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Soluble AXL is ubiquitously present in malignant serous effusions

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HIGHLIGHTS

- Soluble AXL (sAXL) is widely expressed in malignant effusions.
- sAXL is overexpressed in HGSC compared to LGSC and its levels are lower following exposure to chemotherapy.
- sAXL levels in HGSC effusions are not informative of chemoresponse or survival.

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ABSTRACT

Objective. The objective of this study was to analyze the expression level and clinical role of soluble AXL (sAXL) in cancers affecting the serosal surfaces, with focus on ovarian carcinoma.

Methods. sAXL protein expression by ELISA was analyzed in 572 effusion supernatants, including 424 peritoneal, 147 pleural and 1 pericardial specimens.

Results. sAXL was overexpressed in peritoneal effusions compared to pleural and pericardial specimens ($p < 0.001$). sAXL levels were additionally significantly higher in effusions from patients with ovarian carcinoma, malignant mesothelioma and breast carcinoma compared to specimens from patients with other cancers (predominantly carcinomas of lung, gastrointestinal or uterine corpus/cervix origin) or benign reactive effusions ($p < 0.001$). sAXL was further overexpressed in high-grade serous carcinoma (HGSC; $n = 373$) compared to low-grade serous carcinoma (LGSC; $n = 32$; $p = 0.036$). In HGSC, sAXL levels were significantly lower in post-chemotherapy effusions compared to primary diagnosis pre-chemotherapy specimens ($p = 0.002$). sAXL levels in HGSC were unrelated to chemoresponse at diagnosis, progression-free survival or overall survival. Levels were similarly unrelated to survival in LGSC and breast carcinoma.

Conclusions. sAXL is widely expressed in malignant effusions, particularly in ovarian and breast carcinoma and in malignant mesothelioma. sAXL is overexpressed in HGSC compared to LGSC and its levels are lower following exposure to chemotherapy. However, sAXL levels are not informative of chemoresponse or survival.

1. Introduction

The serosal cavities, i.e. the pleural, pericardial and peritoneal spaces, are frequent sites of cancer metastasis at diagnosis or at disease recurrence, particularly from carcinomas of genital, pulmonary, breast and gastrointestinal origin. They are additionally the primary site of malignant mesothelioma. The detection of cancer cells in effusion carries grave clinical implications, placing the majority of patients in a palliative rather than curative treatment category [1]. Tumor cells in effusion are additionally recognized as a chemoresistant population prone to relapse [2]. Identifying molecular targets on cancer cells in effusions in the aim of prolonging survival is consequently an important challenge.

The receptor tyrosine kinase (RTK) AXL is member of the tumor-associated macrophage (TAM) family, together with TYRO-3 and MER. TAM receptors contain Immunoglobulin-like (Ig) and fibronectin type III domains present on other RTKs. The *AXL* gene on chromosome 19q13.2 encodes a 120 or 140 kDa protein, depending on the degree of post-translational glycosylation. The AXL protein is activated by its ligand growth arrest specific 6 (GAS6). AXL is widely expressed in normal tissues and overexpressed in several cancer types. AXL regulators in cancer include specificity proteins 1 and 3 (SP1, SP3), Myeloid Zinc Finger 1 (MZF1), the hypoxia inducible factors HIF-1 and HIF-2 and the microRNA miR-34a. AXL additionally undergoes proteolytic cleavage of its extracellular domain by the metalloproteinases ADAM10 and ADAM17, leading to shedding of soluble AXL (sAXL) [3,4]. The multiple

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biological effects mediated by AXL in cancer include promotion of proliferation, cell survival, anoikis/apoptosis/autophagy resistance, invasion and metastasis, and epithelial–mesenchymal transition (EMT), as well as immune response suppression, promotion of stem cell phenotype and chemoresistance. This had led to growing interest in targeting this protein in cancer [3–7].

Despite the extensive research published to date with respect to the role of AXL in cancer, data for sAXL are more limited. Higher serum sAXL levels were reported to be associated with the presence of cirrhosis and hepatocellular carcinoma (HCC) in the differential diagnosis from other liver tumors or conditions [8]. In another study, higher serum sAXL levels were observed in HCC, including in early-stage disease, compared to cirrhosis and healthy controls [9]. Higher serum sAXL levels were significantly related to advanced stage, higher nuclear grade, tumor-thrombus extension and shorter survival in renal cell carcinoma [10].

To the best of our knowledge, the presence and clinical relevance of sAXL in malignant effusions has not been studied to date. In the present study, we analyzed sAXL protein expression in a large series of effusion specimens, with focus on ovarian carcinoma, particularly its most common and clinically aggressive histotype, high-grade serous carcinoma (HGSC).

2. Material and methods

2.1. Patients and specimens

Effusion specimens (n = 572) were submitted to the Department of Pathology at the Norwegian Radium Hospital, for routine diagnostic purposes during the period 1998–2013. Effusions were centrifuged and separated into cell pellets and cell-free supernatants. The latter were frozen at −80 °C. Clinical diagnoses and specimen site are detailed in Table 1.

