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Serum CD95L level correlates with tumor immune infiltration and is a positive prognostic marker for advanced high grade serous ovarian cancer

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Running title: **Serum CD95L and tumor immune infiltration in ovarian cancer**

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30 **Novelty & Impact Statements**

31 High levels of serum CD95L (s-CD95L) is correlated with the number of tumor-infiltrating
32 immune cells in high grade serous ovarian cancer (HGSOC). These findings might therefore
33 be used as a non-invasive marker of tumor immune infiltration. This would avoid tumor
34 biopsy, which is difficult when a patient has relapsed. Immune checkpoint inhibitors such as
35 PD-1/PD-L1 or CTLA4 antibodies are more effective in tumors with immune infiltration.
36 Tomorrow, a personalized approach could be envisaged: after initial treatment, patients with
37 HGSOC are tailored according to immune factors. Thus, s-CD95L as a surrogate of tumor
38 immune infiltration could be used to select HGSOC patients referred for immunotherapy
39 trials. Patients with low s-CD95L levels may be more appropriately directed to clinical trials
40 using molecular therapies.

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Abstract

Soluble CD95L (s-CD95L) is a chemoattractant for certain lymphocyte subpopulations. We examined whether this ligand is a prognostic marker for high grade serous ovarian cancer (HGSOC) and whether it is associated with accumulation of immune cells in the tumor. Serum s-CD95L levels in 51 patients with advanced ovarian cancer were tested by ELISA. Immunohistochemical staining of CD3, CD4, CD8, CD20, CD163, CD31, FoxP3, CCR6, IL-17, Granzyme B, PD-L1, and membrane CD95L was used to assess tumor-infiltrating immune cells. Although the intensity of CD3, CD8, CD4, CD20, and CD163 in tumor tissues remained constant regardless of membrane CD95L expression, tumors in HGSOC patients with s-CD95L levels ≥ 516 pg/ml showed increased infiltration by CD3⁺ T cells ($p = 0.001$), comprising both cytotoxic CD8⁺ ($p = 0.01$) and CD4⁺ ($p = 0.0062$) cells including FoxP3⁺ regulatory T cells ($p = 0.0044$). Also, the number of tumor-infiltrating CD20⁺ B cells ($p = 0.0094$) increased in these patients. Multivariate analyses revealed that low s-CD95L concentrations (<516 pg/mL, hazard ratio (HR), 3.54; 95% confidence interval (CI), 1.13–11.11), and < 1200 activated CD8⁺ (Granzyme B⁺) cells (HR, 2.63; 95% CI, 1.16–5.95) were independent poor prognostic factors for recurrence, whereas >6000 CD3⁺ cells (HR, 0.34; 95% CI, 0.15–0.79) was a good prognostic factor. Thus, low levels of s-CD95L (<516 pg/mL) are correlated with lower numbers of tumor-infiltrating lymphocytes (CD3⁺ and CD8⁺, but also CD4 and FoxP3 T cells) in advanced HGSOC and are a poor prognostic marker.

Implications: Serum s-CD95L is correlated with the number of tumor-infiltrating immune cells in HGSOC and could be used as a non-invasive marker of tumor immune infiltration to select patients referred for immunotherapy trials that evaluate checkpoint inhibitor treatment.

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70 **Keywords:** High grade serous ovarian cancer; soluble CD95 ligand; tumor immune
71 infiltration

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Introduction

Ovarian carcinoma is the seventh most common cancer in women and the eight most common cause of cancer-related death worldwide ¹. At the time of diagnosis, the majority of patients with epithelial ovarian cancer (EOC) present with advanced disease, which is characterized by a high and widespread tumor load in the peritoneal cavity, often accompanied by malignant ascites. Thus, the prognosis of women with ovarian cancer remains poor, with a 5-year overall survival (OS) rate estimated at < 45% for all cancer stages, and only 20–30% for patients with Federation of Gynecologists and Obstetricians (FIGO) stage III or IV disease ^{1,2}.

Patients with EOC and other cancers who exhibit a robust immune response show increased survival rates ³. Recent studies show that numbers of tumor-infiltrating lymphocytes (TILs), which are lymphocytes that extravasate from blood vessels to access the tumor, may be a positive predictive factor for melanoma ⁴, colorectal cancer ⁵, esophageal carcinomas ⁶, breast cancer ^{7,8}, or endometrial cancer ⁹. The presence of TILs affects the outcome of ovarian cancer; high numbers of CD8⁺ T cells in the immune infiltrate are associated with improved overall survival (OS) ¹⁰⁻¹⁵, particularly in patients with high grade serous ovarian carcinoma (HGSOC) ¹⁶⁻¹⁸. By contrast, clinical outcome of ovarian cancers infiltrated by regulatory FoxP3⁺ T cells (Tregs) remains unclear; studies suggest either decreased OS ¹⁹⁻²¹ or improved clinical outcomes ²²⁻²⁴. Furthermore, some works establish that ovarian cancer is often accompanied by systemic immunosuppression ^{25,26}, which correlates with a poor prognosis ^{27,28}. New treatment strategies based on neutralizing antibodies that target checkpoint inhibitors represent a revolution in the fight against cancer; indeed, such treatments have shown survival benefits in patients with melanoma ²⁹ or lung cancer ³⁰. Therapeutic antibodies that block the PD1/PD-L1 checkpoint (such as nivolumab or pembrolizumab) or the CTLA4 checkpoint (such as ipilimumab) show therapeutic responses linked to tumor immune infiltration ^{31,32}. Phase II studies of anti-PD1/PD-L1 therapy in ovarian cancer suggest that it

triggers anti-tumor responses³³⁻³⁵; and, several phases III studies are underway. In such cases, a serum theranostic biomarker would be useful for selecting patients that are eligible for immunotherapy trials.

CD95L (also called FasL), which belongs to the tumor necrosis factor family, binds to the receptor CD95 (also known as Fas). Whereas CD95 is ubiquitously expressed, CD95L shows a more restricted expression pattern; it is expressed mainly on the membrane of lymphocytes, where it plays a pivotal role in eliminating infected and transformed cells³⁶. Binding of membrane-bound CD95L to CD95 recruits the adaptor protein Fas Associated Death Domain (FADD)³⁷, which in turn aggregates caspase-8 and caspase-10 to induce apoptosis³⁸. CD95L is also expressed by endothelial cells lining the blood vessels of patients with tumors and chronic inflammatory disorders³⁹⁻⁴¹. These CD95L-expressing endothelial cells seem to behave as a selective immune barrier, killing CD8 T cells while being permissive for Treg cells⁴¹. CD95L can be cleaved by metalloproteases, thereby releasing soluble CD95L (s-CD95L) into the bloodstream. Binding of s-CD95L to CD95 fails to trigger cell death but rather induces a non-apoptotic signaling pathway that promotes the migration of T cells⁴². Here, we wondered whether s-CD95L plays a role in ovarian cancer and examined its impact on the immune landscape in HGSOC patients.

Material and methods

Cell lines and culture conditions

IGROV-1, OVCAR-3, OVCAR-8, and SKOV-3 cell lines were obtained from American Type Culture Collection (LGC Standards, Molsheim, France) and were authenticated by Short Tandem Repeat. Each month, routine testing was conducted on all cultured cells using the sensor-cell approach Plasmotest™ - Mycoplasma Detection Kit (InvivoGen). O170, O370, O386, and O829 cell lines were established in-house from primary solid tumor or carcinomatosis samples cut into small pieces ($<1\text{ mm}^3$) with a scalpel and then subjected to enzymatic digestion with collagenase (0.23 Wünsch units/ml; Liberase™ research grade; Roche, Indianapolis, IN). These in-house cell lines had more than 20 passages. All cell lines were cultured in DMEM complete medium supplemented with L-glutamine and 8% fetal bovine serum. Cells were used within 6 months after resuscitation of frozen aliquots.

