AN APPROACH TO KILL HIGH-GRADE SEROUS OVARIAN CANCER CELLS BY COMPLEMENT

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Epithelial ovarian cancer is the sixth leading malignancy amongst women. High-grade serous ovarian cancer (HGSOC) is the most common and fatal subtype. Most patients develop ascites, which promotes metastasis through the peritoneal cavity by increased pressure and contains cytokines that stimulate immune escape. However, ascites may also contain components of an active immune system, such as complement factors and tumor-directed antibodies. The tumor cells protect themselves from complement killing through, for instance, overexpression of the complement inhibitors CD59 on membranes and Factor H in the fluid phase. Our goal is to study whether local inhibition of these complement inhibitors could allow antibodies and complement in ascites to kill tumor cells.

We obtained ascites fluid samples (n=101) from ovarian cancer patients treated at the Helsinki University Hospital Women's Clinic. The ascites samples were screened for the presence of tumor-directed IgG antibodies by immunoblotting. The presence of classical pathway (CP) complement factors in ascites was examined by in-house sandwich ELISA. We observed a wide diversity between patients in both levels and types of antibodies and CP activity in ascites samples. Hemolytic assays showed that monoclonal rat IgG2 anti-human CD59 (YTH53.1) allowed about 25% of ascites fluids to kill red blood cells at a comparable level as normal human serum. YTH53.1 and anti-Factor H increased HGSOC cytotoxicity by ascites fluids only slightly, but some ascites fluid samples, rich in tumor-directed IgG seem to result in higher cytotoxicity in combination with classical pathway complement-containing NHS.

In summary, ascites fluids differ strongly in levels of HGSOC-directed IgG and classical complement activity. Ascites fluids with strongly reactive HGSOC-directed IgG seem to have more HGSOC cytotoxic capacity, especially in combination with NHS, which is slightly increased by anti-CD59 and anti-FH antibodies.

Laymen's Summary

Ovarian cancer is a prevalent disease with over 300,000 patients being diagnosed every year. Due to unspecific symptoms, such as fatigue and pain in the stomach, it is often diagnosed only at a late stage, leading to high mortality. Most patients develop ascites, which is a build-up of fluid in the abdomen. Ascites has the property to both stimulate and inhibit tumor growth. The current standard treatment for ovarian cancer consists of a combination of tumor-removing surgery and chemotherapy. Initially, this cures most patients. However, in 80% of patients, the disease comes back with metastases within 5-years. By then, the tumor often does not respond to chemotherapy anymore, so currently there is no good treatment for these patients. This leads to over half of the patients dying of ovarian cancer within the first 5 years after diagnosis. Therefore, we will aim to study the therapeutic potential of the so-called complement system, which is a specific part of the immune system, involved in the lysis of bacteria and tumor cells.

The complement system's main activation pathway is called the 'classical pathway'. This gets activated when antibodies recognize foreign structures on the surface of bacteria or, in this case, tumor cells. Proteins of the complement system in turn recognize these antibodies. Finally, through a complicated cascade of protein cleavages, complement proteins will form pores in the tumor cell surface, called the Membrane Attack Complexes. When enough of these pores are formed, the tumor cell is lysed. This is a very efficient way of killing cells, so it must be well regulated. At several steps of the complement cascade, complement regulatory proteins act as inhibitors of the cascade. The levels of complement regulators are elevated on ovarian cancer cells. Hence, we will inhibit these complement regulators, thereby aiming to stimulate the killing of ovarian cancer cells.

Unfortunately, the ovarian cancer cells seem to be very resistant against killing, even when blocking several complement regulators, killing was only increased slightly. However, we saw more killing when using ascites fluids, which contain antibodies against the ovarian cancer cells, probably because they are able to initiate the complement activation. We would like to study the properties of these antibodies in more detail. Ideally, we would find antibodies that efficiently initiate complement activation, and only bind to cancer cells. That way, the complement activation would be directed towards the cancer cells specifically. This is important as we would block the complement regulators in general, so the healthy cells would be less protected against complement killing.

To conclude, our attempts to increase killing of ovarian cancer cells by blocking complement regulators only led to very modest stimulation of killing. Antibodies directing complement activation towards the tumor cells seems to be a key element in stimulating further killing. Therefore, we need to do more research on these antibodies and specifically study the killing capacity of those in combination with other factors, such as inhibitors of complement regulators.

Introduction

Ovarian cancer is a prevalent gynaecological disease with over 300,000 cases being diagnosed per year worldwide (Huang *et al*., 2022). In 80% of cases, diagnosis takes place only at an advanced stage due to unspecific symptoms, resulting in a high mortality rate (Lisio *et al*., 2019). Since the 1980s, the standard treatment for ovarian cancer has been surgery combined with platinum-based cytotoxic chemotherapy, mainly carboplatin. This is initially effective in most cases, but cannot prevent relapses in more than 80% of patients (Lisio *et al*., 2019). These relapses often developed chemoresistance, leading to a five-year survival rate below 50% for the most prevalent subtype high-grade serous ovarian cancer (HGSOC) (Aquino-Acevedo *et al*., 2022 ; Lisio *et al*., 2019). Serous ovarian tumors have their origin most likely in the serous membrane of the fallopian tube rather than the ovary (Lisio *et al*., 2019). The high-grade classification comes from the presence of p53 mutations, leading to a more aggressive spread and is therefore associated with a poorer prognosis compared to low grade tumors (Rosen *et al*., 2010).

In advanced stages of the disease, over 90% of HGSOC patients accumulate fluid in the peritoneum, called ascites, a unique tumor microenvironment (TME) for ovarian cancer, in which tumor and immune cells interact closely through both physical and biochemical signals (Rickard *et al*., 2021). The ascites volume can increase up to several liters, thereby promoting metastasis through the peritoneal cavity by a higher peritoneal pressure (Asem *et al*., 2022 ; Huang *et al*., 2013). Additionally, immune evasion is promoted by secretion of cytokines and chemokines in ascites (Aquino-Acevedo *et al*., 2022). On the other hand, ascites can contain elements contributing to an anti-tumor response, such as active complement and tumor-directed antibodies (Bjorge *et al*., 2005).

Antibodies are the initiators of the classical pathway of the complement system, which is highly potent in the lysis of bacteria, but also tumor cells. Through a cascade of proteolytic cleavages, the central protein C3 is activated, initiating further proteolytic cleavages eventually leading to the formation of pores referred to membrane attack complexes (MACs) that together induce cell lysis (Xie *et al*., 2020). Complement regulatory proteins (CRPs), including CD59, CD46 and Factor H interfere with the proteolytic cascade at different steps to protect mammalian cells from complement-mediated lysis (Xie *et al*., 2020). CD59, also known as protectin, is a membrane-bound protein that protects cells from complement-mediated lysis by preventing the final assembly of the membrane attack complex (MAC) by capturing C5b9 complexes (Davies *et al*., 1989). CD46 (membrane cofactor protein; MCP) acts earlier in the complement pathway by serving as a cofactor for Factor I, thereby allowing for degradation of C3b and C4b (Liszweski *et al*., 1991). Factor H, a soluble CRP, inhibits the alternative pathway activation by binding C3b with its N-terminal domain and polyanions on the cell membrane with mainly its C-terminal domain, preventing the assembly of the alternative pathway C3 convertase, thereby inhibiting deposition of more C3b on the cell surface (Moore *et al*., 2021; Ferreira *et al*., 2006).

CD59, CD46 and Factor H are often overexpressed in various tumors including ovarian tumors (Surowiak *et al*., 2006; Barilla-LaBarca *et al*, 2002; Junnikkala *et al*., 2002; Bjorge *et al*., 1997; Liszweski *et al*., 1991). The elevated levels of CRPs on tumor cells can protect the tumor cells from complement-mediated killing, as Surowiak *et al*. (2006) described a lower relapse-free survival for ovarian cancer patients that express higher levels of CD46. Therefore, one could propose that inhibition of CD46, and other CRPs, could lead to complement-induced lysis of tumor cells, thereby reducing tumor growth. Indeed, Geis *et al*. (2010) showed that siRNA-mediated knock-down of CD59, CD55 and CD46 in breast and prostate tumor cells, increased complement-mediated cytotoxicity up to 66% when all three were inhibited, in which CD59 has the highest individual contribution with an elevation of up to 55%. Moreover, Campa *et al*. (2015) showed that auto-antibodies against Factor H increase cytotoxicity of non-small cell lung cancer (NSCLC) cells. Based on these findings, we speculate that inhibition of CRPs such as CD59, CD46 and Factor H also increase complement-mediated cytotoxicity of HGSOC tumor cells.

Therefore, we will aim to increase complement-mediated cytotoxicity of HGSOC tumor cells by antibody-mediated inhibition of the complement regulators CD59, Factor H and CD46. To mimic the true local tumor microenvironment, we will use ascites fluid as a source of active complement and tumor-directed antibodies to induce classical pathway complement activity.

Materials & Methods

Cell lines

The OVCAR8 cell line, identified as likely HGSOC cell line by Haley *et al*. (2016), were kindly provided by Evgeny Kuleskiy. The cell lines were grown at 37°C and 5% CO_2 in RPMI 1640 (Gibco) medium supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS), 50mM L-glutamine, 10IU/mL penicillin, 100µg/mL streptomycin, and 1% HEPES (Gibco). The cells were passaged every 2-3 days before full confluency was reached. The cells were used in experiments until a maximum of 25 passages.