Effusions were diagnosed by an experienced cytopathologist (BD) based on morphology in Diff-Quik-stained and PAP-stained smears, as well as cell block H&E sections. Immunohistochemistry was applied using established panels of antibodies against epithelial and mesothelial epitopes [1].

The majority of breast carcinomas were of no special type (NST). Lung specimens were adenocarcinomas. Metastases of uterine corpus origin were from high-grade tumors, i.e. serous carcinomas, clear cell carcinomas, carcinomas of mixed type with a serous component or carcinosarcomas metastasizing as adenocarcinoma. Mesothelioma specimens were all of the epithelioid or biphasic type.

A total of 373 HGSC effusions from 299 patients were included in this study. One effusion was available from 244 patients, 2 effusions from 43 patients, 3 effusions from 7 patients, 4 effusions from 3 patients, 5 effusions from 2 patients. Clinical data for 295 patients with HGSC (4 patients with missing data) are detailed in Table 2. In the absence of data regarding the status of the fallopian tube in many of these specimens, these cases are grouped together with ovarian carcinomas (OC).

Informed consent was obtained according to national and institutional guidelines. Study approval was given by the Regional Committee for Medical Research Ethics in Norway (REK # S-04300).

2.2. Enzyme-linked immunosorbent assay (ELISA)

Human Axl DuoSet® ELISA (Cat no. DY154, R&D Systems, Minneapolis, MN) was used to measure the concentration of soluble AXL in the effusion supernatants according to the manufacturer's protocol. Samples were diluted 1:50 in reagent diluent and were related to a sample standard of two-fold dilutions from 4000 pg/mL to 62.5 pg/mL. Samples were measured in technical duplicates. Soluble AXL concentrations were quantitated using a second order polynomial standard curve by GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA).

2.3. Statistical analysis

Statistical analysis was performed applying the SPSS-PC package (Version 25). Probability of <0.05 was considered statistically significant. The association between sAXL levels and anatomic site, tumor origin and histological type was performed using the Mann-Whitney *U* test (2-tier analyses) or the Kruskal Wallis H test (>2-tier analyses). The same tests were applied to analysis of the association between sAXL expression in HGSC effusions and clinicopathologic parameters. For this analysis, clinicopathologic parameters were grouped as follows: age: ≤60 vs. >60 years; effusion site: peritoneal vs. pleural; FIGO stage: III vs. IV; chemotherapy status: pre- vs. post-chemotherapy specimens;

Table 1
Specimens studied (n = 572).

Organ	Histology	Anatomic site			Total
		Peritoneum	Pleura	Pericardium	
Ovary	HGSC	329	44	0	373
	LGSC	29	3	0	32
	CCC	10	1	0	11
	EC	4	0	0	4
	MC	3	0	0	3
	Mixed type	6	0	0	6
	CS	7	0	0	7
	Undifferentiated	1	0	0	1
	Total	389	48	0	437
Non-ovarian					
Breast		6	44	1	51
Malignant mesothelioma		10	14	0	24
Reactive		6	15	0	21
Uterine corpus		10	1	0	11
Lung		0	10	0	10
Other[a]		3	15	0	18
Total		35	99	1	135

Abbreviations: HGSC = high-grade serous carcinoma; LGSC = low-grade serous carcinoma; CCC = clear cell carcinoma; EC = endometrioid carcinoma; MC = mucinous carcinoma; CS = carcinosarcoma.

[a] Including 5 uterine cervical, 5 esophageal, 2 colon, 2 prostate, 1 gastric, 1 urothelial and 1 pancreatic carcinoma, as well as 1 melanoma.

Table 2
Clinicopathologic parameters of the HGSC effusion cohort (295 patients).

Parameter	Distribution
Age (mean)	23–88 years (63)
FIGO stage	
I	2
II	8
III	172
IV	110
NA	3
Residual disease[a]	
0 cm	26
≤1 cm	68
>1 cm	56
CA 125 at diagnosis (range; median)	10–43,800 (1188)[b]
Chemoresponse after primary treatment	
CR	139
PR	72
SD	22
PD	24
NA[c]	38

Abbreviations: NA = not available; CR = complete response; PR = partial response; SD = stable disease; PD = progressive disease.

[a] For 150 patients who received surgery as upfront treatment.

[b] Available for 222 patients.

[c] Not available (missing data or disease response after chemotherapy could not be evaluated because of normalized CA 125 after primary surgery or missing CA 125 information and no residual tumor).

residual disease (RD): 0 cm vs. ≤1 cm vs. >1 cm; response to chemotherapy: complete response vs. partial response/stable disease/progressive disease. The association with CA 125 levels at diagnosis was analyzed using a two-sided *t*-test.

