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Article:

Willenbrock, F, Cox, CM, Parkes, EE et al. (10 more authors) (2021) Circulating biomarkers and outcomes from a randomised phase 2 trial of gemcitabine versus capecitabine-based chemoradiotherapy for pancreatic cancer. *British Journal of Cancer*, 124 (3). pp. 581-586. ISSN 0007-0920

<https://doi.org/10.1038/s41416-020-01120-z>

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Circulating Biomarkers and Outcomes from a Randomised Phase II Trial of Gemcitabine versus Capecitabine-based Chemoradiotherapy for Pancreatic Cancer

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Running title:

Circulating CCL5 predicts outcomes in pancreatic cancer.

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Keywords:

Pancreatic cancer; biomarker; CCL5; chemoradiation; chemotherapy; immunotherapy; prognostic.

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ABSTRACT

Background: The phase II SCALOP trial compared gemcitabine with capecitabine-based consolidation chemoradiotherapy (CRT) in locally advanced pancreatic cancer (LAPC).

Methods: Thirty-five systematically identified circulating biomarkers were analysed in plasma samples from 60 patients enrolled in SCALOP. Each was measured in triplicate at baseline (prior to three cycles of gemcitabine-capecitabine induction chemotherapy) and, for a subset, prior to CRT. Association with overall survival (OS) was determined using univariable Cox regression and optimal thresholds delineating low to high values identified using time-dependent ROC curves. Independence from known prognostic factors was assessed using Spearman correlation and the Wilcoxon rank sum test prior to multivariable Cox regression modelling including independent biomarkers and known prognostic factors. The SCALOP trial was registered with ISRCTN, number 96169987.

Results: Baseline circulating levels of C-C motif chemokine ligand 5 (CCL5) were significantly associated with OS, independent of other clinicopathological characteristics. Patients with low circulating CCL5 (CCL5^{low}) had a median OS of 18.5 (95%CI 11.76-21.32) months compared to 11.3 (95%CI 9.86-15.51) months in CCL5^{high}; hazard ratio 1.95 (95%CI 1.04-8.65; p=0.037).

Conclusions: CCL5 is an independent prognostic biomarker in LAPC. Given the known role of CCL5 in tumour invasion, metastasis and the induction of an immunosuppressive micro-environment, targeting of CCL5-mediated pathways may offer therapeutic potential in pancreatic cancer.

INTRODUCTION

Pancreatic cancer is a leading cause of cancer-related death and is predicted to be the second largest cause of death from cancer worldwide by the end of the current decade.(1) Inoperable, non-metastatic, locally advanced pancreatic cancer (LAPC) has an intermediate prognosis (median overall survival (OS) 12-18 months) between metastatic and operable disease, and is treated with combination chemotherapy or chemoradiotherapy (CRT). The use of potent combination chemotherapy has delivered modest improvements in clinical outcomes, though its application in clinical practice is limited by toxicity.(2,3) Recently, a greater understanding of tumour biology has driven the development of novel therapies.(4-6) One example is the recently reported POLO trial of maintenance olaparib in patients with germline BRCA mutations, which is the first successful trial of biomarker-guided therapy in pancreatic cancer.(7)

Tissue and blood samples collected during clinical trials provide an unparalleled resource from which tumour biology can be further interrogated and correlated with clinical outcomes, thereby facilitating the generation of evidence-based hypotheses for biomarker-driven treatments. SCALOP was a multi-centre randomised phase II trial (n=114) which compared gemcitabine versus capecitabine as the radiosensitiser of choice for LAPC.(8) Patients received three cycles of gemcitabine and capecitabine (GEMCAP) induction chemotherapy and eligible patients with responding or stable disease (n=74) were randomised to a further cycle of GEMCAP followed by gemcitabine or capecitabine-based CRT to a total radiation dose of 50.4Gy in 28 fractions. Survival analyses have previously been reported and demonstrate that capecitabine-based CRT was superior in terms of survival and toxicity profile.(8,9) To facilitate later biomarker analysis, blood samples were collected from individual patients at baseline (prior to GEMCAP) and, for a subset, at week 17 (prior to consolidation CRT), as summarised in **Supplementary Figure 1**.