Patients' ascites samples

Ascites samples (n=100) were collected from naïve to chemotherapy HGSOC patients undergoing surgery at the Helsinki University Hospital Women's Clinic from October 2020 to July 2023. Patients gave informed consents and donation of ascites was approved by an ethical permission from the Helsinki University Hospital. To remove cells and debris, the samples were centrifuged at 700g after which the supernatant containing cell-free ascites fluid was frozen at -80°C until use. Part of the cell pellets were treated with RNA later (Invitrogen) or RIPA lysis buffer (ThermoScientific) with 1:1000 Halt protease & phosphate inhibitor (ThermoScientific) and frozen at -80°C, or dissolved in FCS containing 10% DMSO to freeze cells at -130°C.

Antibodies

The hybridoma cell line producing the monoclonal rat IgG2b-anti-CD59 antibody YTH53.1, generated by Hale *et al*. (1983) was grown in IMDM (Gibco) supplemented with 10% HI-FBS and penicillin/streptomycin. Presence of YTH53.1 was checked by concentration of the antibodies in the supernatant using Protein G Dynabeads (Invitrogen), according to the manufacturer's instructions, and running a Western blot as described in the next section with rabbit-anti-rat-HRP (1:3000 ; DAKO) as detection antibody. The YTH53.1-containing culture supernatant was filtered at 0,22µm and precipitated overnight at 4°C with 1:1 saturated Ammonium Sulfate Solution (4.32M). The precipitate following 30min centrifugation at 2,250g was dissolved in 0,2M sodium phosphate buffer (pH=7,0) and dialyzed overnight at 4°C against the same buffer. Finally, YTH53.1 antibodies were purified using HiTrap Protein G HP column (Cytiva), eluted with 0,1M glycine-HCl (pH=2,7) and neutralized to pH=7,0 with 1M Tris-HCl (pH=9,0). After overnight dialysis against PBS, the sample was checked for contamination by Western blot and aliquoted at 1mg/mL at -20°C.

Reactivity against CD59 was confirmed by hemolysis assay as described later and YTH53.1 binding to OVCAR8 cells was confirmed by flow cytometry. For which 100,000 cells were incubated for 30min with 50µg/mL YTH53.1 diluted in PBS/1%BSA. After washing, cells were incubated with goat-anti-rat-IgG-AF488 (10µg/mL; Invitrogen) for 30min, after which the cells were protected from light continuously and washed twice with PBS. The cells were fixed in PBS/1%PFA and events acquired with BD LSR Fortessa flow cytometer. All incubation and centrifugation steps were performed at 4°C. Analysis was done in FlowJo.

Polycloncal Factor H IgG was isolated by Protein G purification as described for YTH53.1 from serum donated by a patient (referred to as p95) with aHUS-like symptoms producing auto-antibodies against the C-terminal domain of Factor H. As a control, IgG from pooled normal human serum (NHS) from healthy lab workers was purified in the same way. The polycloncal rabbit IgG against human CD46 was purchased from Abcam.

Classical pathway complement ELISA

Standard ELISA procedure was used to detect C5b-9 complex formation representing classical complement pathway activity in ascites fluid samples. Briefly, Nunc maxisorp 96-well plates (ThermoFisher) were coated overnight with 2µg/mL human IgM (Biorad) in 50mM Carbonate buffer (pH=9,3). Blocking was performed with 1%BSA/TB, followed by subsequent incubation with samples diluted in TBT++, mouse mAb anti-human C5b9-biotin (clone aE11; 0,4µL) in 1%BSA/TB and SA-HRP (R&D Systems) in 1%BSA/TB. All incubations were done for 1 hour at 37°C with heavy washes with PBS/0,05%Tween in between each step. Finally, 1-step ultra TMB (ThermoFisher) at room temperature was added, after which the reaction was stopped with 0,5M H_2SO_4 and absorbance was measured at 450nm at Hidex. Results of ascites fluid samples were normalized to NHS.

Hemolysis assay

Red blood cells were isolated from lithium heparin blood from healthy volunteers by 3 washes with ice-cold VBS buffer supplemented with 0,15mM $CaCl_2$ and 0,5mM MgCl (VBS++) by centrifuging for 10min at 2,000g at 4°C. Normal human serum (14,3% v/v, unless otherwise stated) from the same donor or ascites fluid (14,3% v/v, unless otherwise stated) was mixed with YTH53.1 in VBS++ (1µg/mL, unless otherwise stated) and/or p95 IgG or NHS IgG in VBS++ in a roundbottom 96-well plate. When p95 or NHS IgG was added, this mixture was pre-incubated for 30min at 4°C before addition of RBCs to a final concentration of 1% of the original blood volume and incubation for 30 minutes at 37°C. The plate was centrifuged for 10 minutes at 2,000g after which the supernatant was diluted in PBS in a flatbottom plate and absorbance was measured at 405nm using Hidex. As a positive control, RBCs were incubated with 1% Triton-X100 to reach full hemolysis, based on which the percentage of hemolysis by ascites fluids was calculated. Alternatively, hemolysis by ascites fluids was normalized to hemolysis by NHS with 1µg/mL YTH53.1. Experiments were performed in duplicates and at least during two separate days.

Detection of antibodies in ascites samples by Western blotting

Ovarian cancer cell lines were lysed by RIPA Lysis Buffer and subsequent sonication at 50% amplitude (Branson Digital Sonifier). Samples were centrifuged at 13,000g for 5 minutes at 4°C and supernatant containing cell lysate was stored at -20°C until further use. The cell lysates were diluted 1:10 with loading dye (1:4; Invitrogen) and Sample Reducing Agent (1:10; ThermoFisher) in MQ, and incubated at 70°C for 10 minutes. These were run in 4-12% Bis-Tris gels (Invitrogen) in 1X MES buffer (Invitrogen) for 30min at 165V, together with Precision Plus Protein Dual Color Standard (BioRad). Afterwards, the gel was transferred to a nitrocellulose membrane (Invitrogen) using iBlot 2NC regular transfer stacks (Invitrogen) for 7min at 20V. The membrane was blocked during 1 hour with 5% milk powder in 0,05%Tween/PBS and incubated overnight at 4°C with ascites fluid samples 1:1000 diluted in PBS. The membrane was extensively washed with 0,05%Tween/PBS and incubated with rabbit anti-human IgG-HRP (1:10.000; Dako) for 1h, followed by more extensive washing with 0,05%Tween/PBS. A light reaction with HRP was induced with 30 seconds incubation with buffer containing 10% Tris-HCl (pH=8,5), 0,5% luminol (250mM), 0,22% coumaric acid (90mM) and 30% H_2O_2, and developed on ultracruz autoradiography films.

Killing assay OVCAR8 cell line

OVCAR8 cells were seeded in a 96-well plate with 100,000 cells per well and allowed to attach overnight. Antibodies against the membrane-bound complement regulators CD59 (clone YTH53.1), CD46 (rabbit polyclonal anti-human CD46; abcam) and CD55 (mouse IgG1 anti-CD55, clone BRIC230) were pre-incubated with the cells for 30min at RT, while antibodies against the secreted Factor H were pre-incubated with NHS or ascites fluid for 30 min at 4°C. Different combinations and concentrations of CRP-directed antibodies were used. LDH release, representing cell death both through apoptosis and necrosis, was measured using the CyQuant LDH cytotoxicity kit (ThermoFisher) according to the protocol. Briefly, LDH reaction mixture was added 1:1 to supernatant of the cell culture and incubated in the dark for 30min at RT, after which the same volume of Stop solution was added. Absorbance was measured at 490nm and 680nm and cytotoxicity percentages were calculated according to the following formula after subtracting the background LDH already present in NHS or ascites fluid.

$$\% \text{ Cytotoxicity} = \left[\frac{\text{Compound-treated LDH activity} - \text{Spontaneous LDH activity}}{\text{Maximum LDH activity} - \text{Spontaneous LDH activity}} \right] \times 100$$

Results

Production of YTH53.1 antibody

Anti-human CD59 rat IgG2b antibody (clone: YTH53.1) was produced and its presence was confirmed by immunoblot (Figure 1a). To determine the concentration of YTH53.1 sufficient to allow for lysis of red blood cells, the antibody was titrated in hemolysis assays with NHS (n=2), based on which a concentration of 1µg/mL was selected to use in further hemolysis experiments. Subsequently, binding of YTH53.1 to OVCAR8 cells was confirmed by flow cytometry, with about 85% of cells being positive for goat-α-rat IgG-AF488 signal after pre-incubation with YTH53.1 (Figure 1c). The gating on single OVCAR8 cells can be found in Supplementary Figure 1a-c, including the negative controls without YTH53.1 pre-incubation.