Spheroid formation assays

Cells (1000/well) were seeded in 96-well ultra-low attachment plates (Corning) and cultured for 7 days in serum-free culture medium (Mammoth™ Human Medium Kit, STEMCELL) supplemented with heparin (STEMCELL) and hydrocortisone (STEMCELL). At Day 7, the number of spheres per well was counted manually by taking five large field pictures of each well using a $\times 4$ objective lens.

Generation of cleaved CD95L

HEK/293T cells cultured in 1% FCS-containing medium were transfected with 3 μg of empty plasmid or a wild-type CD95L-containing vector using the calcium/phosphate precipitation method. Medium containing s-CD95L and exosome-bound full-length CD95L was harvested 5 days after transfection. Dead cells and debris were removed by centrifugation ($2 \times 4,500$

139 rpm/15 min). Exosomes were pelleted by ultracentrifugation at 100,000 g for 2 h. Finally,
140 debris- and exosome-free supernatants were concentrated (Centricon; 10 kDa cut-off) and
141 dialyzed against PBS.

142 ***Western blot analysis***

143 Cells were lysed for 30 min at 4°C in lysis buffer (25 mM HEPES pH 7.4, 1% v/v Triton X-
144 100, 150 mM NaCl, 2 mM EGTA supplemented with a mix of protease inhibitors; Sigma-
145 Aldrich). Protein concentration was determined using the bicinchonic acid method (PIERCE,
146 Rockford, IL, USA). Proteins were separated on an 8% SDS-PAGE gel and transferred to a
147 nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK). The membrane was
148 blocked for 30 min with TBST (50 mM Tris, 160 mM NaCl, 0.05% v/v Tween 20, pH 7.8)
149 containing 5% w/v dried skimmed milk or bovine serum albumin (BSA) and incubated
150 overnight at 4°C with primary antibodies (mouse anti-E-Cadherin, clone 36, BD Pharmingen;
151 mouse anti-Vimentin, clone V9, DAKO; and mouse anti- α -actin, clone AC-74, Sigma)
152 diluted in the same buffer used for saturation (milk for anti-E-cadherin and anti- α -actin
153 antibodies and BSA for anti-vimentin antibodies). The membrane was washed with TBST,
154 followed by incubation for 1 h with a peroxidase-conjugated anti-mouse antibody
155 (SouthernBiotech, Birmingham, Alabama, USA). Proteins were visualized using the enhanced
156 chemiluminescence substrate kit (ECL, GE Healthcare).

157 ***Flow cytometry analysis***

158 Cells were stained for 30 min at 4°C with anti-CD24 (clone ML5, BD Pharmingen), CD44
159 (clone G44-26, BD Pharmingen), and anti-CD95 (clone DX2, BD Pharmingen) monoclonal
160 antibodies (mAbs). Isotype-matched murine fluorochrome-conjugated immunoglobulins (PE

mouse IgG2a, APC mouse IgG2b, and PE mouse IgG1, respectively; BD Pharmingen) were used as negative controls.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from the cells using the NucleoSpin® RNA Isolation Kit (Macherey-Nagel) and cDNA was generated from 1 µg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quality and quantity of total RNA and cDNA was measured using a DS-11 Spectrophotometer (DeNovix). Expression of mRNA was measured using the TaqMan Gene Expression Assay Kit (Applied Biosystems), which contains primers and TaqMan MGB probes specific for the following genes: FAP1 (assay #: Hs00196632_m1), ZEB1 (Hs00611018_m1), ZEB2 (Hs00207691_m1), CDH1 (Hs00170423_m1), SNAIL (Hs00195591_m1), TWIST1 (Hs01675818_s1), and human GAPDH (Hs99999905_m1; endogenous control). The probes targeting SLUG (ENST00000020945.3|ENSG00000019549.9) were designed using the Universal Probe Library's Assay Design Center Tool (Roche), and the forward (TGGTTGCTTCAAGGACACAT) and reverse (GCAAATGCTCTGTTGCAGTG) primers were purchased from Eurogentec. Expression of SLUG mRNA was measured using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems), with human GAPDH used as an endogenous control. Fifty nanograms of cDNA were used for each qPCR reaction. All qPCR experiments were performed in a QuantStudio5® machine (Applied Biosystems) and the data were analyzed using Thermo Fisher Connect™ software (Thermo Fisher). Relative expression of each gene was calculated using the $2^{-\Delta\Delta CT}$ method and expressed as a fold change.

Cell death assay

Cell viability was measured in an MTT assay. Briefly, 4×10^4 cells were cultured for 24 h in flat-bottom, 96-well plates along with the indicated concentrations of apoptosis inducer (final volume, 100 μ L). Next, 15 μ L MTT (5 mg/ml in PBS) solution was added and incubated at 37°C for 4 h. Absorbance was measured using the Infinite F200 Pro (TECAN) at a wavelength of 570 nm.

Cell migration assay

To determine whether s-CD95L contributed to T cell migration, T cells were exposed to s-CD95L or control medium in Boyden chambers. This migration assay was previously described⁴³. Briefly, to measure trans-endothelial migration of activated T cells, use a 3 μ m-sized porous membrane of a Boyden chamber. HUVEC cells are plated to form a monolayer mimicking endothelial barrier. Membranes are first hydrated in sterile PBS, then, CD3/CD28-activated peripheral blood lymphocytes (PBLs) isolated from healthy donors (3×10^5 cells/300 μ L) are added to the top chamber covered with a monolayer of HUVEC in a low serum (1%)-containing RPMI. Bottom chamber contains 500 μ L of RPMI 1% FBS in presence or absence of s-CD95L (100 ng/mL). Cells are cultured in a CO2 incubator at the same conditions as adherent cells for 24 h. Transmigrated cells are then counted in the lower reservoir.

Patients

All clinical investigations were conducted in accordance with the principles outlined in the Declaration of Helsinki. Blood samples were collected from patients diagnosed with ovarian cancer after written informed consent was obtained. The study was approved by the local institutional review board (CEROG 2016-GYN-1003). Samples collected prospectively from patients diagnosed with ovarian cancer between January 2010 and December 2013 were

reviewed retrospectively. All samples were obtained at the time of diagnosis and before chemotherapy treatment. Fifty-one patients with advanced stage ovarian cancer were analyzed: 37 with HGSOC, six with endometrioid subtype, four with mucinous subtype, two with clear cell subtype, and two with low grade serous ovarian cancer. Thirty-six patients received neoadjuvant chemotherapy after tumor sample plus interval debulking surgery, and 15 underwent primary debulking surgery followed by adjuvant chemotherapy. The surgical specimens were evaluated histologically at the Department of Pathology. All patients were staged according to the FIGO staging system⁴⁴. After surgery, all patients received standard chemotherapy comprising carboplatin plus paclitaxel. For the current study, the main inclusion criterion was ovarian cancer with FIGO stage IIIC and IV (i.e., carcinomatosis stage or higher) and a serum sample in which the s-CD95L level before chemotherapy could be measured. Progression-free survival (PFS) was defined as the time from diagnosis of ovarian cancer to the time of recurrence or death. OS was defined as the time from diagnosis of ovarian cancer to the time of death. Observation time was defined as the interval between diagnosis and time of last contact (death or last follow-up). Data were censored at death or at the last follow-up for patients without recurrence.