Survival data were available for 291 of the 295 patients with HGSC. Progression-free and overall survival (PFS; OS) were calculated from the date of the last chemotherapy treatment/diagnosis to the date of recurrence/death or last follow-up, respectively. Univariate survival analyses of PFS and OS were executed using the Kaplan-Meier method and Breslow test. Multivariate survival analysis was performed using the Cox Regression Model (Enter function). Platinum resistance was defined as PFS ≤ 6 months according to guidelines published by the Gynecologic Oncology Group (GOG) and progressive disease or recurrence was evaluated by the Response Evaluation Criteria In Solid Tumors (RECIST) criteria. For survival analyses, sAXL levels were classified as high vs. low based on the median value.

3. Results

3.1. sAXL is differentially expressed in cancers affecting the serosal cavities

sAXL was detected in all 572 effusions, with levels ranging from 23 pg/mL to 138.325 ng/mL. Comparative analysis of peritoneal (n = 424) vs. pleural/pericardial (n = 148) effusions showed significantly higher sAXL levels at the former anatomic site (median = 1555 vs. 1221 pg/mL, respectively; $p < 0.001$). This difference was retained when the analysis was limited to all ovarian carcinomas ($p = 0.002$) or to HGSC ($p = 0.004$).

Comparative analysis of different cancers and benign reactive specimens showed significantly higher sAXL levels in effusions from patients with ovarian carcinoma, malignant mesothelioma and breast carcinoma compared to specimens from patients with other cancers or benign reactive effusions (Table 3; $p < 0.001$).

3.2. sAXL is expression is significantly related to grade and chemotherapy exposure in ovarian carcinoma

Comparative analysis of sAXL protein levels in OC of different histology was limited to HGSC, low-grade serous carcinoma (LGSC) and clear cell carcinoma (CCC) in view of the small number of cases in the other entities. sAXL protein levels were higher in HGSC compared to LGSC and CCC, though not significantly (mean rank = 212.91, 166.67 and 180.73, respectively; $p = 0.084$). This difference became significant when the analysis was limited to HGSC vs. LGSC (mean rank = 206.57 vs. 161.42, respectively; $p = 0.036$).

Analysis of the association between sAXL levels and previous exposure to chemotherapy performed for all OC effusions (n = 427; 10 effusions with no data regarding chemotherapy status) showed overexpression in pre-chemotherapy primary diagnosis specimens compared to post-chemotherapy effusions (mean rank = 230.30 vs. 189.84, respectively; $p = 0.001$). Limiting the analysis to HGSC alone (364 specimens, 9 effusions with no data regarding chemotherapy status) generated comparable results (mean rank = 197.11 vs. 161.66, respectively; $p = 0.002$).

Table 3
sAXL levels per tumor origin.

Tumor type	# cases	Range; median (pg/mL)
Ovarian	437	49–138,325; 1550
Breast	51	373–3108; 1435
Malignant mesothelioma	24	671–2467; 1519
Reactive	21	23–3456; 165
Other[a]	39	50–1729; 323

Values for lung carcinomas (n = 10): range: 181–1345; median: 326 pg/mL.
[a] Values for uterine carcinomas (n = 11): range: 104–1729; median: 343 pg/mL.

3.3. sAXL levels in HGSC are unrelated to clinicopathologic parameters, chemotherapy response or survival

sAXL levels in HGSC supernatants were unrelated to patient age, FIGO stage, RD volume, primary chemoresistance or chemoresponse at diagnosis ($p > 0.05$; data not shown).

The follow-up period for the 291 patients with HGSC effusions with survival data ranged from 1 to 295 months (mean = 39 months, median = 29 months). PFS ranged from 0 to 233 months (mean = 12 months, median = 7 months). At the last follow-up, 269 patients were dead of disease, 13 were alive with disease and 4 were with no evidence of disease. Four patients died of complications, and 1 died of unrelated cause.

In univariate survival analysis of all cases, sAXL protein expression was unrelated to OS ($p = 0.366$; Fig. 1-A). Among clinical parameters, older age ($p < 0.001$; Fig. 1-B) and FIGO stage IV disease ($p < 0.001$; Fig. 1-C) were significantly related to shorter OS. The same was observed for non-optimal (RD > 0 cm) debulking for patients who received upfront surgery ($p = 0.007$; Fig. 1-D). Similarly, sAXL levels were not informative of PFS ($p = 0.25$; Fig. 2-A). Older age ($p = 0.001$; Fig. 2-B) and FIGO stage IV disease ($p < 0.001$; Fig. 2-C) were significantly related to shorter PFS, with a similar finding for non-optimal debulking for patients who received upfront surgery ($p < 0.001$; Fig. 2-D).

Survival analysis limited to patients who received upfront surgery similarly failed to detect significant association between sAXL levels and survival of patients with HGSC ($p > 0.05$; data not shown).

In multivariate analysis of all patients with HGSC, in which sAXL protein level, age and FIGO stage were entered, FIGO stage ($p < 0.001$) and patient age ($p = 0.045$) were independent prognosticators of OS, with no such role for sAXL levels ($p = 0.143$). FIGO stage was the only parameter informative of PFS ($p = 0.001$), with $p = 0.263$ and $p = 0.344$ for age and sAXL levels, respectively.