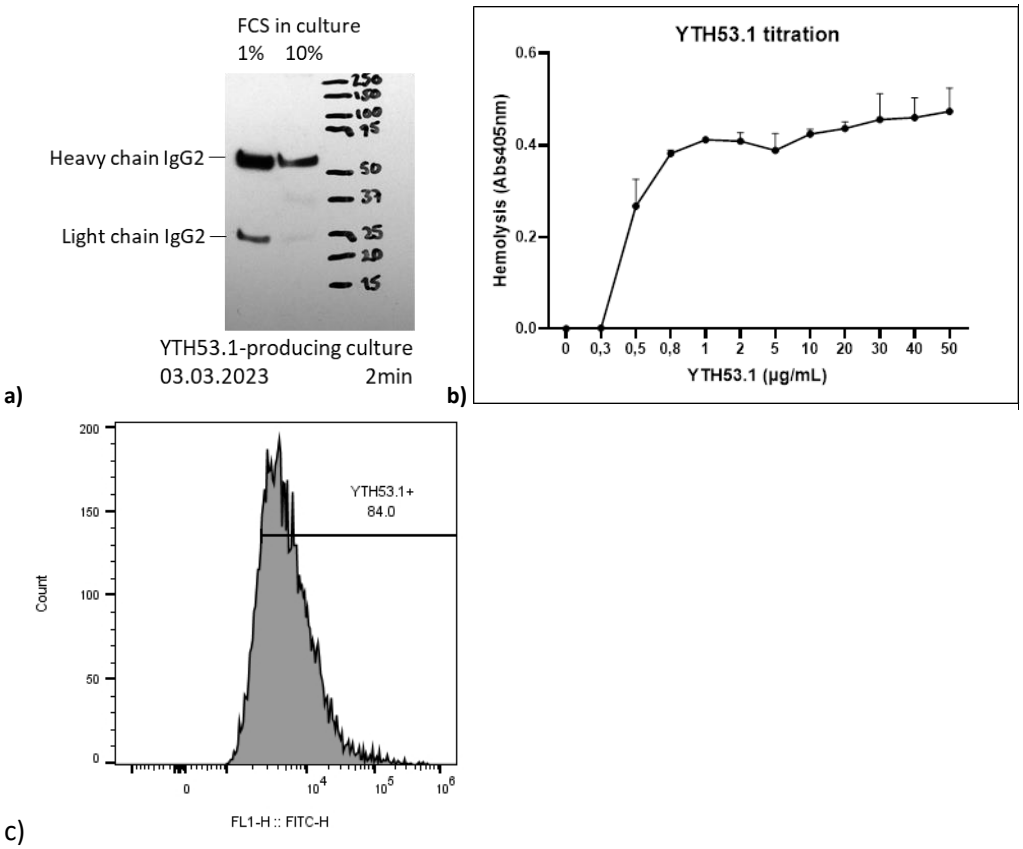

***Figure 1. Assessment of the presence and functionality of YTH53.1**. **a)** Immunoblot detecting YTH53.1 in the supernatant of hybridoma culture with 10% FCS and 1% FCS which was used as final source to isolate YTH53.1 from. **b)** Titration of YTH53.1 in hemolysis assay (n=2). **c)** Confirmation of YTH53.1 binding to OVCAR8 cells by flow cytometry.*

Complement activity and HGSOC-directed IgG in ascites fluid

To analyze the potential of classical pathway complement-mediated cytotoxic activity by ascites fluid, the presence of HGSOC-directed IgG and activity of the classical complement pathway was determined in ascites fluid samples by Western blot and ELISA, respectively. The average classical pathway complement activity in ascites fluid (n=99) was about 80% of the activity in NHS (Figure 2a) with a huge diversity between samples with a 5-95% interval ranging from 25% to 115%. The complement activity for the individual ascites fluid samples are shown in Supplementary Figure 2a.

Subsequently, the presence of HGSOC-directed antibodies was detected in ascites fluid samples. About half of the ascites fluid samples contain IgG antibodies against the tested HGSOC cell lines, of which examples are given in Figure 2b-c. However, the ascites fluids positive for HGSOC-directed IgG differ both in the antigens they detect and the concentration of HGSOC-directed IgG in the sample, which can be observed in Supplementary Figure 3, containing an overview of immunoblots of all measured (n=68) ascites fluid samples. In Figure 2d, a representative example of an ascites fluid without HGSOC-directed IgG is shown. In general, the samples containing HGSOC-directed IgG also contain HUVEC-directed IgGs to likely the same antigens. However, Asc17, Asc19 and Asc64 seem to contain HGSOC-selective antibodies, as they recognize HGSOC antigens of size 70mWCO, 55mWCO and 100mWCO, respectively, but do not, or to a lower extent, detect an antigen of the same size in HUVEC lysates (Supplementary Figure 3). The ascites fluid samples containing relatively high levels of active complement and antibodies against OVCAR8 cell lines seem promising to analyze further in cytotoxicity assays using OVCAR8 cell lines, representing HGSOC.

Hemolytic activity of ascites with CRP-inhibiting antibodies

Subsequently, the complement-mediated cytotoxic activity of ascites fluids in combination with YTH53.1 was tested in hemolytic assays on two different donors. An average hemolytic activity of about 60% of NHS (Figure 3a) was observed for the ascites fluid samples, with a broad range from 0 to 125% of hemolytic activity by NHS. The hemolytic activity for each ascites fluid sample can be found in Supplementary Figure 2b. For both NHS and all ascites fluid samples, the addition of 1µg/mL YTH53.1 was required for hemolysis (Figure 3a & 3c). Hemolytic activity of ascites fluid samples seemed to be slightly positively correlated with classical pathway complement activity in ascites fluid with a slope of 0,3772, as can be observed in Figure 3b.

To observe if complement-mediated cytotoxicity can be increased by αFH-containing p95 IgG we performed hemolysis assays with both NHS and ascites fluids. Addition of p95 IgG next to YTH53.1 increased hemolytic capacity of NHS with approximately 23%, which was not observed when IgG isolated from NHS was added (Figure 3c). This effect seemed not to be concentration dependent (Figure 3c). We proceeded with hemolysis by different ascites fluid samples in presence of 1µg/mL YTH53.1 and/or 10µg/mL IgG isolated from p95 serum or NHS. For two out of four ascites fluid samples, the addition of both p95 and NHS IgG to YTH53.1 led to higher hemolysis up to around 30%, but no hemolysis was observed with only one of these three antibodies (Figure 3d-e). For the ascites fluid samples for which only YTH53.1 induced already 40% and 80% hemolysis, hemolysis was not increased further by p95 or NHS IgG, or was even decreased (Figure 3f-g). Concluding, ascites fluid samples differ widely in their hemolytic capacity, which might be partly related to the activity of the classical pathway complement. YTH53.1 is required to allow for hemolysis by NHS and ascites fluid, whose effects can be supplemented by p95 or for some ascites fluids also NHS IgG, both of which cannot induce hemolysis on their own.

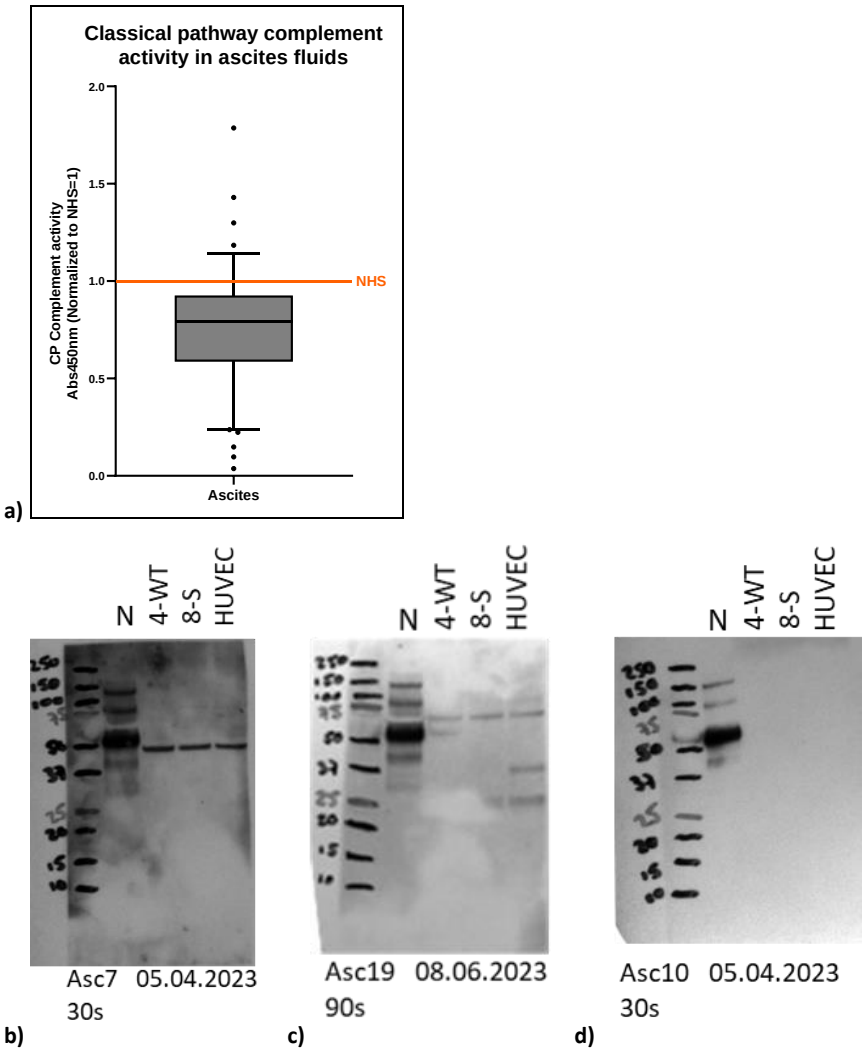

***Figure 2. Properties of ascites fluids: Classical pathway complement activity and presence of HGSOC-directed IgG**. **a)** Boxplot showing the 5-95% interval of classical pathway complement activity in ascites fluid samples (n=99; mean=76,2% ; 25-75% percentile=58,7% - 92,6%), normalized to the classical pathway complement in NHS. **b-d)** Representative examples of immunoblots of ascites fluids **b-c)** with HGSOC-directed IgG and **d)** without HGSOC-directed IgG. Legend of cell lysates: N = normal ovary tissue from ovarian cancer patient, 4-WT = OVCAR4-Wild type, 8-S = OVCAR8-Sensitive to chemotherapy, HUVEC = Healthy Umbilical Vein Endothelial Cord. 4-WT and 8-S represent HGSOC cell lines.*