In a prior systematic review, we identified a number of cytokines reported to be of diagnostic, prognostic or predictive significance in PDAC.(10) Here, we report the results of a detailed prognostic biomarker analysis, including correlation with clinical outcomes, assessing a panel of 35 of these systematically identified cytokines in samples from the SCALOP trial cohort.

MATERIAL AND METHODS

This study report aligns with the Reporting Recommendations for Tumour Marker prognostic studies (**REMARK**) criteria.(11)

Patients

The design and results of the SCALOP trial have been reported elsewhere.(8,9) Briefly, patients with histologically/cytologically confirmed inoperable locally advanced pancreatic cancer with maximum diameter 7cm or less, performance status (PS) 0-2, were eligible. Response was assessed following three cycles of GEMCAP chemotherapy, and those with responding or stable disease (according to RECIST criteria), PS 0-1 and tumour diameter 6cm or less were eligible for randomisation to a further cycle of GEMCAP followed by gemcitabine (300mg/m² weekly) or capecitabine (830mg/m² twice daily on days of radiotherapy) concurrent with radiotherapy (50.4Gy in 28 fractions), as summarised in **Supplementary Figure 1**.

Sample collection and analysis

Peripheral venous blood was collected in ethylenediaminetetraacetic acid (EDTA) anticoagulant vacutainers. Each was centrifuged at 3000 × g for 10 minutes and plasma aliquoted into cryovials then snap frozen. Samples were stored at -80°C at investigating centres until the end of the trial and subsequently shipped on dry ice to Wales Cancer Bank (WCB) for centralized storage. Centres that were unable to store frozen blood samples or which did not have a Human Tissue Authority (HTA) license were required to send samples to WCB for processing using pre-paid safe boxes. All samples were processed on the same day of collection and, where possible, within an hour of collection.

Biomarker analysis was undertaken at the molecular laboratory at Oxford Institute for Radiation Oncology, University of Oxford. Assays were performed while blinded to clinical outcome. Assayed cytokines are listed in **Table 1**. Insulin-like growth factor 1 (IGF-1), transforming growth factor-β (TGF-β) and β-nerve growth factor precursor (β-NGF) were quantified using DuoSet® enzyme-linked immunosorbent assay (ELISA) (R&D Systems, USA) and the signal detected using a POLARstar® Omega microplate reader. The remaining 32 cytokines were assessed by Human Multiplexed Magnetic Luminex assay (R&D Systems, USA) and measured using the Luminex MAGPIX® fluorescent detection system.

Prior to assay, all plasma samples were thawed on ice and centrifuged at 10,000 × g for 5 minutes at 4°C to remove precipitate. Samples were limited to two freeze-thaw cycles. Supernatants were loaded onto ELISA or Luminex plates at the recommended dilutions (i.e. 50-fold for CCL-5 and PDGF, each at 1µl per well, and two-fold for all other cytokines, which were loaded at 25µl per well) with standard protein controls. The manufacturer's protocol was followed in each case with no modifications. For

ELISA assays, standard curves were constructed and used to quantify each marker. For Luminex assays, biomarker quantification was derived from analysis of raw data using xPONENT 4.2® software.

All biomarkers were measured in triplicate across all patients at each timepoint. If a run contained $\geq 50\%$ out of range values for a given biomarker across all patients ($>1SD$ from the mean) then the entire run was removed for that biomarker. Any remaining values above the upper limit of detection or below the lower limit of detection were substituted with the highest or half the lowest value of that biomarker respectively for a given run. The mean value across the remaining runs was then used for analysis. This method for handling measurement errors has been used elsewhere.(12)