Measurement of s-CD95L by ELISA

S-CD95L concentrations in the serum of HGSOC patients and healthy donors were measured in an ELISA (Diacclone, Besançon, France). All blood samples were harvested at the time of diagnosis.

Tissue specimens and immunohistochemical staining

Surgical specimens were fixed in 4% formalin, embedded in paraffin, and stained with hematoxylin-eosin-safran (HES). Sections from each histological specimen were reviewed by

two experienced pathologists (University Hospital, Rennes, France) to confirm the diagnosis and grade according to the method of Silverberg or Malpica (35). For each patient, a representative HES slide and the corresponding paraffin block were selected. The selected slide had to contain both tumor and adjacent stroma. The formalin-fixed paraffin-embedded blocks were cut into 5 µm slices and mounted on SuperFrost Plus microscope slides (Menzel Gläser, Braunschweig, Germany). Expression of CD3 (clone SP7; ThermoFisher, Brignais, France), CD4 (clone SP35; Cell Marque, Brignais, France), CD8 (clone C8/144B; DAKO, Les Ulis, France) CD20 (clone L26; DAKO), CD163 (clone 10D6; LEICA, Rungis, France), CD31 (clone JC70A; DAKO), Podoplanin (clone D2-40, INVITROGEN, Saint Aubin, France), FoxP3 (clone SP97; EURO BIO, Les Ulis, France), Granzyme B (clone GrB-7; Millipore), IL-17 (clone bs-2140R; BIOSS antibodies), and PD-L1 (clone E1L3N; Cell Signaling Technology) was assessed by immunohistochemistry (Ventana Discovery XT® automaton, Ventana Roche, Suisse). CD95L (clone G247-4, BD Pharmingen, France) was immunostained manually. After deparaffinization with toluene and rehydration with ethanol, sections were incubated at 95°C and bathed in Tris-EDTA at pH 8 prior to staining for CD4, CD8, FoxP3, CD20, CD3, CD31, CD163, CCR6, and Granzyme B. For CD95L, sections were deparaffinized and rehydrated, incubated at 95°C, and immersed in EDTA (pH 9). For all preparations, endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide. The reactivity of all antibodies, except CD95L, was revealed with an HRP-labeled polymer-conjugated secondary antibody followed by diaminobenzidine (DAB; OmniMap DAB® Roche). CD95L was revealed with an anti-mouse HRP-labeled polymer-conjugated secondary antibody followed by DAB (DAB® Dako).

TIL count

According breast cancer guidelines ⁴⁵, Tumour Infiltrating Lymphocytes can be subdivided according to their location within the tumor: 1) stromal TILs when located in the peritumoral space, and 2) intra-epithelial TILs, when they have penetrated the tumor islets. These recommendations are for breast cancer, and yet, there is no standardized approach to evaluate TILs in Epithelial Ovarian Cancer. Thus, in order to avoid a biased evaluation, we decided to perform a representative HES slide, which had to contain both tumor and adjacent stroma, in order to evaluate overall immune cells whatever their localization in tumor. Each immunostained slide was scanned with a NanoZoomer (HAMAMATSU). For each patient, five large field pictures were taken using a $\times 5$ objective lens and HAMAMATSU's software (NDPview). All fields were analyzed using NIS-Elements software (Nikon) and positively stained cells were counted; for analysis, a specific threshold was applied for each antibody.

Statistical analysis

Statistical analysis was performed using SAS, v.9.4 (SAS Institute, Cary, NC, USA) and R logical Version 3.4.1 software programs. Quantitative results were expressed as the statistical mean \pm standard deviation and qualitative results as percentages (%). The Mann–Whitney–Wilcoxon test was used to compare the distribution of quantitative variables between two groups (abnormal statistical distributions). The Chi square or Fisher's exact test was used to compare the distribution of qualitative variables between two groups with theoretical headcounts < 5 . The method of Contal and O'Quigley was used to determine cut-off values for the continuous variables used to examine prognosis ⁴⁶. The correlation between TILs and levels of cleaved CD95L was assessed using Spearman's correlation. The Kaplan–Meier method was used to compare survival curves between groups. All tests were two-sided and a p value < 0.05 was deemed significant.

Results

Serum CD95L is a prognostic marker for high grade serous ovarian cancer

The clinical data and outcomes of our current HGSOC cohort match those of previously described cohorts,^{47,48} indicating that, although the number of patients was relatively small, the cohort is likely to be representative of larger HGSOC cohorts (Table S1). In agreement with known clinical prognostic factors^{2,48,49}, univariate analysis identified involved lymph nodes (HR, 4.87; 95% confidence interval (CI), 1.67–14.16) and residual disease after surgery (HR, 5.74; 95% CI, 2.40–13.74) as clinical parameters significantly associated with disease-free survival and OS (Table 3). Multivariate analysis revealed that residual disease after surgery (HR, 6.16; 95% CI, 2.27–16.67) correlated with recurrence rate (Table 4). Because we showed that serum CD95L is a poor prognosis marker in triple negative breast cancer women (26), we next investigated whether the concentration of serum CD95L in women with advanced ovarian cancer was associated with clinical outcome. Serum CD95L concentrations in ovarian cancer patients showed a more heterogeneous distribution than in healthy donors (Figure 1a), and there was no statistically significant difference between serum CD95L concentrations in ovarian cancer patients and healthy subjects ($234,9.6 \pm 28.1$ pg/mL vs. 359 ± 73.8 pg/mL, respectively).

The Contal and O'Quigley method⁴⁶ revealed that a serum CD95L concentration of 516 pg/mL showed the strongest prognostic value for HGSOC patients (Figure 1c, e). We found that this cut-off failed to discriminate patients with long-term disease-free survival (DFS) from the whole population of ovarian cancer patients (Figure 1b). Nonetheless, for patients with HGSOC, this concentration discriminated poor-responders from long-term survival patients (Figure 1c). In agreement with this, the median concentration of s-CD95L in patients with DFS < 12 months was significantly lower than that in patients with a DFS >12 months (236 (0–480) vs. 317 (0–738), respectively; $p=0.039$); Figure 1d), and the OS of patients with

serum CD95L concentrations > 516 pg/ml was significantly longer than that of patients with a lower concentration (Figure 1e). Furthermore, univariate analysis pointed out that s-CD95L concentrations below 516 pg/mL were significantly correlated with node involvement (28% vs. 46%, respectively; $p=0.0409$) (Table 1) and node involvement is strongly associated with tumor relapse (Table 3).

S-CD95L does not affect survival or proliferation of tumor cells or their sensitivity to chemotherapy

Because high concentrations of s-CD95L in HGSOC patients were associated with a better clinical outcome, we asked whether this ligand had a direct effect on death, proliferation, and/or differentiation of ovarian cancer cells. CD95 can induce cell death upon binding to membrane-bound CD95L (mCD95L)³⁸. Therefore, we examined whether CD95L could kill ovarian cancer cells. All tested ovarian cancer cell lines (OVCAR-3, OVCAR-8, SKOV-3, and IGROV-1) and cells established from patients with HGSOC (O170, O370, O386, and O829) expressed CD95 (Supplementary data Figure 1a). These cells were classified as epithelial (OVCAR3, O370, O170, and 0386) or mesenchymal (O829, IGROV1, SKOV-3, and OVCAR-8) cells based on the vimentin/cadherin ratio (supplementary Figure 1b). Using a high s-CD95L concentration (*i.e.*, 100 ng/mL), we did not observe any cytotoxic effect of s-CD95L alone or in combination with doxorubicin and carboplatin chemotherapy drugs (Supplementary data Figure 2a and b).