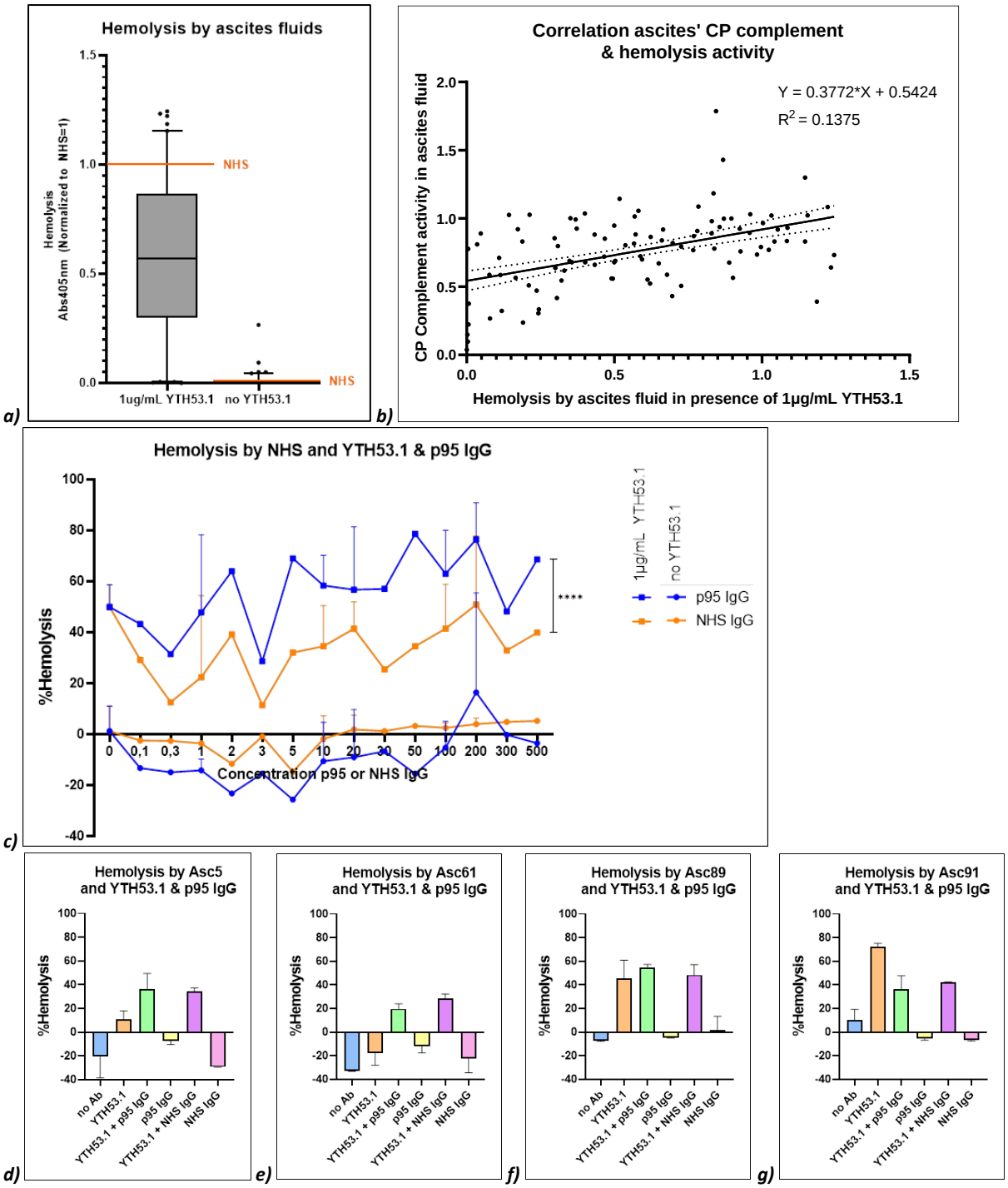

Figure 3. Hemolytic activity of NHS or ascites fluids in presence of YTH53.1 and/or p95 IgG. a) *Boxplot showing the 5-95% interval of hemolytic activity by ascites fluid samples (n=100; mean=57,4%% ; 25-75% percentile=29,8% - 86,6%), normalized to the hemolytic activity of NHS.* ***b)*** *F-test showed a significant correlation (p<0,0001) between ascites' CP complement activity and hemolysis activity with a formula of y = 0,5424 + 0,3772x .* ***c)*** *Hemolytic activity of NHS in the presence of 1µg/mL YTH53.1 and/or a range of concentrations of p95 IgG or NHS IgG. Significantly a higher percentage (difference: 23% ; p-value < 0,001) of hemolysis was reached when combining YTH53.1 with p95 IgG compared to NHS IgG, as confirmed by a paired t-test.* ***d-g)*** *Hemolytic activity of* ***d)*** *Asc5,* ***e)*** *Asc61,* ***f)*** *Asc89 and* ***g)*** *Asc91 in the presence of 1µg/mL YTH53.1 and/or 10µg/mL p95 IgG or NHS IgG.*

Cytotoxicity of OVCAR8 by ascites fluids with CRP-inhibiting antibodies

Next, we proceeded to cytotoxicity tests of the cell line OVCAR8, representing HGSOC, by ascites and NHS in combination with CRP-inhibiting antibodies, detecting LDH release as measure for cytotoxicity. Ascites samples with high hemolytic activity were selected first. Generally, the percentages of cytotoxicity were low, with around 5% cytotoxicity induced by NHS and even lower cytotoxicity percentages for the tested ascites fluid samples (Figure 4a-d). The combination of ascites fluid and NHS only increased cytotoxicity by Asc89, up to 8,5% without CRP-inhibiting antibodies and up to 15-21% with YTH53.1 and/or p95 IgG (Figure 4c). Additionally blocking CD55 and CD46, did not affect the cytotoxicity by both NHS and Asc89 (Figure 4e). The effect of p95 IgG, and 196X or OX24 seems to be comparable, containing antibodies against the C- and N-terminal domain of Factor H, respectively (Figure 4e-f).

Moreover, some pilot experiments were performed to see possible increases in cytotoxicity. Sublytic concentrations of Triton-X100 (0,01%), to initiate first killing of OVCAR8 cells, seemed to be neutralized by NHS and Asc89, as Triton-X100 induced less killing in the presence of NHS or Asc89 (Figure 5a). Preliminary results suggest that adding fresh NHS every hour, which would more realistically represent the true situation, can increase killing by ascites fluid, specifically Asc101, with more than 30% (Figure 5b). Blocking CD59 and Factor H by YTH53.1 and p95 IgG did not have any effect on killing after 5 hours (Figure 5b). As OVCAR8 cells might protect themselves with sialic acids and a mucinous layer, we added pneumolysin (PLY) and neuraminidase (NanA) to the cells to remove this sialic acid layer. Both PLY and NanA did not increase cytotoxicity more than NHS alone (Figure 5c).

Overall, blocking CD59 and/or factor H by p95 only stimulated cytotoxicity of OVCAR8 by ascites fluids slightly. Especially the combination of Asc89 and NHS with antibodies against CD59 and FH resulted in elevated levels of cytotoxicity.