Statistical Analysis

Statistical analyses were conducted using R v3.5.2 according to a pre-specified analysis plan. Univariate Cox proportional hazard models were used for each of the 35 biomarkers as continuous variables to determine the association with OS. OS was measured from randomisation until death from any cause. Multiple comparisons were accounted for by using the False Discovery Rate (FDR). Those found to be significant at the q value <0.2 were then further investigated for independence from existing prognostic clinical characteristics (i.e. cancer antigen 19-9 (CA19-9), treatment (capecitabine CRT vs gemcitabine CRT), PS (0 vs 1) and age (<65 vs ≥ 65 years)) using Spearman correlations ($r \leq 0.7$ shows independence) and Wilcoxon rank sum tests ($p \geq 0.05$ shows independence). PET-CT data were unfortunately unavailable for a majority of patients and could not therefore be included in these analyses. Those clinical characteristics found to be independent were then split into tertiles and associated with OS using univariate Cox regression to ensure that any associations with the biomarker as a continuous variable were linear. Optimal thresholds delineating low to high values were identified using the R “survivalROC” package based on time-dependent receiver operating characteristic (ROC) curves from censored survival data and their corresponding area under the curve (AUC). Both continuous and dichotomised biomarkers were then associated with OS using multivariable Cox proportional hazard models, along with existing prognostic clinical characteristics, to determine whether or not novel biomarkers maintained prognostic value. The proportional hazards assumption was tested by calculating Schoenfeld residuals, and the linearity assumption was assessed by plotting deviance residuals.

RESULTS

Patient Characteristics

Cytokine data were available from 63 patients in total. No significant differences in clinicopathological characteristics were identified comparing patients who did and did not have cytokine data available (**Supplementary Table 1**). Measurements of the full panel of cytokines and corresponding clinical outcome information were available for 60 patients. These data were assessed further in order to identify correlations between circulating cytokines and clinical outcomes.

Prognostic factor identification

Raw biomarker levels at both baseline and, where measured, at week 17 are shown in **Table 1**, as is an analysis of the association with OS of each of the 35 assayed cytokines. . Of the biomarkers tested, two (C-C Motif Chemokine Ligand 5, CCL5 and interferon- γ , IFN γ) measured at baseline (prior to commencement of GEMCAP chemotherapy) had significant associations with OS at the $q < 0.2$ FDR level. IFN γ levels significantly correlated with age ($p = 0.019$). However, CCL5 was independent of existing clinical characteristics, including age ($p = 0.859$), PS ($p = 0.660$) and CA19-9 ($r = 0.339$) (**Table 2**). No biomarkers were associated with progression at the univariate level at the $q < 0.2$ FDR level, as illustrated in **Supplementary Table 2**. Consequently, no further analyses on progression were undertaken.

CCL5 is an independent prognostic biomarker in LAPC

CCL5 data was available at baseline for 60 patients, with no differences identified in patient characteristics between those with and without CCL5 data (**Table 3**).

We identified a linear association of CCL5 with OS. When associated with OS in a multivariable Cox proportional hazards model as a continuous variable, patients with high circulating CCL5 were found to have a HR of 1.01 for each ng/ml unit increase (95% CI 1.00-1.03; $p = 0.013$, $n = 54$) (**Table 4**). A time-dependent ROC curve was constructed to identify the optimal threshold for CCL5 (**Supplementary Figure 2**). Dichotomisation of CCL5 at its optimal threshold of 25.4 ng/ml was significantly associated with OS, with a HR of 1.95 (95% CI: 1.04-3.65; $p = 0.037$) in the Cox multivariable model. Median OS was 18.5 months in patients with CCL5^{low} (21/60) (95% CI: 11.76-21.32) and 11.3 months (95% CI: 9.86-15.51) in patients with CCL5^{high} (39/60); as demonstrated in **Figure 1**. Diagnostic tests showed that there was no evidence of departure from proportionality or violations of the linearity assumption, and that there were no extreme outliers or influential points.

CCL5 circulating levels were additionally assessed in the context of other clinicopathological characteristics, including age, PS and CA19-9 ($n = 54$ for CA19-9 data). A signature utilising

dichotomised CCL5 was determined (**Supplementary Figure 3**) including age, PS and CA19-9. The CCL5 signature was created for each patient using the beta coefficients from the multivariable survival model and multiplying them by their corresponding covariate ($CCL5*0.6678 - Age*0.4072 + WHO\ PS*0.7807 + CA199*0.0001663$). With the optimal threshold determined at 0.398, 17 patients were classified as CCL5^{low} and 37 as CCL5^{high}. Using this approach, patients classified as CCL5^{low} had a median OS of 19.68 months (95% CI 16.3-27.79) and CCL5^{high} of 11.2 months (95% CI 8.25-13.24); hazard ratio 2.69 (95% CI 1.40-5.17; p=0.003) (**Supplementary Table 3**).