Tumor heterogeneity is explained mainly by the hierarchical organization of tumor tissues, in which several subpopulations of self-renewing cancer stem cells (CSCs) sustain the long-term oligoclonality of the neoplasm⁵⁰. Moreover, CSCs are thought to be the seeds required to establish distant metastasis, which is responsible for poor clinical outcome⁵¹. Addition of s-CD95L, with or without chemotherapy agents, did not affect the number of ovarian CSCs,

defined as CD24^{Low}CD44^{High}ALDH1⁺ (Figure 2a, 2c and supplementary data Figure 2c). Moreover, neither the epithelial profile of OVCAR-3 nor the mesenchymal profile of OVCAR-8 varied in the presence of s-CD95L (Figure 2b). Finally, in all tested ovarian cancer cells, the rate of proliferation did not change in the presence of s-CD95L (Supplementary data Figure 1 d, e and f). Overall, these findings indicated that soluble CD95L has no effect on cell death, proliferation, or differentiation of ovarian cancer cells and does not enhance the anti-tumor effects of chemotherapeutic drugs. This leads to the hypothesis that s-CD95L may affect the tumor microenvironment, specifically the number/composition of immune tumor-infiltrating cells, in HGSOC patients.

Membrane CD95L is expressed on endothelial cells lining the blood vessels of HGSOC tumors

CD95L is expressed by endothelial cells lining the vessels of some ovarian cancers ⁴¹. Therefore, we wondered whether this was also the case for HGSOCs. Immunohistochemical (IHC) analysis of tumor sections identified m-CD95L in 29 (79.2%) patients, but not in eight (20.8%). Using serial tumor slices, we validated that m-CD95L was expressed by CD31-expressing endothelial cells (Figure 3a), but not lymphatic endothelium (D2-40+). Interestingly, the intensity of m-CD95L expression in endothelial cells was correlated with the quantity of s-CD95L dosed in serum of these patients (supplementary Figure 3a, b). Of note (and unlike s-CD95L), we found no significant correlation between the intensity of staining for m-CD95L in tumor tissue and PFS or OS (table 3), suggesting that metalloprotease-mediated release of s-CD95L in ovarian cancers could exert an important biological function that impacts prognosis.

m-CD95L and the immune landscape in HGSOC

Next, because tumor-infiltrating immune cells^{10,11,18} are associated with a good clinical outcome in HGSOC, we examined tissues for the presence of tumor-infiltrating T cells (CD3/CD4/CD8/Th17), B cells (CD20), and macrophages (CD163). The cytolytic activity of CD8⁺ T cells was monitored by staining for Granzyme B. Checkpoint inhibitors are promising therapeutic regimens for HGSOC patients; therefore, we also stained tissues for PD-L1. Although the intensity of CD3, CD8, CD4, CD20, and CD163 markers did not change regarding the staining of membrane CD95L in tumor tissues, transmembrane CD95L expression was significantly associated with the number of tumor-infiltrating FoxP3 T cells (Table 2). This finding is in agreement with results published by Coukos et al⁴¹, who suggested that m-CD95L is a selective immune barrier; it killed CD8⁺ cells but spared FoxP3-T cells. Nonetheless, we found no significant correlation between the intensity of m-CD95L staining and PFS or OS, indicating that unlike metalloprotease-cleaved s-CD95L, its membrane-bound counterpart is not a prognostic marker for HGSOC patients and that a yet unidentified metalloprotease plays a pivotal role in the disease progression. Because s-CD95L promotes T cell trafficking in lupus patients⁵² (supplementary figure 4C) and expression of matrix-metalloproteinase-7 (MMP7) is higher in these patients⁵³, MMP7 might be a good candidate for the metalloprotease involved in CD95L cleavage in HGSOC. Although the number of tumor-infiltrating CD8⁺ T cells did not correlate with the expression of m-CD95L, we found that the number of activated (GZB⁺) CD8⁺ T cells was higher in tumor tissues with greater expression of m-CD95L (Supplementary data Figure 3c). Because m-CD95L levels were not associated with a good prognosis, but activated (GZB⁺) CD8⁺ T cells were, this observation suggests immunosuppressive activity even in tumors showing high endothelial expression of m-CD95L.

S-CD95L and the immune landscape in HGSOC

Because high levels of serum s-CD95L are associated with a good prognosis in HGSOC patients, we next investigated whether the concentration of s-CD95L was associated with the number of TILs. We observed that the concentration of s-CD95L was correlated with the number of tumor-infiltrating CD3- and CD4-expressing T cells (Supplementary data Figure 4 Panel A; CD3: $r=0.4373$, $p=0.0068$; CD4: $r=0.3284$, $p=0.0472$). Counterintuitively, the number of tumor-infiltrating Treg cells (FoxP3+) in HGSCO patients also correlated with s-CD95L expression ($r=0.4584$, $p=0.0043$); by contrast, the number of infiltrating IL17-producing Th17 cells did not (Figures 3b and supplementary data figure 4a). The number of B cells was associated significantly with the concentration of s-CD95L (Figure 3b and supplementary figure 4a). Finally, we found no correlation between s-CD95L levels and the number of tumor-infiltrating macrophages (CD163+ cells) (Figure 3b and supplementary figure 4a). Also, there was no correlation between s-CD95L and Granzyme B (a marker of CD8⁺ T cell activation) or PD-L1 (Figure 3b and supplementary figure 4a), suggesting that s-CD95L is not involved in tumor recruitment/activation of cytolytic CD8⁺ T cells (GZB) or expression of PD-L1, which contributes to exhaustion of CD3⁺ T cells.

Overall, these findings suggested that the chemoattractant s-CD95L increases recruitment of CD8⁺ T cells and Treg cells, among the CD4⁺ T-cells, to HGSCO tumors, leading to an improved clinical outcome. Of note, we did not find any correlation between serum s-CD95L level and CD3⁺ T-cell count in blood sample of HGSOC patients (supplementary data figure 4, Panel b). To confirm that s-CD95L was able to promote cell motility of activated peripheral blood lymphocytes (PBLs), we incubated CD3/CD28-activated PBLs in the presence or absence of s-CD95L and evaluated cell migration using Boyden chambers. As shown in Figure 4, Panel c, s-CD95L enhanced the migration of activated T-cells.

Discussion

Here, we show that HGSOC patients with high levels of s-CD95L have a better prognosis than those with low levels. In addition, high s-CD95L levels correlate with increased numbers of tumor-infiltrating immune cells, including T-cells (CD8⁺ lymphocytes and Tregs), and B-lymphocytes. These findings suggest that s-CD95L plays a role in regulating tumor immune responses in women with HGSOC and could therefore be a non-invasive marker of tumor immune infiltration. Such a marker would avoid the need for tumor biopsy, which is difficult when a patient has relapsed. Immune checkpoint modulators such as PD-1/PD-L1 or CTLA4 antibodies do not recruit lymphocytes, but break immunosuppression, which lead to restore cytotoxic T cell activity. Indeed, PD-1/PD-L1 blockade is more effective in tumors with immune infiltration. PD-1/PD-L1 antibodies trigger objective tumor responses in only 20–30% of patients with recurrent ovarian cancer^{34,35}. So far, no biomarkers are associated strongly with high response rates, although PD-L1 expression on both tumor and immune cells can be used to select patients that are more likely to respond to PD1/PD-L1 treatment³⁴. However, a pathology sample is needed to assess these biomarkers. Moreover, due to the heterogeneous nature of cancer, such a sample may not reflect the disease. Tomorrow, a personalized approach could be envisaged: after initial treatment, patients with HGSOC are tailored according to expression of immune factors. Thus, s-CD95L, as a surrogate marker of tumor immune infiltration, could be proposed to select HGSOC patients for immunotherapy trials: indeed, patients with high s-CD95L expression could be offered check point inhibitor treatment as a maintenance therapy, and patients with low s-CD95L expression may be more appropriately referred to clinical trials of chemotherapies. Nevertheless, a stronger efficiency of PD1/PD-L1 checkpoint inhibitor have still to be demonstrated for ovarian cancer patients with high infiltrate of immune cells as compared to women showing a low immune response. By now, the results of immunotherapy trials in ovarian cancer remain disappointing. Mesnage