Cox analysis was repeated limited to patients with upfront surgery, with RD volume as fourth parameter. In this patient group, FIGO stage was independently related to OS ($p = 0.003$), with no such role for sAXL ($p = 0.237$), age ($p = 0.858$) or RD volume ($p = 0.135$). RD volume was significantly associated with PFS ($p = 0.028$), with marginal association for FIGO stage ($p = 0.061$), whereas age and sAXL levels were unrelated to PFS ($p = 0.664$ and $p = 0.468$, respectively).

In view of the observed differences in sAXL levels between pre- and post-chemotherapy effusions, separate survival analyses were performed for these groups. sAXL levels were unrelated to OS ($p = 0.225$ and 0.905 for pre- and post-chemotherapy effusions, respectively) or PFS ($p = 0.483$ and 0.314 for pre- and post-chemotherapy effusions, respectively).

Survival analyses for patients with LGSC and breast carcinoma effusions with survival data (n = 31 and n = 42, respectively) similarly showed no association with survival ($p > 0.05$; data not shown).

4. Discussion

The present study constitutes the first large-scale analysis of sAXL expression levels in cancers affecting the serosal cavities. Our data suggest that sAXL expression is highest in OC, breast carcinoma and malignant mesothelioma, particularly in the former, with significantly lower expression in tumors of other origin and benign reactive effusions. Whether these differences have diagnostic relevance is questionable, given the broad distribution of values within these diagnostic groups. Furthermore, unlike serum samples, which may be obtained for normal subjects and thus be used as true controls, the presence of an effusion is always a pathological finding. The benign effusions in the present study were relatively highly cellular specimens from patients with clinical

suspicion of cancer. Analysis of transudates, e.g. from patients with heart failure, may reveal clearer differences.

The difference between the potential diagnostic role of sAXL in HCC [8] and in the present study may reflect differences between these cancers and the differential diagnosis assessed. However, it may additionally be due to a different mechanism of AXL shedding in the blood compared to that in effusions. In addition to tumor cells, the microenvironment of effusions consists of inflammatory cells, mesothelial cells, adipocytes and fibroblasts, all which have been shown to play a role in cellular AXL signaling [11–13] and therefore may contribute to the total levels of sAXL observed in effusions. Further, AXL has been reported to be expressed in extracellular vesicles [14]. The corresponding author's group has previously shown that effusion supernatants contain exosomes [15–17], and one may speculate that the levels of sAXL observed may to some extent reflect its presence in extracellular vesicles.

AXL expression is associated with significantly shorter survival based on analysis of The Cancer Genome Atlas (TCGA) data. In OC cells, reduced expression or inhibition of AXL was shown to suppress proliferation, viability, migration and invasion in vitro, as well as tumor growth and intraperitoneal metastasis formation in vivo [18–20]. In addition, the tumor suppressor opioid-binding protein/cell adhesion molecule-like (OPCML) was recently shown to inactivate AXL [21]. OPCML is silenced in the majority of OC by loss of