Relative change in CCL5 levels between baseline (prior to start of GEMCAP) and week 17 (prior to start of CRT, data available for n=47) did not demonstrate any association with OS (**Supplementary Table 4**). Independent measurement of CCL5 at week 17 did not associate with OS. High levels of circulating CCL5 were identified to be a poor prognostic factor independent of concurrent chemotherapy received during radiotherapy (capecitabine or gemcitabine). Taken together, these suggest that circulating CCL5 levels at baseline relate to intrinsic tumour properties. No benefit of CCL5 as a potential pharmacodynamic biomarker was identified within the context of the CRT treatment delivered in this randomised trial.

DISCUSSION

Interpretation of results in the context of pre-specified hypotheses and other relevant studies

Pancreatic cancer responds poorly to chemotherapy and radiotherapy and the aim of this research was to determine the prognostic value of previously reported circulating biomarkers in patients with LAPC treated with chemotherapy and CRT. To the best of our knowledge this is the first biomarker study from a prospective randomised clinical trial in LAPC. The cytokine panel tested in this study was based on a systematic review that identified cytokines of diagnostic, prognostic or predictive significance in PDAC.⁽¹⁰⁾ This included six cytokines (IL-1 β ; IL-6, IL-8, VEGF, TGF β , IL-10) previously reported as consistently elevated in patients with PDAC compared to healthy controls, all of which have previously been found to have potential prognostic value (carrying higher risk of metastasis and lower OS).^(10,13-19) However, none of these six cytokines correlated with survival in the SCALOP trial.

We identified baseline levels of circulating CCL5 and IFN γ as being significantly associated with OS, of which CCL5 remained significant in multivariable analysis. CCL5 can be secreted by a variety of tumour cells (including pancreatic cancer) as well as non-malignant stromal cells including T regulatory cells (T_{regs}) and macrophages. Engagement of CCL5 with its receptor, C-C chemokine receptor type 5 (CCR5), can favour tumour growth by several mechanisms including induction of the mammalian target of

rapamycin (mTOR) pathway,(20,21) and by recruiting tumour associated macrophages (TAMs), leading to immunosuppression and release of pro-angiogenic cytokines.(22,23) The CCL5-CCR5 axis has also been implicated in tumour migration and metastasis through modulating the activity of the PI3K/Akt, MAPK/ERK and NF- κ B pathways, and via induction of matrix metalloproteinases.(24)

The interaction between pancreatic tumour cells, the immune system and the role of the CCL5-CCR5 axis has been investigated in preclinical studies. Tan et al. demonstrated that both human pancreatic cancer and murine pancreatic tumour (Pan02) secreted CCL5 and correspondingly, within the stroma, CCR5 was preferentially expressed by CD4⁺ Foxp3⁺ T_{regs}.(23) The investigators also showed that T_{reg} infiltration within the tumour could be reduced by systemic administration of CCR5 antagonists, resulting in restricted tumour growth. CCL5 secretion in pancreatic cancer was further investigated by Wang et al in a cohort of 120 resected PDAC tissues. While Foxp3 is typically utilised to identify T_{regs}, here the investigators demonstrated the presence of Foxp3 positive cancer cells. CCL5 was directly trans-activated by cancer-Foxp3, which in turn promoted T_{reg} infiltration.(24) Intriguingly, Jang et al. reported accumulation of T_{regs} around murine pancreatic tumours within one week of implantation, suggesting tumour cell-intrinsic secretion of CCL5 plays an important role in this rapid infiltration.(25) This proposes a model of cell intrinsic CCL5 secretion resulting in autocrine and paracrine signalling promoting a pro-tumourigenic immunosuppressive TME. However, the prognostic implication of circulating CCL5 has not been previously reported in literature.