et al. showed that neoadjuvant chemotherapy increases tumor immune infiltration in some HGSOc patients⁴⁹; thus s-CD95L as a marker of tumor immune infiltration may allow us to monitor immune responses during neoadjuvant chemotherapy, and to select patients for immunotherapy when a strong tumor immune infiltrate is observed after neoadjuvant chemotherapy.

Although our data required an external validation with an independent cohort, one strength of the present study is that we selected only patients with HGSOc and FIGO III and IV stage (i.e., carcinomatosis stage or tumor peritoneal spread), which avoids confusion with other subtypes of ovarian cancer harboring different molecular mutations (e.g., mucinous, endometrioid, or clear cell ovarian cancer) and showing different immune responses^{54,55}. Indeed, we observed no correlation between survival and s-CD95L levels in patients with HGSOc, mucinous, or endometrioid cancer. Furthermore, IHC experiments identified several types of immune cell in tumor tissue from these highly-selected patients: regulatory T cells (FoxP3 T cells), CD8 T cells, B cells (CD20 cells), and macrophages (CD163 cells). Zhang et al. showed a correlation between TIL numbers and OS and PFS¹¹, as did Tomsova et al.¹³, Sato et al.¹⁰, and others^{56,57}. Nevertheless, these studies suffer from lack of immune cell markers and heterogeneity. A counter-intuitive result is the better prognosis in patients with higher rate of tumor-infiltrating regulatory T cells (FoxP3 T cells). The magnitude of the immune reaction including tumor-infiltrating CD4+, CD8+ T cells and FoxP3+ T-cells is associated with a better prognosis. Of note, in multivariate analysis, CD3+ and CD8+ T cells remain independent parameters associated with a better survival, while FoxP3 T cells is not (tables 4 and 5). It is also noteworthy that our IHC analysis fails to discriminate the different subsets of regulatory T cells. Indeed, recent data showed that regulatory T cells are heterogeneous, with five major structurally and genetically distinct cell subsets, each

representing a stage of maturation with distinct functional capacities, which could be pro-inflammation or tolerance⁵⁸.

In other tumor models such as breast cancer, TILs can be subdivided according to their location within the tumor: stromal TILs when located in the peri-tumoral space, and intra-epithelial TILs, when they have penetrated the tumor islets. This classification was widely used in breast cancer and is now recommended in clinical routine⁴⁵. Although these recommendations exist in breast cancer, there is no standardized approach to evaluate TILs in OC. In present study, we decided to perform a representative HES slide, which had to contain both tumor and adjacent stroma, in order to evaluate overall immune cells whatever their localization in tumor. Nonetheless, it would be interesting to perform in the future additional studies to address whether a correlation exists between TIL distribution and mCD95L expression or s-CD95L serum level. Because evaluation of tumor immune infiltration is difficult, the International Immuno-Oncology Biomarkers Working Group advocates evaluating intra-tumor lymphocytes in ovarian carcinoma using standard staining methods such as HES. They suggest a semi-quantitative evaluation of the area occupied by inflammatory mononuclear cells (lymphocytes and plasma cells, excluding polymorphonuclear cells)⁴⁵. These recent recommendations suggest the need for a more reliable method of evaluating tumor immune infiltration and accordingly, we examined TILs in the recommended way using IHC. The serum concentration of s-CD95L may be a reproducible and simple tool for evaluating tumor immune responses in HGSOC patients. Furthermore, we found that s-CD95L is an independent prognostic factor of PFS in HGSOC ($p = 0.0063$). HGSOC patients with high levels of s-CD95L show a good prognosis, which is opposite to that found for those with TNBC⁵⁹. This discrepancy may be due to differences in tumor load, disease history (metastasis for TNBC and carcinomatosis for HGSOC), and different roles played by s-CD95L. In TNBC, s-CD95L triggers a pro-motile signal in tumor

cells, whereas in HGSOC it seems to contribute to the immune landscape through molecular mechanisms that remain to be elucidated.

Although we observed a correlation between expression of m-CD95L (assessed by IHC) in the tumor and serum s-CD95L levels in HGSOC patients, the eight patients lacking m-CD95L in the tumor still express s-CD95L in serum. This discrepancy could be due to intra-tumor heterogeneity; further studies are required to assess m-CD95L expression in a cohort from whom multiple biopsies are obtained from anatomically distinct sites. Another hypothesis could be that the membrane-bound ligand would already have been stripped from the endothelial cell surface.

Motz et al showed that m-CD95L on endothelial cells in ovarian cancer selectively killed cytotoxic CD8 T lymphocytes, without affecting FoxP3+ regulatory T lymphocytes; they postulated that CD95L expression on tumor endothelial cells is a poor prognostic factor due to this mechanism, which generates a tolerogenic microenvironment⁴¹. Our data suggest that the mechanism proposed by Motz et al. is not complete and that the CD95/CD95L system plays a more complex role in cancer. Indeed, we showed that s-CD95L is a good prognostic factor because it could contribute to drive anti-tumor immune responses; however, data obtained with m-CD95L confirm the results of Motz et al. in that a higher number of FoxP3 T cells are accumulated in tumors of patients with endothelial expression of m-CD95L as compared to those in which CD95L is not detected. The metalloprotease that cleaves m-CD95L to generate s-CD95L is the missing link that prevents us from presenting a more complete overview of HGSOC prognosis based on the CD95/CD95L system. Nevertheless, our findings support the utility of s-CD95L as a biomarker for selecting HGSOC patients for checkpoint inhibitor treatment trial.

Conclusion

504 Serum concentration of s-CD95L is associated with an increased number of tumor-infiltrating
505 FoxP3 T cells and a better prognostic in HGSOC women. S-CD95L levels correlate with
506 prognosis. An s-CD95L level > 516 pg/mL is a cut-off value for assessing tumor immune
507 infiltration in HGSOC patients and could be used to select patients for inclusion in clinical
508 trials that evaluate checkpoint inhibitor treatment responses, such as inhibitors that block PD-
509 1/PD-L1.

510

511 **List of abbreviations:**

512 HGSOC : high grade serous ovarian cancer

513 s-CD95L: serum CD95L

514 CD: cluster of differentiation

515 EOC: Epithelial ovarian cancer

516 OS: overall survival

517 PFS: Progression-free survival

518 FIGO: Federation of Gynecologists and Obstetricians

519 TIL: tumor-infiltrating lymphocytes

520 Tregs: regulatory FoxP3⁺ T cells

521 FADD: Fas Associated Death Domain (FADD)

522 BSA: bovine serum albumin

523 HES: Hematoxylin-Eosin-Safran

524 DAB: Diaminodenzidine

525 CI: Confidence Interval

526 HR: Hazard Ratio

527 IHC: Immuno-Histo-Chemistry

528 MMP: metalloprotease

529 GZB: Granzyme B

530

531

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533

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537

538 **Conflict of interest statement:**

539

540 The authors declare no potential conflicts of interest.

541

542 **Data and material:**

543 All data and material are available.

544

545 **Agreement with publication and authors:**

546 All authors agree with this publication.

547

548 **Ethic statement**

549 This study was agreed by local institutional review board and French laws. All patients

550 consent to participate.