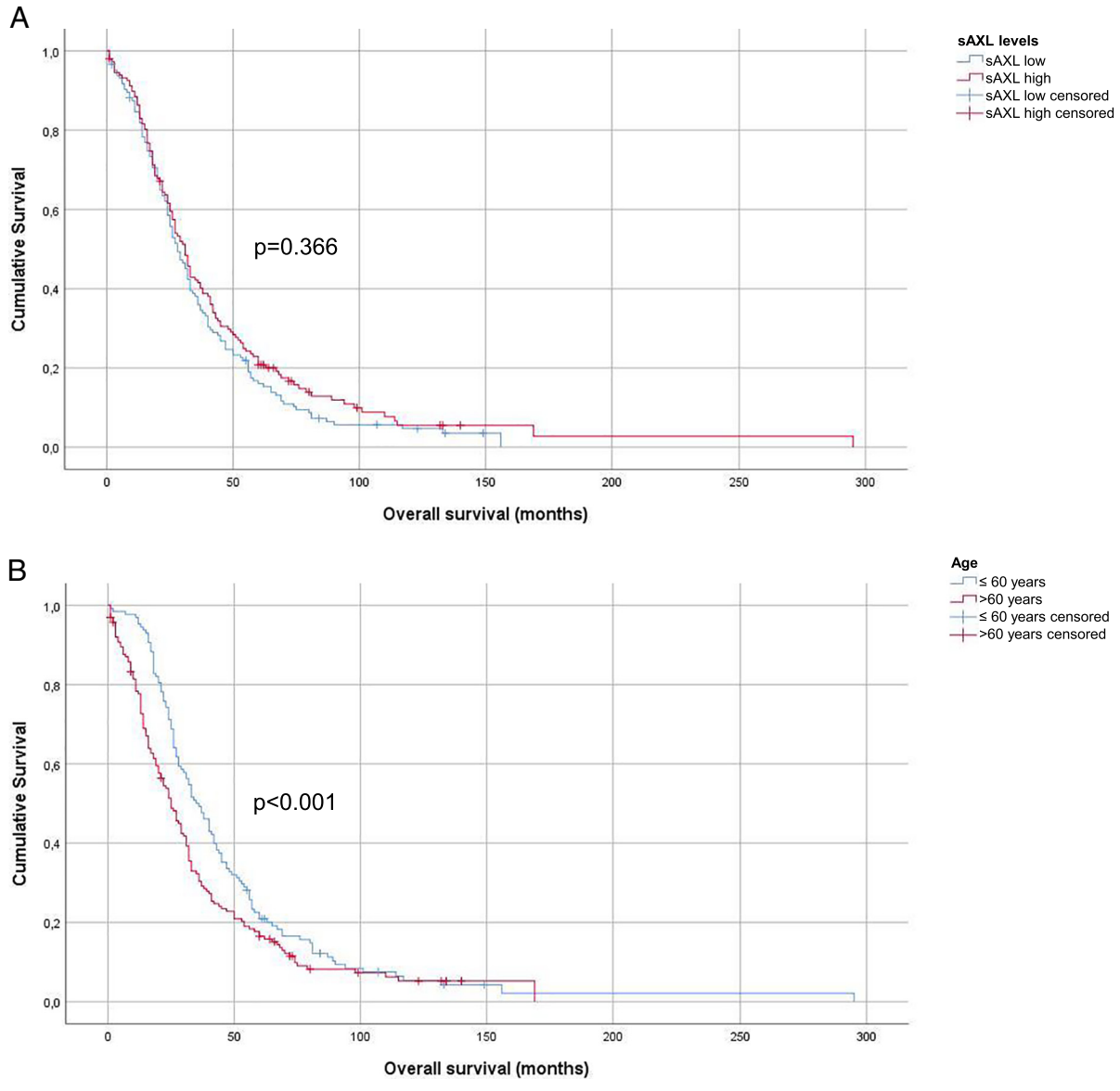

Fig. 1. sAXL expression level is unrelated to overall survival in HGSC. A: Kaplan-Meier survival curve showing the association between sAXL protein level in HGSC effusions (n = 291) and overall survival (OS). Patients with effusions with high (above median) sAXL protein level (n = 147; red line) had mean OS of 47 months compared to 38 months for patients with effusions having low sAXL protein level (n = 144, blue line; p = 0.366). B: Kaplan-Meier survival curve showing the association between patient age and OS. Patients aged >60 years at diagnosis (n = 163; red line) had mean OS of 37 months compared to 49 months for patients aged ≤60 years at diagnosis (n = 128, blue line; p < 0.001). C: Kaplan-Meier survival curve showing the association between FIGO stage and OS. Patients with FIGO stage IV disease (n = 109; red line) had mean OS of 29 months compared to 47 months for patients with FIGO stage III disease (n = 169, blue line; p < 0.001). Thirteen patients with stage I–II disease or no data with respect to FIGO stage were excluded. D: Kaplan-Meier survival curve showing the association between RD volume and OS for patients who received upfront surgery (n = 147). Patients debulked to no macroscopic disease (n = 25; blue line) had mean OS of 86 months compared to 50 and 42 months for patients debulked to 1 cm (n = 68; red line) or 2 cm (n = 54, green line; p = 0.007).