Implications for future research and clinical value of the work

PDAC has been described as an “immune desert” and conventional immunotherapy has failed to impact outcomes in this disease. Low mutational burden, induction of TAMs and myeloid-derived suppressor cells, and exclusion of CD8⁺ and other pro-inflammatory cells are some of the proposed mechanisms for failure of immunotherapy. Mitigating the factors that contribute to immune suppression may enhance response to immunotherapy in PDAC. Several strategies, including combinations of immunotherapy with gemcitabine, radiation, pancreatic cancer vaccine (GVAX) and CSF1R antibody, are being evaluated in clinical trials.(26,27) The biomarker analysis of this clinical cohort is consistent with the pre-clinical hypothesis that the CCL5-CCR5 axis plays an adverse role in pancreatic cancer pathogenesis. Given its immunomodulatory effects, inhibition of the CCL5-CCR5 axis in combination with immunotherapy should be tested in pre-clinical models.

Discussion of limitations

We have been unable to validate a number of previously reported prognostic biomarkers in the prospective trial cohort reported here. It is unclear whether this relates to the trial cohort under study or to limitations in previous studies that identified these candidate biomarkers. In this study, we analysed biomarkers in patients randomised within SCALOP but not in 40/114 patients ineligible for randomisation due to disease progression, poor PS or because of patient or clinician choice. This may have biased the analysed cohort and validation of our findings in a larger cohort is required.

Although clear protocols for sample collection and processing were pre-specified, variations between centres is possible. For example, 18/27 centres did not have facilities or regulatory approvals for sample storage, thereby requiring unprocessed samples to be shipped centrally to WCB for processing. Given that this study predates the widespread adoption of preservative tubes that allow a 14-day window within which samples can be transported and processed, the requirement for many samples to be sent to WCB may have impacted on their quality. Nevertheless, all assays were performed under identical experimental conditions at a single centre using pre-defined protocols.

A final point of note is that the median biomarker levels we have reported here differ from their measurement in previous studies of pancreatic cancer.(17-19) For example, the measured values for IL-6 and IL-8 are a factor of between 3 to 5 higher than in some previous reports. Levels of IL-1 β were also higher here compared with previous reports (a median of 30 pg/ml compared with 0 pg/ml in the existing literature). The median CCL5 reading is also around 200-fold higher than for example reported previously in pancreatic cancer by Farren and colleagues,(19) but at 30.1 ng/ml is similar in magnitude to values that have been reported for ovarian, breast and cervical cancer.(28,29) These variations may reflect differences in the studied patient populations, in the storage and processing of samples, and in the assays used to process these samples. Nevertheless, they do not detract from the standardised conditions used to compare serum biomarker levels for patients within this study, from which we identified clinical support for pre-clinical studies that have previously postulated a role for the CCL5-CCR5 axis in the pathogenesis of pancreatic cancer.

CONCLUSION

Circulating CCL5 is an independent marker for poor prognosis for patients with LAPC treated with combination chemotherapy and consolidation CRT within the SCALOP trial. Further studies are required to validate CCL5 as a tumour marker in LAPC. Blockade of the CCL5-CCR5 axis may provide opportunities to modulate the efficacy of immunotherapy in pancreatic cancer.

ADDITIONAL INFORMATION

Acknowledgements: Not applicable.

Authors' contributions: CNH, EON and SM devised and led the translational component SCALOP trial. FW performed the in vitro measurement of biomarkers and CC led the statistical analysis and interpretation of data, with input from all other authors. FW, CC and SM authored the first draft of the manuscript. All authors contributed to subsequent revisions of the manuscript.

Ethics approval and consent to participate: The trial protocol was approved by the UK Medicines and Healthcare Products Regulatory Agency and a multicentre research ethics committee. Written informed consent was obtained for all patients who participated in SCALOP, as well as for the optional translational sample collection component. The study was performed in accordance with the Declaration of Helsinki.

Consent to publish: No relevant identifiable patient data.

Data availability: Anonymised data available on request from the corresponding author.

Competing interests: The authors declare no potential conflicts of interest.

