cancer cells (100 cells, HT29 and H460) were spiked were spiked in 3,5 cc HD whole blood and ficoll-paqued. Isolated cells were suspended in 600 μl SF media, seeded on 4 wells (150μl/well) of 8-well chamber slide containing 150 μl CLG and allowed to migrate for 16 h. Cells were fixed in 2 % FA-HAase and stained with anti-hCD45-Alexa488 (clone HI30, Biolegend, cat.304017), Alexa594-*anti*-human panCK (clone C-11, Biolegend cat. 628606), pan-and DAPI (1:25.000, sc-3598 Santa Cruz). Spiked cancer cells were identified as DAPI+/pan-CK+/CD45-cells; as control HD blood samples were also CLG-assessed. The same number of blood spiked cells were seeded on collagen/fibronectin functionalized coverslips, centrifuged, 4 % FA fixed and stained with DAPI to assess the precise number of spiked cells.

5.6. Flow cytometry

Surface markers were assessed by flow cytometry with FACSAriaIII cytometer (BD Biosciences, San Jose, CA, USA) and FACS-Diva software 8.1 (BD Biosciences, San Jose, CA USA). $1x10^4$ cells/tube were harvested and incubated with specific or isotype control antibodies for 30 min at 4 °C in PBS-0.25 % Sodium Azide in the dark, washed in PBS and analyzed. PE Anti-Human CXCR4 antibody was purchased from R&D (clone 12G5), PE Mouse Anti-Human EpCAM (clone HEA-125) and PE mouse anti-human E-cadherin (clone 67A4) antibodies were from Milteniy Biotech.

5.7. Real-time-PCR

RNA was extracted with TRIzol (Invitrogen, Carsbald, CA, USA) according to manufacturer's instructions. Quantitative real-time PCR was executed using SYBR Green Master Mix (Sensi mix, Bioline); data were analyzed on QuantStudio™ 5 Real-Time PCR System with $2-\Delta\Delta Ct$ method. Target mRNA expression was normalized on β-actin (BACT) expression. See Supplementary Methods for full Primers list.

5.8. TRAP4MET clinical trial

TRAP4MET is a monocentric, biological, no-profit, clinical trial in which 48 metastatic cancer patients were evaluated for CLG-dependent CTCs isolation and characterization in comparison with Screen Cell filters. Forty-eight advanced solid cancer patients at diagnosis (8 colon (CRC), 8 lung (LC), 8 ovary (OC), 8 renal (RCC), 8 endometrium (EC) and 8 glioblastoma (GMB)) were eligible for the study. 10 cc blood have been collected from each patients and used as follow: a. 7 cc for CTCs isolation using CLG b. 3 cc were used for CTCs isolation using Screen Cell™. When drawing blood, the first milliliter of blood collected was eliminated to avoid cutaneous epithelial cells contamination during the sampling. For CTCs isolation using CLG 7 cc patient's blood were collected in EDTA containing tube and stored at +4 °C until isolation. Blood was Ficoll-paqued within 3 h from drawing and isolated cells were suspended in 1.2 ml SF media. 150 μl of cell mixture were placed on 150 μl CLG in a single well of an 8-well chamber slide. Cells were allowed to migrate 16 h and then fixed in 400ul 2 % FA-HAase and stained with DAPI (1:25.000, sc-3598 Santa Cruz), Alexa488-anti human CD45 (clone HI30, Biolegend, cat.304017) and Alexa594-*anti*-human panCK (clone C-11, Biolegend cat. 628606). Cancer cells were identified as DAPI + pan-CK + $CD45^-$cells and enumerated.

CTCs were isolated with the ScreenCell filters according to manufacture instructions. Briefly, all blood samples were collected in 3 ml EDTA tube, inverted, incubated with 4 ml of fixative buffer (ScreenCell, Paris, France) and then filtered through the Cytology ScreenCell® device. Filters were then separated and captured cells stained with hematoxylin and pan-CK (ICC). All stained slides were evaluated by a consultant pathologist. The clinical study has been designed and developed according to the principles of the Good Clinical Practice guidelines of the International Conference on Harmonization and of the Declaration of Helsinki and approved by the Ethical Committee of the Istituto Nazionale Tumori di Napoli – IRCCS Fondazione G. Pascale (No. 50/20). All patients provided a written informed consent before starting blood samples collection.

5.9. Statistical analysis

The data was entered in a Microsoft excel database and imported to MedCalc (MedCalc Software, 10.6 version). We used Bland and Altman and Passing and Bablok analysis to know the agreement between ScreenCell and CLG. These analysis are employed to identify systematic difference between two different methodologies. In Bland and Altman graph the difference of CTCs count with the two methods (CLG minus SC) are on the y axis while the average on the x axis. The plot also show the mean of difference, 95 % CI for the mean of difference, and the line of equality. Passing and Bablok regression results are showed with a scatter diagram integrating regression line and regression equation (the intercept and slope respectively represents constant and proportional bias). Intercept A and slope B and their 95 % confidence intervals are used to define whether there is fixed or proportional bias. Mann–Whitney test was used to assess non-parametric data difference in distribution between 2 independent groups distribution and Wilcoxon for paired groups distribution.

Ethics approval and consent to participate

The clinical study was approved by the Ethical Committee of the Istituto Nazionale Tumori di Napoli – IRCCS Fondazione G. Pascale

(No. 50/20) and all patients gave a written consent before starting blood samples collection.

Data availability statement

Data that support the findings of this study have been deposited at Zenodo (https://zenodo.org/) with accession numbers n.11658466 (DOI 10.5281/zenodo.11658466″ title = "doi:DOI 10.5281/zenodo.11658466">DOI 10.5281/zenodo.11658466).

CRediT authorship contribution statement

Luigi Portella: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Giulia Bertolini:** Methodology, Conceptualization. **Giuseppe Guardascione:** Visualization, Validation, Methodology, Investigation, Data curation. **Dario Guido Di Febbraro:** Methodology, Formal analysis. **Caterina Ieranò:** Validation, Investigation, Conceptualization. **Crescenzo D'Alterio:** Validation, Conceptualization. **Giuseppina Rea:** Resources, Methodology, Investigation, Data curation. **Maria Napolitano:** Resources, Methodology, Investigation, Data curation. **Sara Santagata:** Resources, Methodology, Data curation. **Anna Maria Trotta:** Methodology, Conceptualization. **Rosa Camerlingo:** Methodology, Data curation. **Emilia Scarpa:** Resources, Project administration. **Sabrina Chiara Cecere:** Resources, Methodology. **Alessandro Ottaiano:** Methodology. **Giuliano Palumbo:** Resources, Methodology. **Alessandro Morabito:** Supervision, Resources. **Teresa Somma:** Supervision, Resources, Methodology. **Giuseppe De Rosa:** Writing – review & editing, Conceptualization. **Laura Mayol:** Writing – review & editing, Conceptualization. **Roberto Pacelli:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Sandro Pignata:** Writing – review & editing, Supervision, Funding acquisition. **Stefania Scala:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e35524.

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