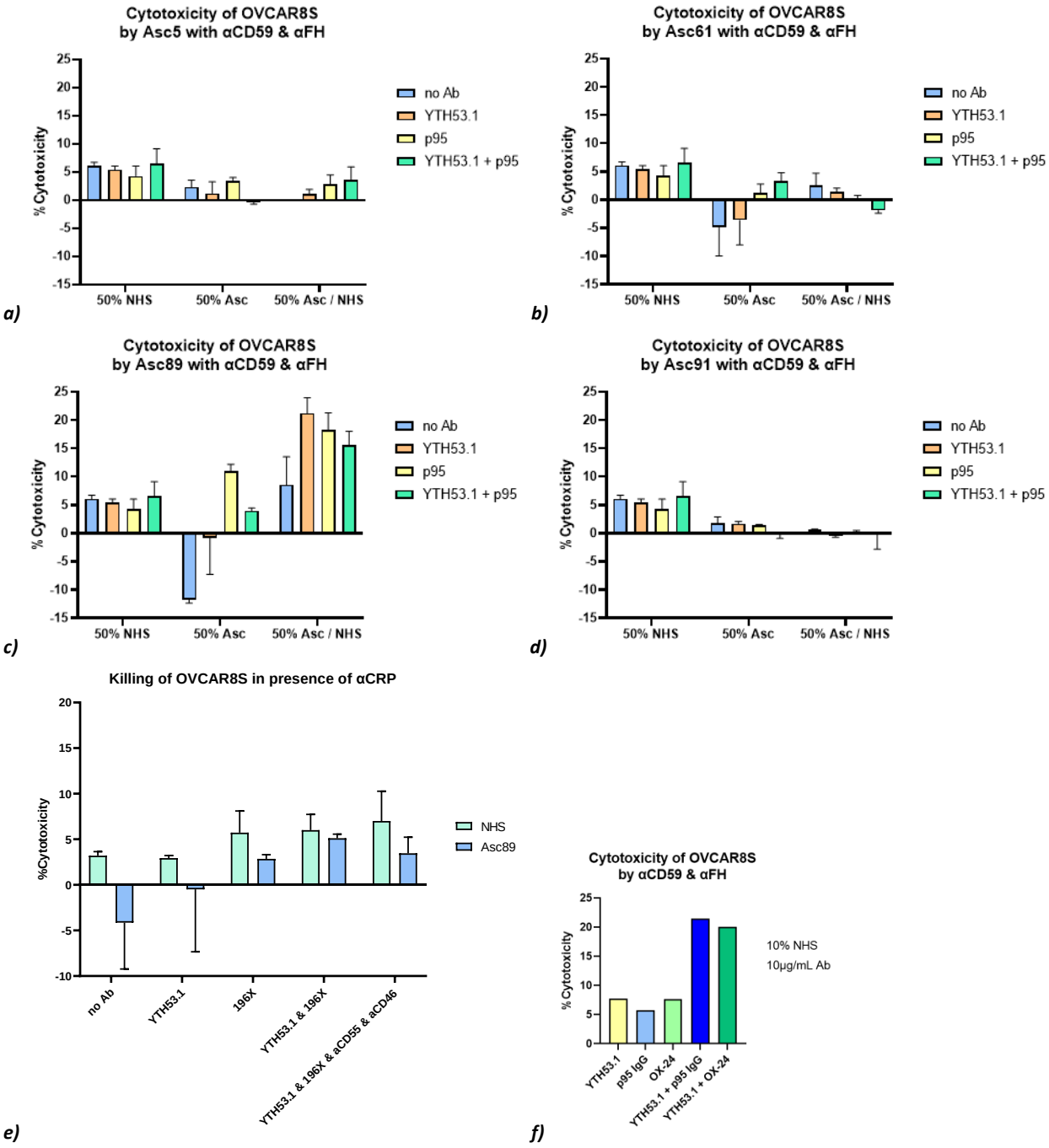

Figure 4. Cytotoxic activity of ascites fluids in presence of YTH53.1 and/or p95 IgG. a-d) *Cytotoxicity of 100,000 cells OVCAR8/well by 50%* ***a)*** *Asc5,* ***b)*** *Asc61,* ***c)*** *Asc89 and* ***d)*** *Asc91 and/or NHS (**a-d**) with 50µg/mL YTH53.1 and/or 50µg/mL p95 IgG.* ***e)*** *Cytotoxicity of 100,000 cells OVCAR8/well by 50% NHS or Asc89 with 20µg/mL YTH53.1, p95 IgG and/or αCD55 and αCD46.* ***f)*** *Cytotoxicity of 100,000 cells OVCAR8/well by 10% NHS with 10µg/mL YTH53.1 and/or p95 IgG or OX-24.*

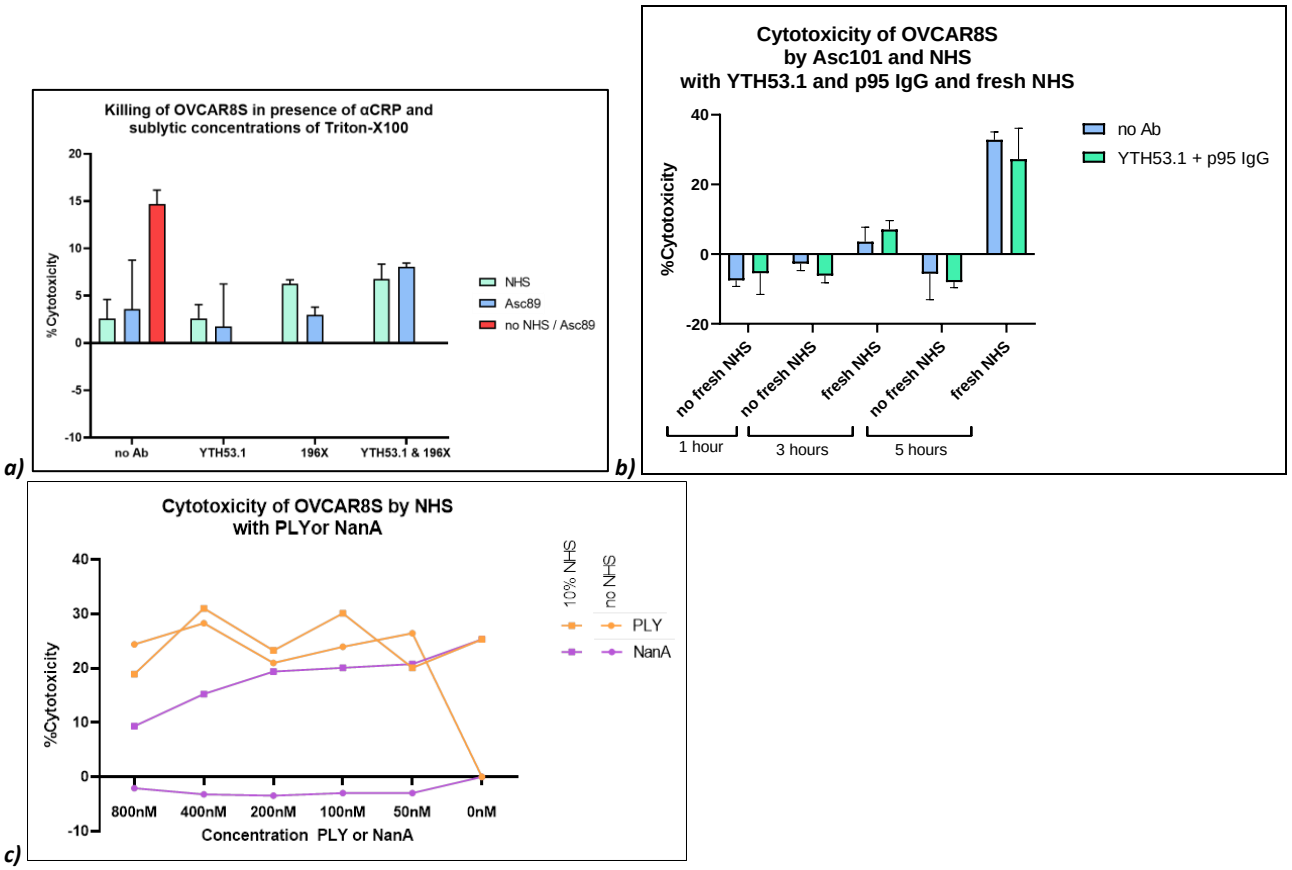

Figure 5. Pilot experiments of cytotoxic activity of NHS and ascites fluids in different conditions. a) *Cytotoxicity of OVCAR8 by NHS or Asc89 with sublytic concentrations of Triton-X100 (0,01%).* ***b)*** *Cytotoxicity of OVCAR8 by the combination of Asc101 and NHS over 5 hours, adding fresh NHS every hour.* ***c)*** *Cytotoxicity of OVCAR8 by NHS in the presence of different concentrations of PLY or NanA.*

Discussion

The main aim of this study was to analyze the classical complement pathway as a therapeutic approach for HGSOC, using HGSOC patients' ascites fluid and CRP-blocking antibodies. In summary, we found that ascites fluids differ a lot between HGSOC patients, with different targets and levels of HGSOC-directed IgG and classical pathway complement activity. This also contributes to high diversity of cytotoxic potential of ascites fluids against the HGSOC cell line OVCAR8, which generally seems to be largely resistant against complement-mediated cytotoxicity. Antibodies blocking the CRPs CD59, Factor H and CD46 had only limited effect in increasing HGSOC cytotoxic capacity of ascites fluid or NHS. However, the combination of NHS and ascites fluid with potent HGSOC-directed IgG did increase HGSOC cytotoxicity up to 20% in the presence of CD59- and/or Factor H blocking antibodies. In conclusion, the classical complement pathway seems to have potential as a therapeutic approach for HGSOC, but requires more extensive research on how the HGSOC cytotoxicity can be stimulated further.

Firstly, the diversity between ascites fluids should be studied in more detail to understand the difference in cytotoxic potential better. The ascites fluid that showed the highest cytotoxicity, Asc89, seemed to have prominent HGSOC-directed antibodies (Supplementary Figure 3bg). We speculate that these could be responsible for the observed cytotoxicity, especially in combination with NHS-derived complement factors. Therefore, the target and complement-activation potential of these antibodies should be analyzed. Additionally, continuous supply of complement-containing serum might stimulate HGSOC cytotoxicity, as the regular addition of fresh complement-containing serum increases cytotoxicity (Figure 5b). The relevance of complement levels is also reflected in the correlation between complement and hemolytic activity of ascites fluids (Figure 3b). If the relapse-free survival of patients would be monitored, we could check whether the diversity in complement activity and HGSOC-directed IgG in ascites fluids would be reflected herein (Figure 2a & 3a).