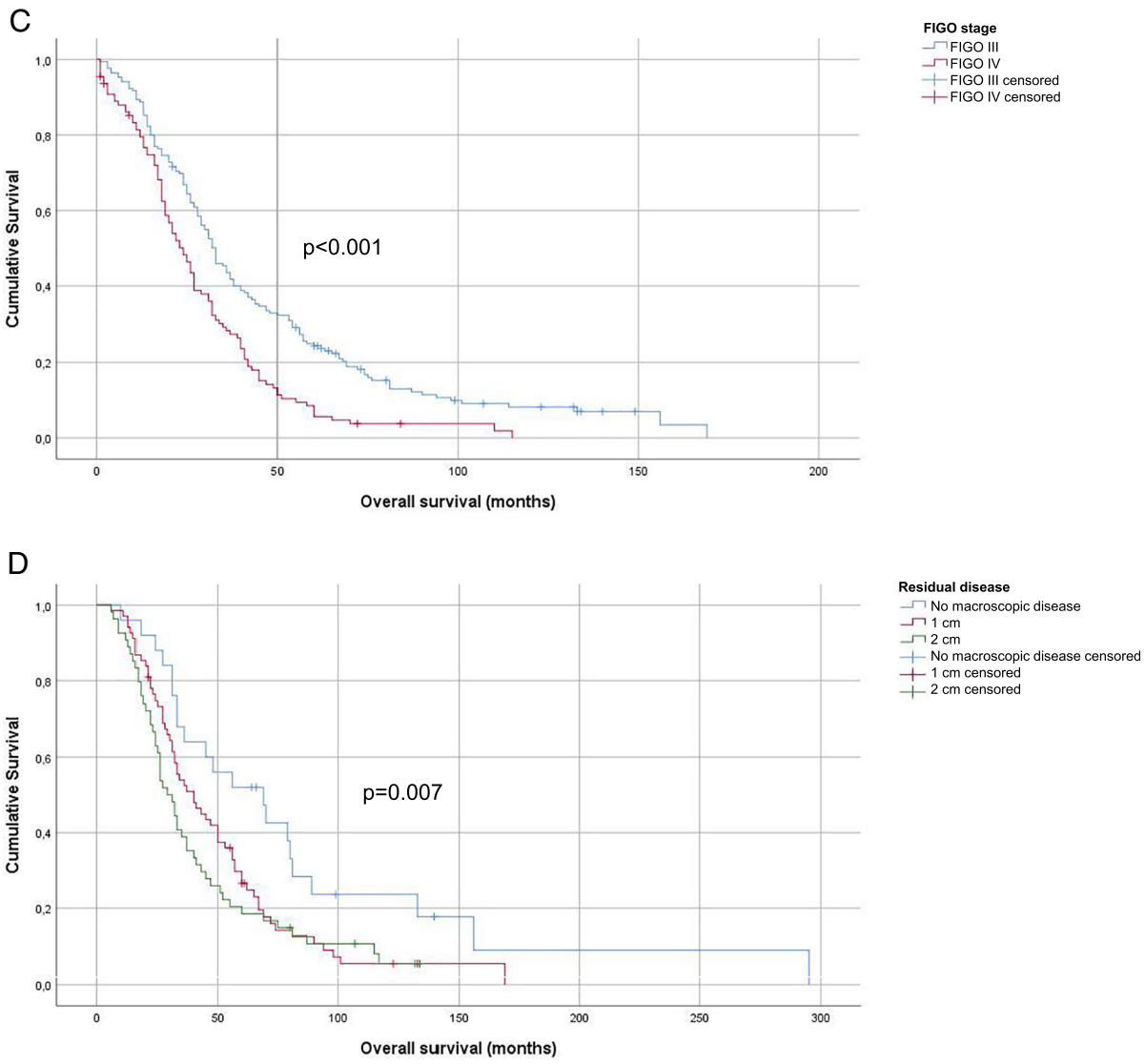

Fig. 1 (*continued*).

heterozygosity and epigenetic mechanisms. Data regarding the biological and clinical role of AXL in OC are mainly available from studies of its cellular fraction, primarily in the experimental setting. Thus, the biological role of sAXL in OC in the clinical setting is still undefined, in addition to the manner in which AXL cleavage is altered during cancer progression. sAXL levels may mirror cellular AXL expression, or alternatively the cleavage process may be regulated by other signaling pathways. In addition, ADAM10 and ADAM17, the matrix metalloproteinases responsible for AXL cleavage, have been reported to be upregulated in cancer and are implicated in metastatic progression [22–24]. As AXL is involved in EMT [25], its cleavage may be involved in the metastatic process. In line with what has been reported for cellular AXL, we found higher sAXL levels in the highly aggressive HGSC compared to the less aggressive LGSC, suggesting a link between the expression of AXL on cells and in effusions. Further evidence supporting an association with aggressive clinical behavior is the fact that sAXL levels were highest in effusions from patients with OC and malignant mesothelioma, two highly metastatic and often incurable cancers, and in breast carcinoma. The latter cancer is in general associated with better survival, but the presence of breast carcinoma cells in effusions is associated with very poor survival [1].

Unexpectedly, HGSC patients with higher sAXL levels had 9 months longer mean OS compared to those with lower levels. Although not significant, this result is in contrast to reported data showing that higher AXL levels are related to shorter OS [19]. The trend towards higher levels in HGSC patients with longer survival may suggest that sAXL has a tumor-suppressing rather than tumor-promoting effect by acting as a decoy for Gas6 when its level reaches a certain threshold.

AXL mRNA and protein levels were higher in platinum-resistant IGROV-1/Pt1 and IGROV-1/OHP OC cells compared to the parental IGROV-1 cells, though not in the resistant variants of the A2780 and OVCAR-5 OC cell lines compared to their parental lines. IGROV-1/Pt1 had higher migration and invasion capacity than the parental cell line and this was suppressed by AXL silencing using siRNA. However, AXL silencing in IGROV-1/Pt1 cells had no effect on response to cisplatin or taxol [26]. On the other hand, AXL was reported to be overexpressed in $PEO1^{CDDP}$ cells, the cisplatin-resistant variant of the PEO1 OC cell line, compared to the parent line by microarray analysis [27]. Also, AXL inhibition combined with paclitaxel treatment enhanced antitumor efficacy [19]. In the present series, sAXL expression levels were higher in pre-chemotherapy compared to post-chemotherapy specimens, the latter predominantly tapped at disease recurrence. As AXL was reported to

be upregulated in cells after chemotherapy and mediates resistance [28], one may speculate that the reduced sAXL levels in the effusions may stem from less AXL cleavage as a way of regulating its cellular expression.

One potential limitation of our study is the broad span of storage period, which may have affected sAXL levels. It has nevertheless been shown that sAXL is a stable molecule that has long-term stability during freeze-thaw cycles and incubation under temperature stress conditions [29], which may suggest limited effect of this fact.