Funding: CC, CWB and CNH are supported by Cancer Research UK Clinical Trials Unit core funding. CMJ is supported by a Wellcome Trust Clinical Research Fellowship. EON receives funding from the Kidani Memorial Trust and Cancer Research UK. SM is supported by funding from the NIHR Oxford Biomedical Research Centre.

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FIGURES

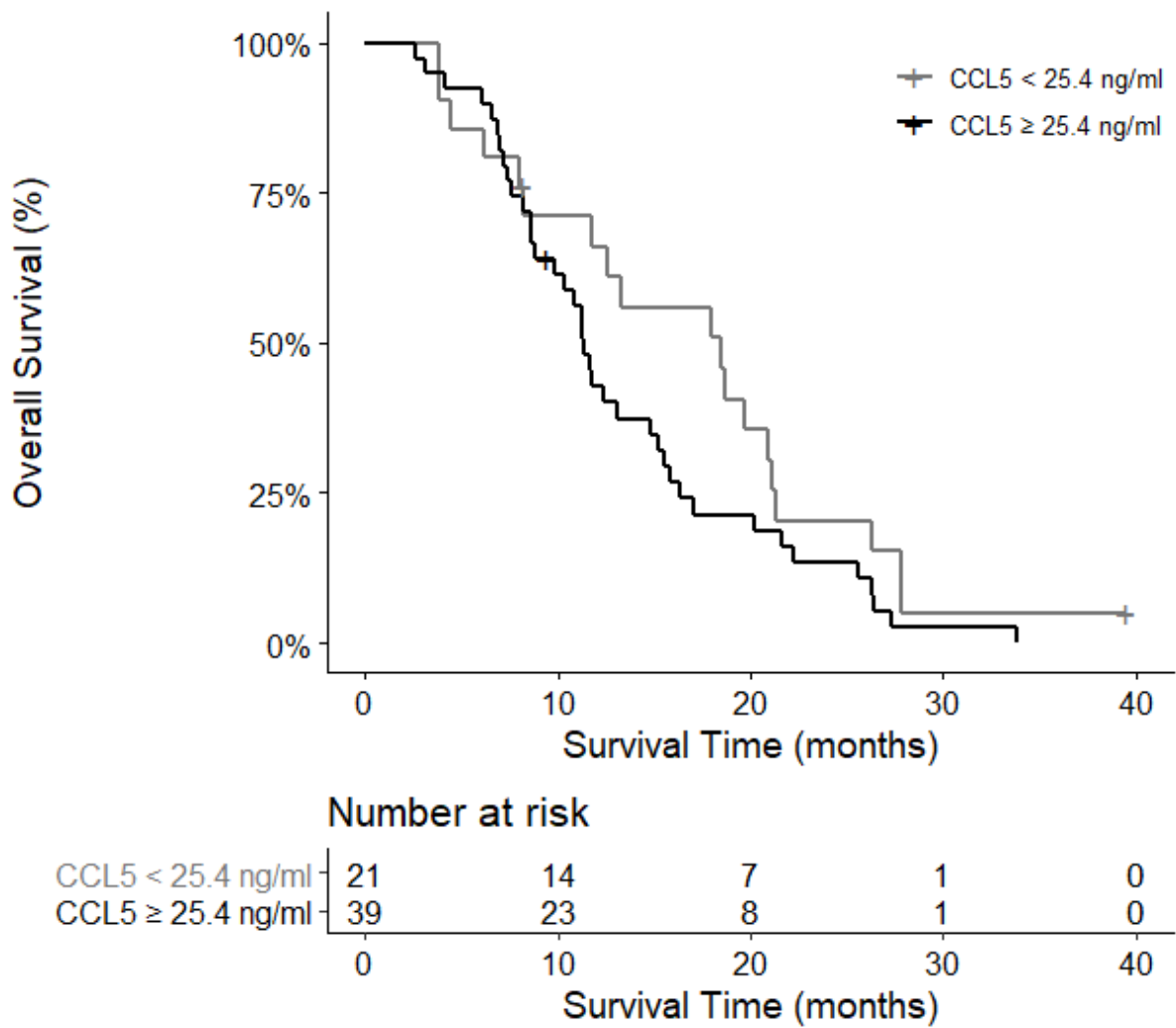


Figure 1: Kaplan-Meier estimates of overall survival for dichotomised serum C-C chemokine ligand 5 (CCL5) concentration in patients randomised within the SCALOP trial