In addition to fluid containing complement and antibodies, ascites also contains tumor cells and lymphocytes, which currently gets excluded from our experiments by centrifugation. However, the antibodies with the strongest binding capacity might be missed in our approach, as these are already bound to the tumor cells. For this reason, we would examine the ascites' cell pellets for bound IgG by flow cytometry and add the full ascites sample rather than the isolated fluid. Furthermore, ascites' own tumor cells could be used in cytotoxicity experiments rather than HGSOC cell lines, as more antibodies might be produced against antigens on the patients' own tumor cells. Moreover, lymphocytes in ascites samples might contribute to complement-mediated killing of HGSOC cells by continuous production of antibodies. Additionally, we could isolate B-lymphocytes from ascites samples with lysis-inducing antibodies to generate a B-cell clone producing these efficient antibodies in higher quantities for therapeutic approaches.

In contrast to our research, previous studies to breast and prostate tumors found that inhibition of CRPs increased cytotoxicity significantly (Geis *et al*., 2010). Supposedly, siRNA inhibition which they used might be more effective than inhibition by antibodies. In our approach, we might initiate too little classical pathway complement activation by antibody deposition, as we used diluted ascites fluid as only source of antibodies. Therefore, antibodies of ascites fluids showing effective cytotoxicity, like Asc89, could be isolated by for instance Protein G purification and supplemented to the HGSOC culture in higher concentrations. Alternatively, breast and prostate tumors could be more sensitive to CRP inhibition than HGSOC tumors. Possibly, the ascites samples from ovarian cancer patients contain more tumor-promoting properties, causing that CRP-inhibition cannot overcome the tumor-growth promotion. To study this, the presence of immune inhibitors, specifically complement-inhibitors should be measured in the ascites fluid samples by for instance ELISA. Differences in CRP concentrations in ascites fluids could also partly explain differences in cytotoxic activity by different ascites fluids, when the used anti-CRP concentration is not enough to neutralize CRP in some ascites fluids.

To gain more insight in which specific step in the complement cascade should be counteracted to enhance complement-mediated HGSOC killing, we should study the deposition of complement factors in several steps of the cascade to observe at which step the cascade is halted, thereby preventing complement-mediated killing. If we find an increase in MACs, but not sufficient to lyse tumor cells, a combination of low

dosage of classical chemotherapy with anti-CRP and ascites fluid could be applied. We would hypothesize that carboplatin enters the cells through these pores. Potentially, a lower concentration of carboplatin would be sufficient for similar HGSOC cytotoxicity, leading to less side-effects.

Obviously, when HGSOC killing is stimulated efficiently, damage to the surrounding healthy tissue should be prevented as much as possible. To this purpose, we should direct antibodies against antigens that are uniquely or at higher levels expressed at HGSOC cells compared to healthy cells. To identify tumor-selective antigens, we will analyze the antigens that we found to be present in HGSOC cell lines, but not in the healthy HUVEC cell line (Supplementary Figure 3m, 3o & 3aw). For analysis by mass spectrometry, these antigens should be concentrated by 2D Western Blot. Additionally, we will perform cytotoxicity experiments with healthy endothelial cell lines next to HGSOC cell lines to determine HGSOC-specificity. Finally, undesired damage to healthy tissues would be prevented as much as possible by only adding the potential therapeutic antibodies locally in the ascites fluid, only reaching the peritoneal cavity.

The final aim to study local therapy using CRP-blocking antibodies by enabling ascites fluids' immune components to locally allow for killing of HGSOC cells requires more extensive studies as so far we found HGSOC to be very resistant against complement-mediated killing. However, the combination of HGSOC-directed IgG-containing ascites fluid and complement containing NHS allows for increased cytotoxicity of HGSOC cells, with a small elevation by αCD59 and αFH antibodies. We hope to stimulate complement-mediated killing of HGSOC further by increasing concentrations of HGSOC-directed antibody, complement, or anti-CRP antibody, simultaneously blocking more CRPs or combining CRP-blocking antibodies with low concentrations of chemotherapeutic drugs.

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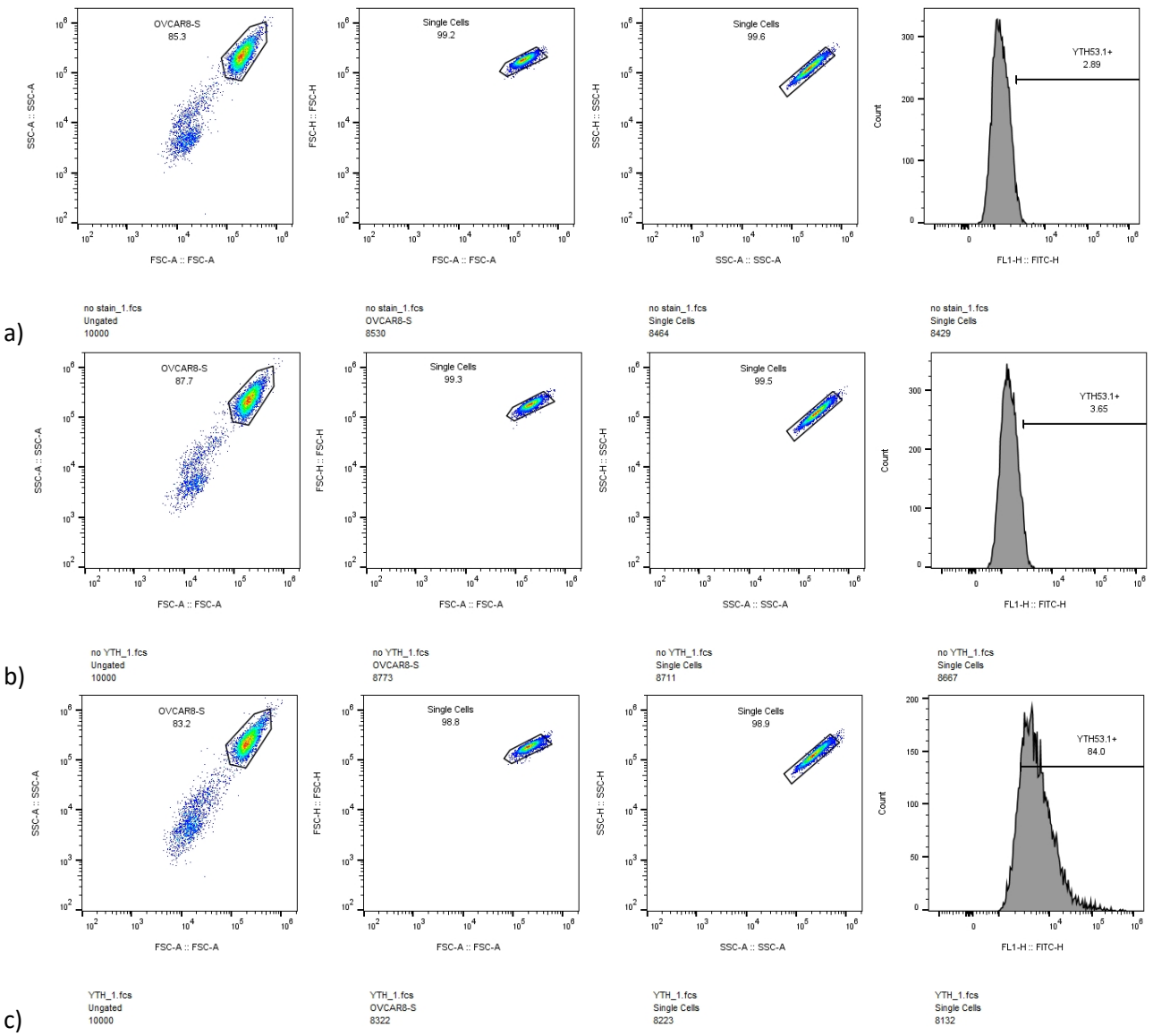
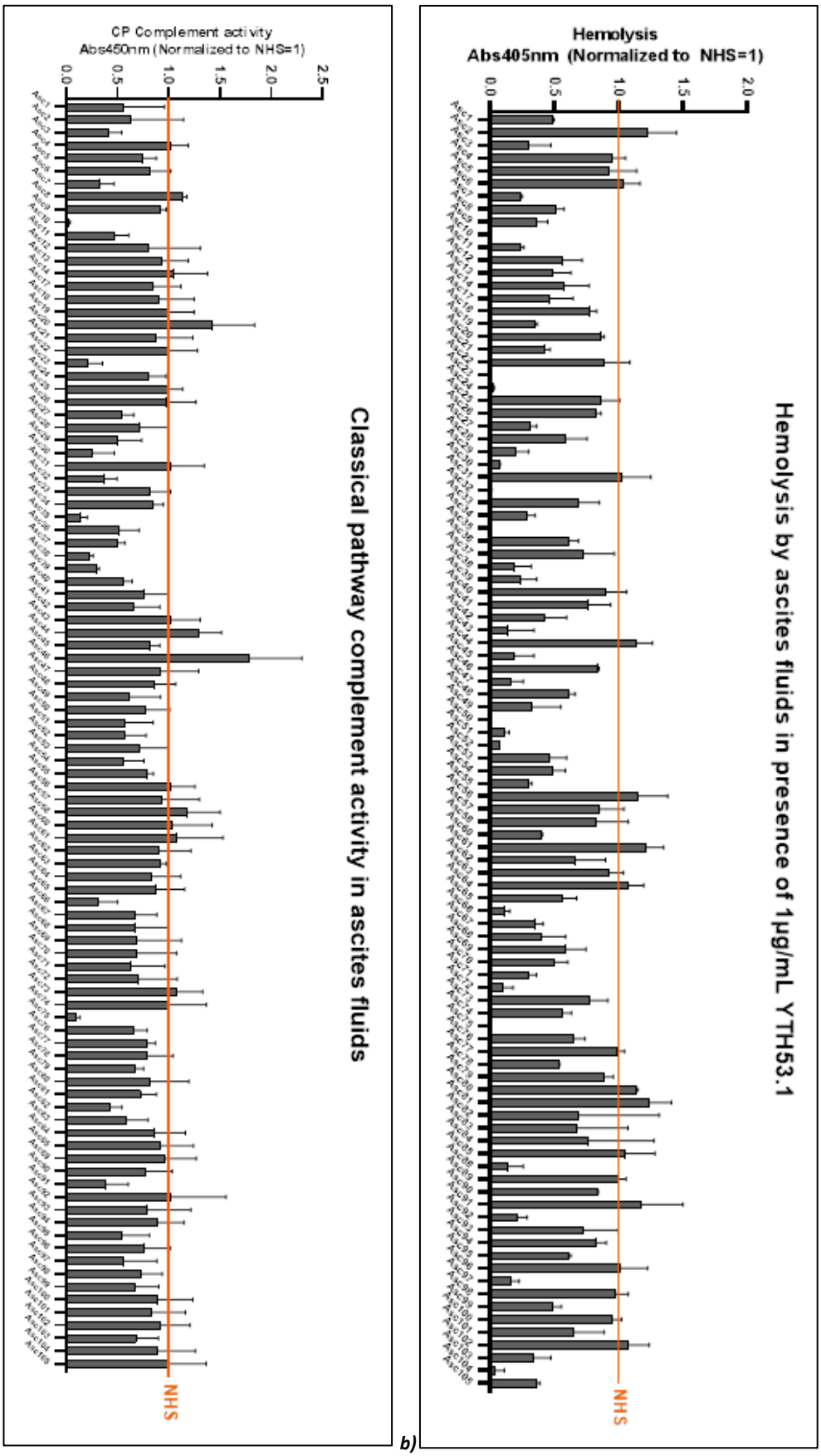
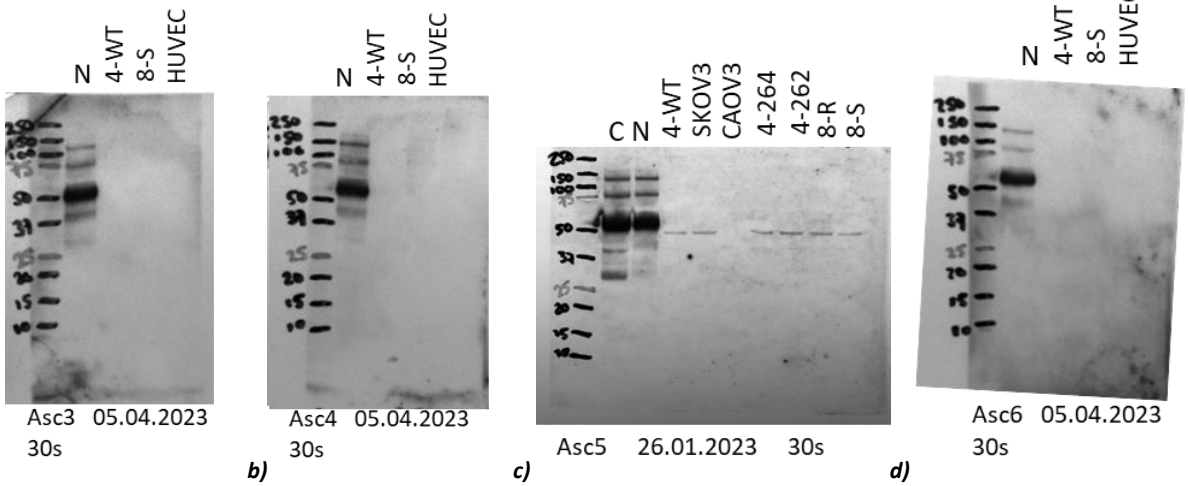