551

552 **Authors' contributions**

553 TDLMR, JC, AC, MLB, CP, PL and VL conceived and planned the experiments. TDLMR,

554 JC, AC, MLB, CP, and SH carried out the experiments. SH., NRL, ELP, BL, VC, JL, and

555 MLG contributed to sample preparation. TDLMR, SH, NRL, ELP, BL, PL and VL

556 contributed to the interpretation of the results. VL took the lead in writing the manuscript. All

557 authors provided critical feedback and helped shape the research, analysis and manuscript.

558

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Figure legends:

Figure 1: Serum cleaved CD95L is increased in High grade Serous Ovarian Cancer (HGSOC) patients with better prognosis.

A, Serum s-CD95L level in all ovarian cancer cohort compared with healthy donor

B, Kaplan-Meier analysis of recurrence ovarian cancer patients with s-CD95L higher (thick line) or lower (dotted line) than 516pg/mL

C, Kaplan-Meier analysis of recurrence in High Grade Serous (HGS) Ovarian Cancer patients with cleaved CD95L higher (thick line) or lower (dotted line) than 516pg/mL.

D, Serum cl-CD95L level according Disease free survival (DFS) (< or > 12 months) in HGS Ovarian Cancer patients

E, Kaplan-Meier analysis of overall survival in High Grade Serous (HGS) Ovarian Cancer patients with cleaved CD95L higher (thick line) or lower (dotted line) than 516pg/mL.

Figure 2: s-CD95L does not display any functional modifications on HGSOC tumor cells.

A, CD24 and CD44 expression and ALDH1 activity on different ovarian cancer cells lines with or without s-CD95L (100 ng/mL).

B, Comparison of mRNA level expression of mesenchymal (FAP1, ZEB1, ZEB2, SNAIL, SLUG, TWIST1) or epithelial (CDH1) genes in OVCAR 8 (mesenchymal cell) and OVCAR 3 (epithelial cell). Evaluation of mRNA level expression of mesenchymal (ZEB1, ZEB2, SLUG, TWIST1) or epithelial (CDH1) genes in OVCAR 8 and OVCAR 3 (epithelial cell) with or without s-CD95L (100 ng/mL).

C, Ovarosphere formation on ovarian cancer cells with or without s-CD95L (100 ng/mL).

Figure 3: concentrations of serum s-CD95L are associated with increased tumor-infiltrating immune cells

A, Immunohistochemical staining analysis of consecutive sections of high grade serous ovarian cancer tissues stained with markers of blood (CD31+), or lymphatic endothelium (D2-40+) and CD95L. Black arrowheads depict a blood vessel (CD31 staining and CD95L staining) and red arrowheads depict immune cells near vessels. (picture from three patients) (data representative of 37 analyzed patients)

B, Immunohistochemistry of immune cells in tumor sample of high grade serous ovarian cancer patient with high level of s-CD95L (851.57 pg/mL) and low level of s-CD95L (40pg/mL).

Table 1. Clinical characteristics of patients with high-grade serous ovarian cancer patients according to a serum s-CD95L threshold of 516 pg/mL.

Variable(s)*	s- CD95L ≥ 516pg/ml n=7	s-CD95L < 516pg/ml n=30	p
Age (years) (mean (± SD))	65.1 ± 11.6	63.3 ± 11.0	0.6002
Menopause			
No	1 (14.3%)	1 (3.8%)	0.3845
Yes	6 (89.7%)	25 (96.2%)	
Missing data	0 (0%)	4 (7.7%)	
Ca125 level (UI/mL) (mean (± SD))	1032 ± 1036	4155 ± 9370	0.6399
FIGO stage			
IIIc	7 (100.0%)	22 (73.3%)	0.4400
IV	0 (0%)	8 (26.7%)	
Nodes Involved			
Yes	2 (28.6%)	14 (46.7%)	0.0409
No	5 (71.4%)	7 (23.3%)	
Not removed	5 (20.8%)	9 (30.0%)	
BRCA gene mutation carrier			
No	2 (28.5%)	9 (30.0%)	0.4231
Yes	1 (14.2%)	1 (3.3%)	
Not known	4 (57.1%)	20 (66.6%)	
Neoadjuvant chemotherapy			
No	1 (14.2%)	9 (30.0%)	0.6471
Yes	6 (85.7%)	21 (70.0%)	
Residual disease after surgery			
No	7 (100.0%)	22 (73.3%)	0.3079
Yes	0 (0.8%)	8 (26.7%)	

SD: Standard deviation; FIGO: International Federation of Gynaecology and Obstetrics; BRCA: Breast Cancer gene

Table 2. Tumor infiltrating cell count in high-grade serous ovarian carcinoma patients based on immunohistochemical analysis of CD95L expression

Variable(s)	Endothelial membrane CD95L expression (n=29)	No membrane CD95L expression (n=8)	p
s-CD95L serum level	358.20 ± 350.12	226.04 ± 205.28	0.4485
CD3	9022.7 ± 7832.4	9945.1 ± 13658	0.4277
CD4	3841.0 ± 4760.1	1161.1 ± 935.65	0.1555
CD8	4262.3 ± 5955.5	4187.9 ± 6739.0	0.4719
CD20	2766.7 ± 3933.3	1411.6 ± 2610.8	0.1006
FoxP3	717.62 ± 750.59	276.25 ± 353.65	0.0528
CD163	8809.8 ± 5430.0	9270.6 ± 7968.3	0.5927
IL17	789.03 ± 937.34	545.25 ± 669.34	0.3660
PD-L1	904.41 ± 1360.1	527.63 ± 563.54	0.5927
GRANZYMEB	1722.6 ± 1332.2	762.88 ± 794.99	0.0173

Table 3. Univariate analysis of risk of recurrence or death

Data	Risk of recurrence		Risk of death	
	HR [95% CI]	P	HR [95% CI]	P
Age > 65 years	1.03 [0.52–2.06]	0.9332	1.09 [0.52–2.06]	0.8419
Serum Ca125 level (UI/mL) <1200	0.89 [0.42–1.86]	0.7519	0.85 [0.35–2.56]	0.7321
Stage FIGO IV (vs IIIc)	1.50 [0.63–3.56]	0.3583	1.97 [0.77–5.05]	0.2645
Involved nodes				
No	1	0.0209	1	0.0072
Yes	2.08 [0.90–4.82]		2.85 [0.90–9.00]	
Not removed	3.80 [1.48–9.75]		7.03 [2.05–24.12]	
Residual disease after surgery	5.74 [2.40–13.74]	< 0.0001	6.31 [2.40–13.74]	0.0002
Neoadjuvant chemotherapy	1.29 [0.60–13.74]	0.2937	2.69 [0.90–8.04]	0.0762
s-CD95L <516 pg/mL	3.44 [1.29–9.15]	0.0135	4.45 [1.03–19.10]	0.0449
CD3 >6000	0.32 [0.15–3.76]	0.0040	0.28 [0.11–0.70]	0.0061
CD8 >750	0.45 [0.20–1.01]	0.0532	0.35 [0.37–1.68]	0.0242
Granzyme B >1200	1.62 [0.81–3.24]	0.1733	2.52 [1.08–5.92]	0.0335
CD4 >803	0.51 [0.25–1.05]	0.0670	0.42 [0.18–0.99]	0.0468
IL17 >740	1.58 [0.75–3.33]	0.2318	1.14 [0.47–2.81]	0.7687
Foxp3 >700	0.46 [0.21–1.05]	0.0639	0.76 [0.31–1.86]	0.5506
CD163 >5500	0.56 [0.27–1.13]	0.1048	0.62 [0.27–1.43]	0.2577
PD-L1 >1000	0.36 [0.12–1.02]	0.0550	0.37 [0.09–1.60]	0.1858
CD20 >1060	0.39 [0.19–0.82]	0.0134	0.33 [0.13–0.82]	0.0174
Membrane CD95L expression	0.64 [0.31–1.89]	0.2842	0.56 [0.23–1.38]	0.2061