In conclusion, sAXL is frequently expressed in malignant effusions, with highest levels in OC, breast carcinoma and malignant mesothelioma. sAXL is overexpressed in HGSC compared to LGSC and in pre-chemotherapy compared to post-chemotherapy OC specimens. However, its levels are unrelated to chemoresponse or survival, suggesting a limited role for this molecule in identifying patients with clinically aggressive disease.

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Conflict of interest statement

We have no conflict of interest.

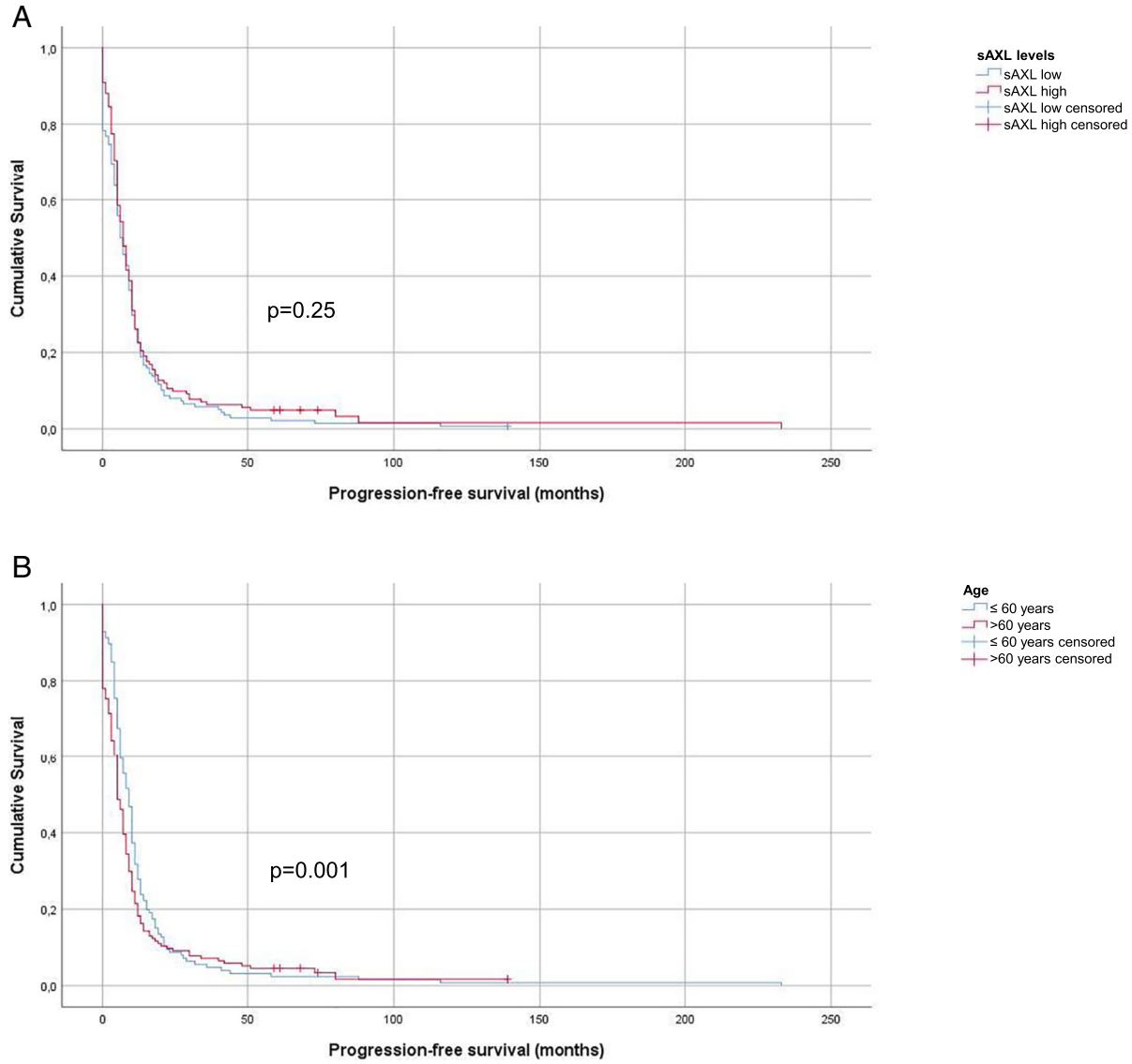

Fig. 2. sAXL expression level is unrelated to progression-free survival in HGSC. A: Kaplan-Meier survival curve showing the association between sAXL protein level in HGSC effusions and progression-free survival (PFS; n = 280; 11 patients with missing PFS data). Patients with effusions with high (above median) sAXL protein level (n = 142; red line) had mean PFS of 15 months compared to 11 months for patients with effusions having low sAXL protein level (n = 138, blue line; p = 0.25). B: Kaplan-Meier survival curve showing the association between patient age and PFS. Patients aged >60 years at diagnosis (n = 154; red line) had mean PFS of 12 months compared to 14 months for patients aged ≤60 years at diagnosis (n = 126, blue line; p = 0.001). C: Kaplan-Meier survival curve showing the association between FIGO stage and PFS. Patients with FIGO IV disease (n = 104; red line) had mean PFS of 7 months compared to 14 months for patients with FIGO III disease (n = 164, blue line; p < 0.001). Twelve patients with stage I-II disease or no data with respect to FIGO stage were excluded. D: Kaplan-Meier survival curve showing the association between RD volume and PFS for patients who received upfront surgery (n = 145; 2 patients with missing PFS data). Patients debulked to no macroscopic disease (n = 25; blue line) had mean PFS of 42 months compared to 13 and 15 months for patients debulked to 1 cm (n = 67; red line) or 2 cm (n = 53, green line; p < 0.001).