Supplementary Figure 1. Gating of single OVCAR8 cells to check for YTH53.1 binding*. Gating strategy to include only single OVCAR8 cells for the negative controls* ***a)*** *without secondary antibody goat-α-rat IgG-AF488,* ***b)*** *without pre-incubation with YTH53.1 and* ***c)*** *the experimental condition with pre-incubation with 50µg/mL YTH53.1.*



a) ***b)***

Supplementary Figure 2. Classical pathway complement and hemolytic activity of individual ascites fluid samples. ***a)*** *Classical pathway complement activity in individual ascites fluids, as measured by ELISA, normalized to NHS=1.* ***b)*** *Hemolytic activity in individual ascites fluids with 1µg/mL YTH53.1, normalized to NHS=1.*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b)
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Asc5 26.01.2023 30s
c)
N 4-WT 8-S HUVEC
Asc6 05.04.2023 30s
d)
N 4-WT 8-S HUVEC
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e)
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g)
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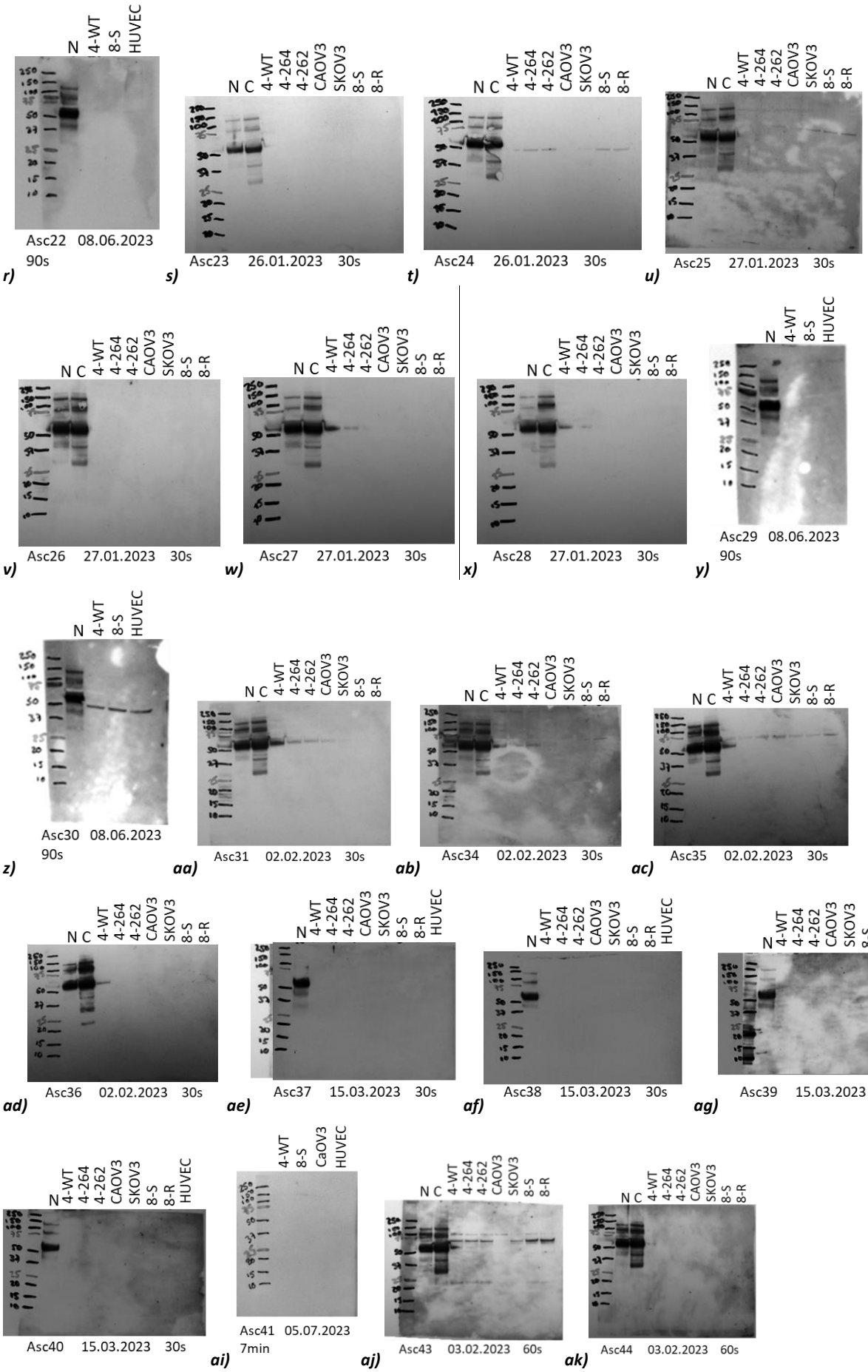
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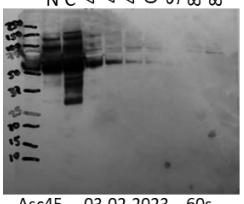