Biomarker	n	Value (pg/ml)					p	FDR
		Mean	SD	95% CI	Median	IQR		
Beta-nerve growth factor (bNGF)	29	18.6	33.8	-47.6, 84.7	6.0	3.15, 14.0	0.192	0.8295
C-C motif chemokine ligand 11 (CCL11)	60	217.4	178.7	-132.8, 567.5	159.2	98.2, 257.2	0.5539	0.8858
C-C motif chemokine ligand 2 (CCL2)	60	240.8	135.4	-24.6, 506.0	209.0	154.9, 310.9	0.3209	0.8295
C-C motif chemokine ligand 27 (CCL27)	60	629.4	212.6	212.7, 1046.0	611.8	517.3, 746.7	0.8368	0.9414
C-C motif chemokine ligand 4 (CCL4)	60	596.5	121.3	358.7, 834.0	599.8	532.5, 746.7	0.3164	0.8295
C-C motif chemokine ligand 5 (CCL5)	60	37205.9	24871.1	-11541.3, 85953.2	30108.5	20941.1, 48637.9	0.0133	0.1772*
C-C motif chemokine ligand 7 (CCL7)	60	117.8	39.9	49.7, 195.9	114.3	86.9, 141.7	0.7038	0.8858
Interleukin-2 receptor alpha chain (CD25/IL2RA)	60	1156.2	416.6	339.8, 1972.6	1093.6	843.6, 1373.2	0.2481	0.8295
C-X-C motif chemokine ligand 1 (CXCL1)	44	177.4	90.3	0.4, 354.4	167.1	127.3, 213.7	0.8965	0.9449
C-X-C motif chemokine ligand 10 (CXCL10)	60	63.4	50.5	-35.5, 162.3	50.8	5.8, 74.1	0.4322	0.8295
C-X-C motif chemokine ligand 8 (CXCL8)	60	152.6	403.2	-637.5, 942.8	17.8	9.7, 72.5	0.2613	0.8295
C-X-C motif chemokine ligand 9 (CXCL9)	60	712.5	174.6	370.4, 1054.5	670.9	616.7, 773.5	0.6452	0.8858
Granulocyte colony stimulating factor (G-CSF)	60	17.8	11.7	-5.2, 40.7	16.2	12.6, 18.9	0.8654	0.9414
Hepatocyte growth factor (HGF)	60	442.8	364.7	-271.9, 1157.5	381.4	60.1, 548.0	0.4276	0.8295
Intracellular adhesion molecule 1 (ICAM1)	60	998988.5	532408.5	-44532.5, 2042510	879960.3	704481.1, 1065006.5	0.9861	0.9882
Interferon γ (IFN γ)	60	87.6	124.3	-156.0, 331.1	69.2	55.4, 84.9	0.0136	0.1772*
Insulin-like growth factor-1 (IGF-1)	46	398.4	984.7	-1631.6, 2328.4	156.4	84.0, 279.6	0.7268	0.8858
Interleukin-10 (IL-10)	60	4.6	3.2	-1.6, 10.7	4.1	2.9, 4.6	0.4466	0.8295
Interleukin-13 (IL-13)	60	782.2	172.0	445.2, 1119.2	766.4	624.6, 940.6	0.7106	0.8858
Interleukin-16 (IL-16)	60	1905.9	2163.1	-2333.7, 6145.4	1149.3	139.5, 3354.6	0.2311	0.8295
Interleukin-18 (IL-18)	60	2405.4	3401.7	-4261.8, 9072.6	1133.9	348.1, 3253.5	0.5726	0.8858
Interleukin-1 β (IL-1 β)	60	45.3	44.7	-42.2, 132.8	30.4	18.5, 55.5	0.4451	0.8295
Interleukin 1 receptor antagonist (IL-1RA)	60	6762.5	7899.3	-8720.0, 22245.0	4510.9	894.4, 11339.7	0.4949	0.8774
Interleukin-3 (IL-3)	60	57.4	42.9	-188.0, 335.5	50.7	32.8, 70.9	0.0616	0.8858
Interleukin-4 (IL-4)	60	74.8	20.9	33.8, 115.6	75.2	59.2, 90.4	0.442	0.8295
Interleukin-6 (IL-6)	60	7.2	5.1	-2.7, 17.0	5.7	4.9, 8.85	0.7172	0.8858
Interleukin-7 (IL-7)	60	8.2	3.5	1.3, 15.0	7.7	6.0, 9.3	0.3565	0.8295
Macrophage colony stimulating factor (M-CSF)	60	952.2	1065.2	1135.6, 3039.9	553.0	416.1, 1069.8	0.2069	0.8295
Macrophage inhibitory factor (MIF)	60	44434.9	69633.7	-92047.2, 180926.9	25334.0	7080.3, 50172.5	0.2781	0.8295
Osteopontin (OPN)	60	59412.9	20243.5	19735.7, 99090.0	59664.0	46423.2, 72998.8	0.869	0.9414
Platelet-derived growth factor (PDGF)	60	3790.5	2508.2	-1125.5, 8706.6	3535.8	1986.7, 5284.2	0.3548	0.8295
Stem cell factor (SCF)	60	92.6	31.2	31.4, 153.7	90.7	74.2, 106.8	0.1321	0.8295
Transforming growth factor- β (TGF- β)	46	32.4	16.5	-0.0, 64.7	31.5	21.7, 41.8	0.2677	0.8295
TNF-related apoptosis-inducing ligand (TRAIL)	60	65.9	47.4	-27.0, 158.7	54.3	38.3, 85.1	0.6925	0.8858