Table 4. Multivariate analysis of risk of recurrence

Data	Risk of recurrence HR [95% CI]	P
Residual disease after surgery	6.16 [2.27–16.67]	0.0003
CD3 > 6000	0.34 [0.15–0.79]	0.0124
Granzyme B <1200	2.63 [1.16–5.95]	0.0207
s-CD95L <516 pg/mL	3.54 [1.13–11.11]	0.0301

Table 5. Multivariate analysis of risk of death

Data	Risk of death HR [IC95%]	P
Residual disease after surgery	9.99 [2.61–38.19]	0.0035
CD3 >6000	0.27 [0.10–0.71]	0.0082
CD8 >6000	0.33 [0.12–0.87]	0.0252

Figure 1

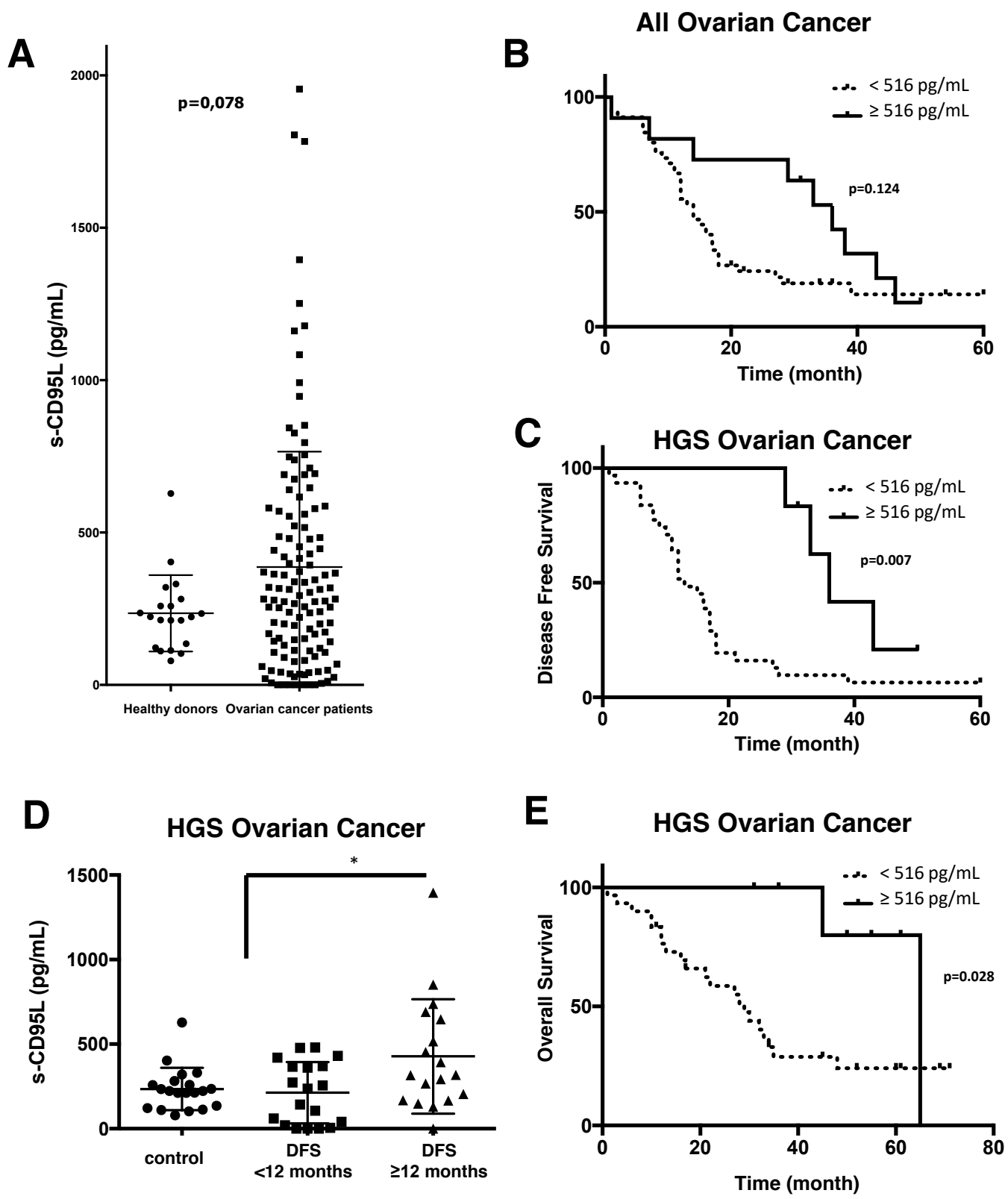
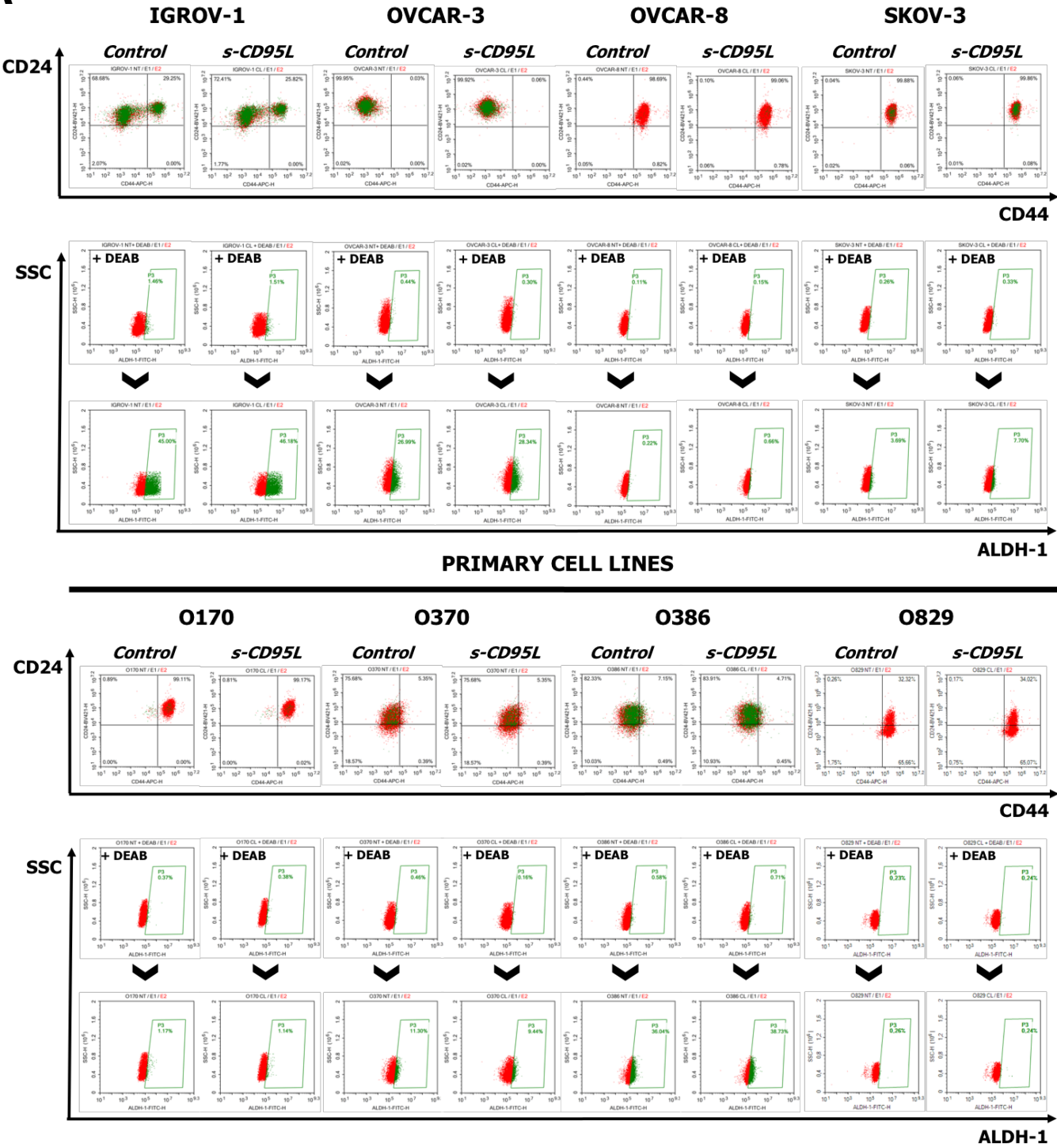