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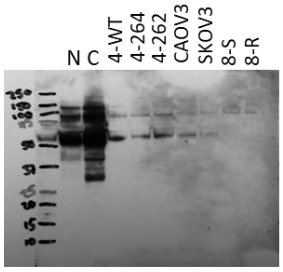

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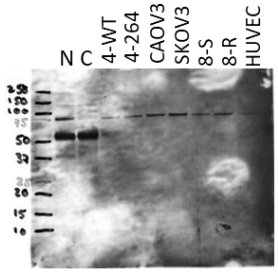

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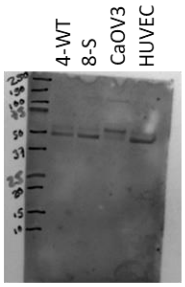

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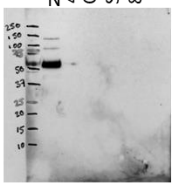

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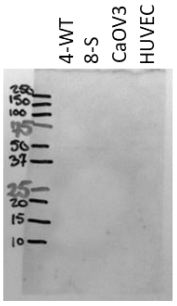

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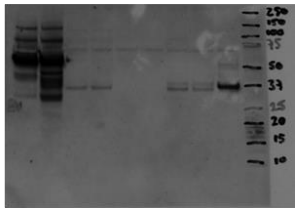

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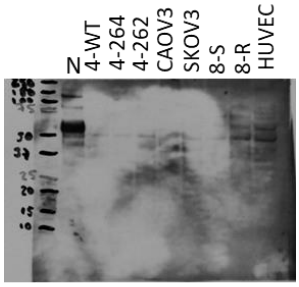

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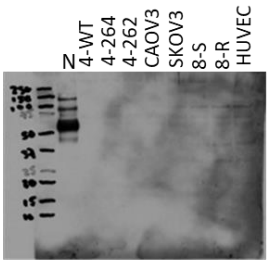

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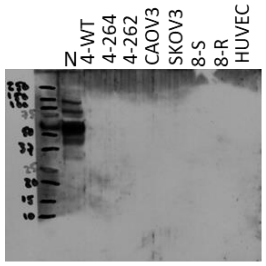

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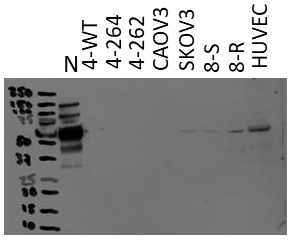

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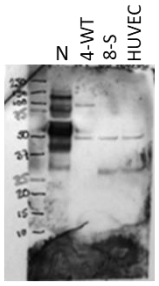

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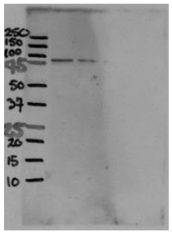

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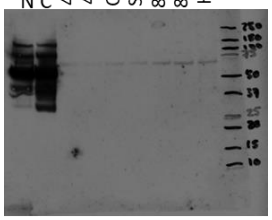

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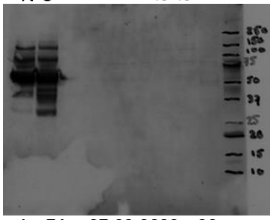

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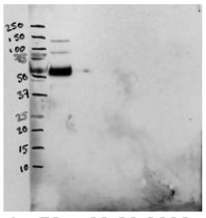

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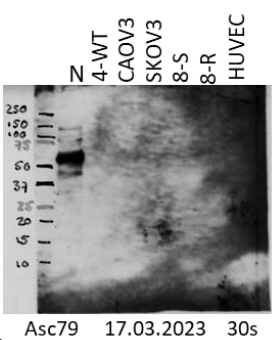

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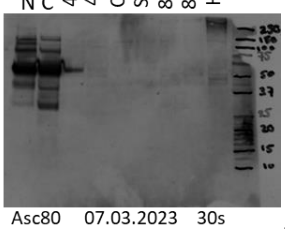

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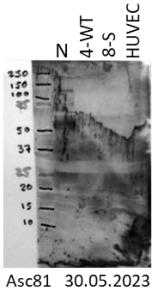

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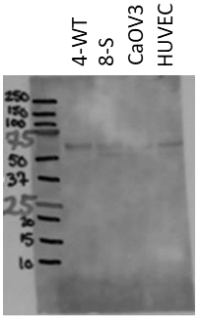

Asc83 05.07.2023 3min

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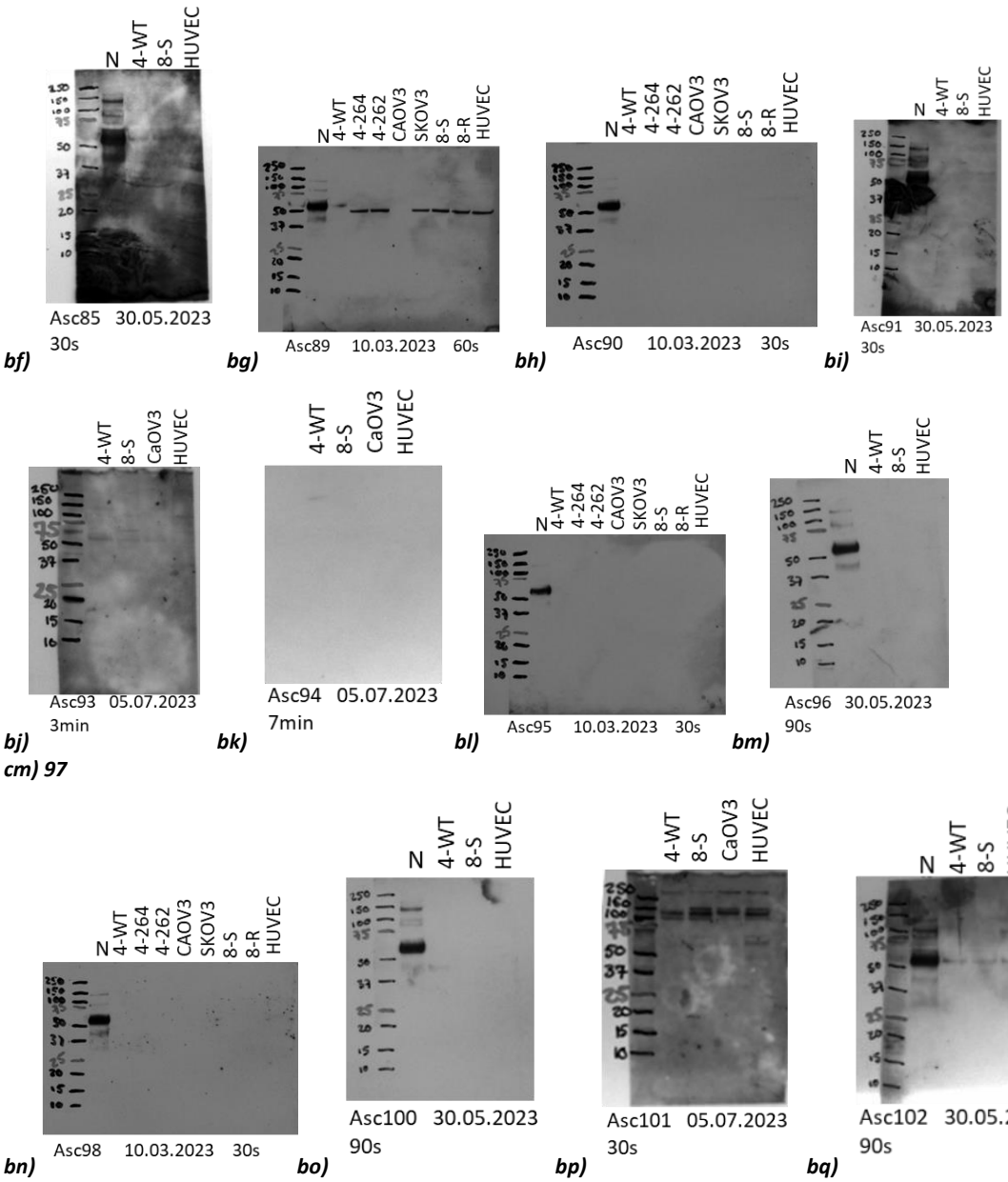

Supplementary Figure 3. Presence of HGSOC-directed IgG in individual ascites fluid samples*. **a-bq)** Immunoblots showing the presence of IgG against the used cell line lysates in individual ascites fluid samples from patient **a)** 1 to **bq)** 102. Legend of cell lysates: N = normal ovary tissue from ovarian cancer patient, C= ovarian tumor tissue from ovarian cancer patient, 4-WT = OVCAR4-Wild type, 4-264 = Crispr-Cas BRCA-2 mutated OVCAR4 (HR deficient; clone 264), 4-262 = Crispr-Cas BRCA-2 mutated OVCAR4 (HR deficient; clone 262), 8-S = OVCAR8; Sensitive to chemotherapy, CAOV3 = CAOV3, SKOV3 = SKOV3, HUVEC = Healthy Umbilical Vein Endothelial Cord.*