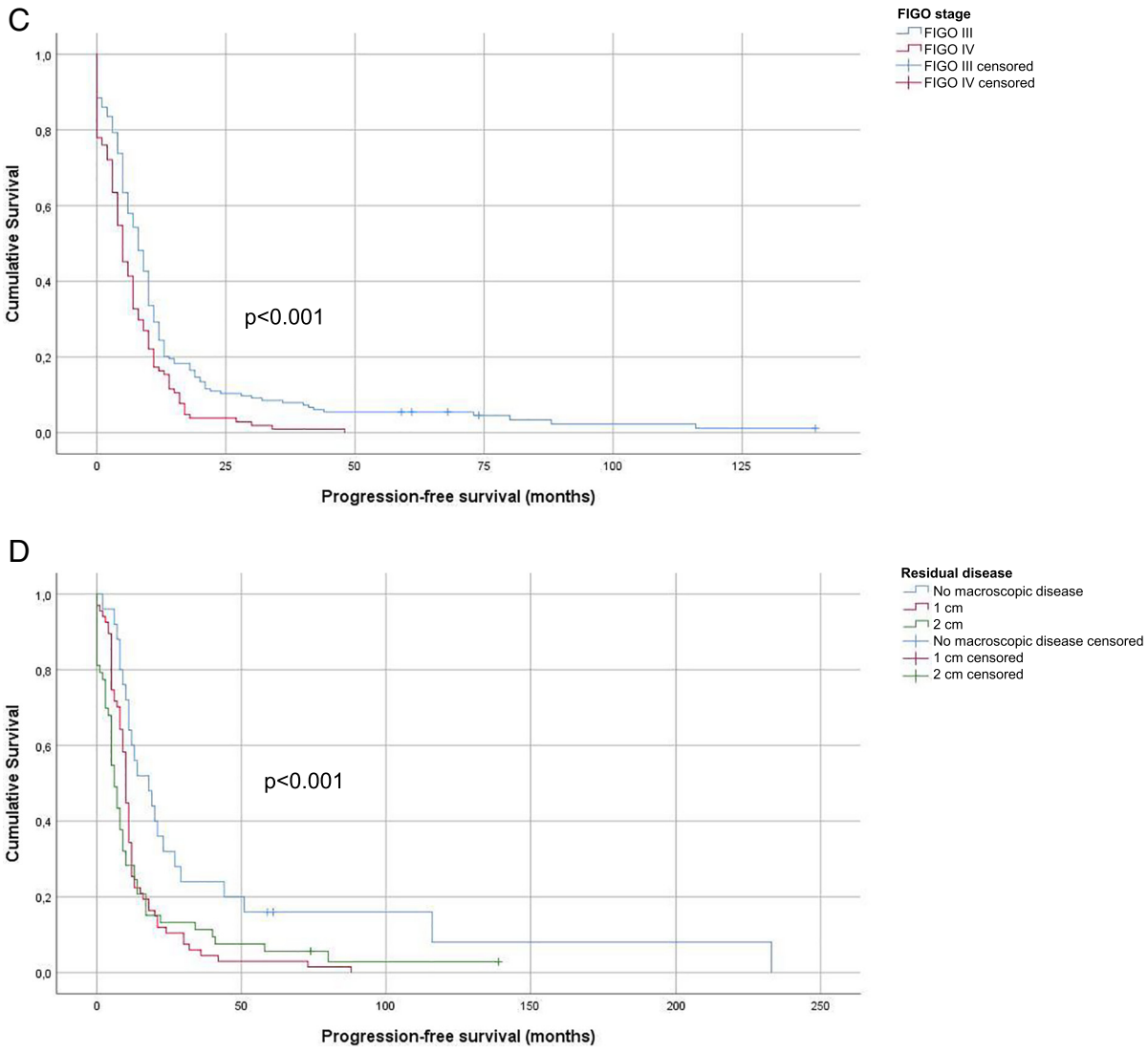

Fig. 2 (*continued*).

CRediT authorship contribution statement

Karine Flem Karlsen: Investigation, Methodology, Writing - original draft. **Erin McFadden:** Investigation, Methodology, Writing - review & editing. **Vivi Ann Flørenes:** Supervision, Writing - review & editing. **Ben Davidson:** Conceptualization, Data curation, Funding acquisition, Resources, Supervision, Writing - review & editing.

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