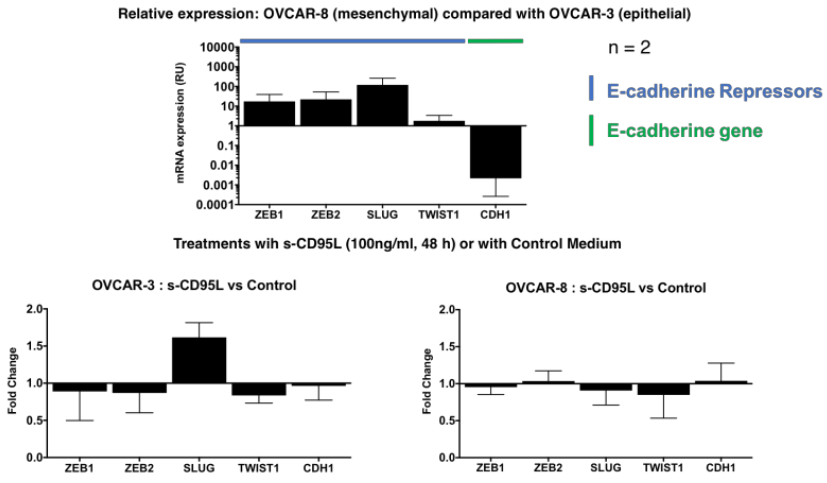
Figure 2

COMMERCIAL CELL LINES

A



B



C

OVCAR 8

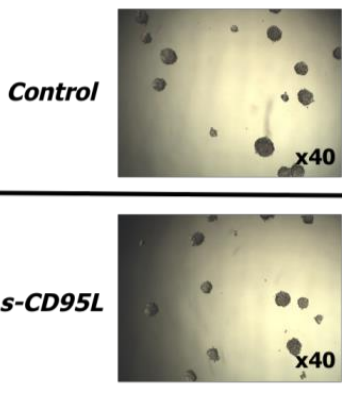
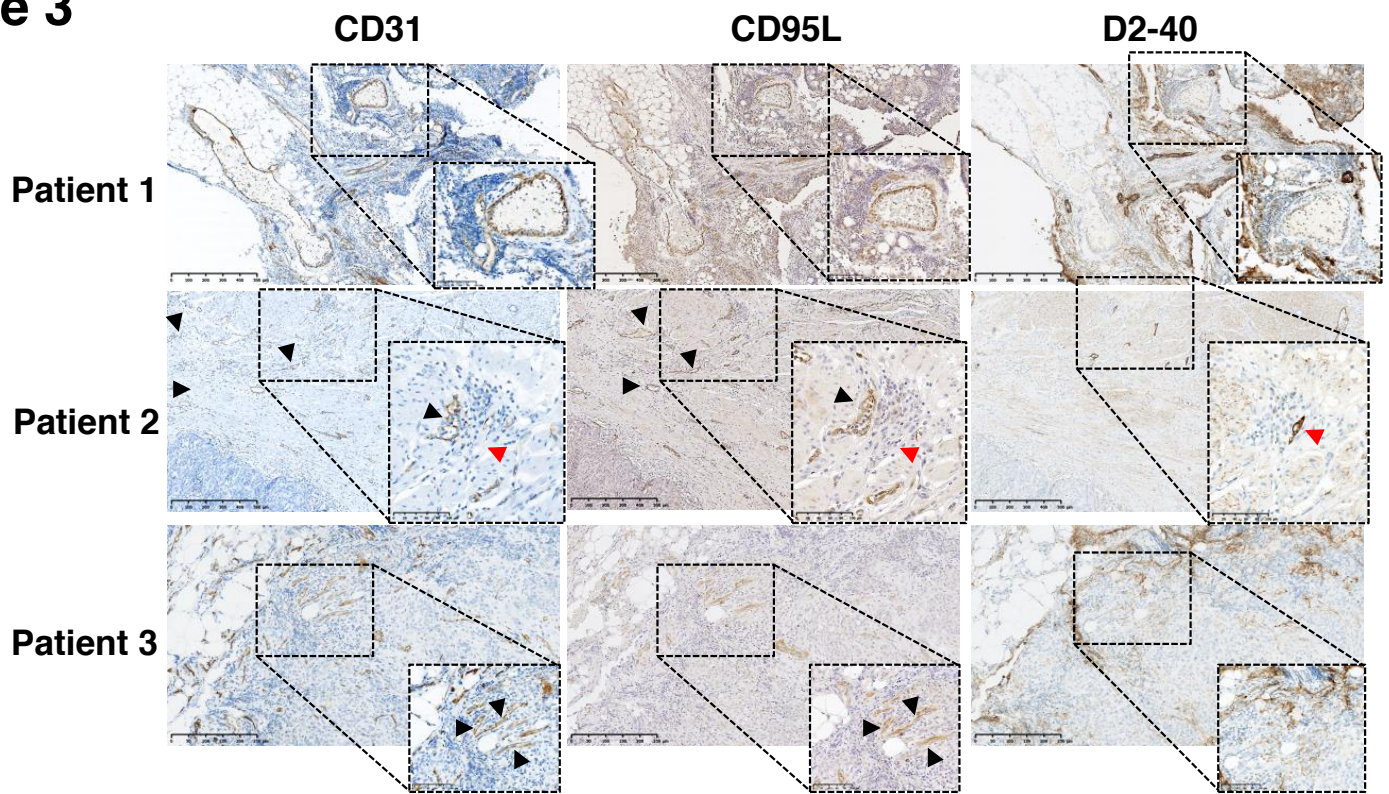


Figure 3

A



B

s-CD95L^{High}
(851,67 pg/ml)

s-CD95L^{Low}
(40 pg/ml)