Vascular cell adhesion protein 1 (VCAM-1)	60	1381170.6	598701.2	207716.2, 2554625.0	1289218.1	1022870.6, 1562745.6	0.5633	0.8858
Vascular endothelial growth factor-A (VEGF-A)	48	92.2	77.0	-58.6, 243.0	68.9	0, 119.7	0.6493	0.8858
Week 17								
Beta-nerve growth factor (bNGF)	50	11.8	5.2	1.65, 22.0	10.8	7.7, 14.3	0.9882	0.9882
C-C motif chemokine ligand 5 (CCL5)	47	80240.0	86870.0	-90030, 250510	50620.0	33360, 92560	0.847	
Insulin-like growth factor-1 (IGF-1)	49	1066.3	2390.4	-3618.8, 5751.4	337.5	131.0, 616.7	0.4341	0.8295
Transforming growth factor- β (TGF- β)	50	12.9	9.1		9.9		0.8377	0.9414

Table 1. Association of each biomarker with overall survival. Results are shown for baseline for all biomarkers and additionally for week 17 for an additional subset. Association with overall survival was determined using univariable Cox regression. Biomarkers significantly associated with survival at the $q < 0.5$ level are highlighted by *. FDR, false discovery rate; n, number.

	CCL5		IFN γ	
	Median (IQR) μ g/ml	p-value	Median (IQR) pg/ml	p-value
Age				
<65 (n=33)	34.0 (19.2, 49.8)	0.859	59 (51.5, 79)	0.019
\geq 65 (n=27)	28.2 (21.4, 45.8)		79 (62, 92.5)	
WHO PS				
0 (n=34)	29 (21, 45.6)	0.660	151 (109, 168)	0.929
1 (n=26)	32.4 (21, 51)		127 (113, 172)	
Treatment				
Capecitabine (n=33)	33.6 (22.8, 49)	0.438	54 (31.4, 76)	0.679
Gemcitabine (n=27)	28.2 (18, 47)		50.5 (39.5, 67.5)	
CA19-9 (n=54)	r = 0.339	0.012	r = 0.012	0.9337
Longest disease diameter (n=58)	r = 0.091	0.498	r = -0.087	0.5160

Table 2. Correlation between biomarkers significantly associated with overall survival by Cox univariable regression and clinical characteristics known to be associated with overall survival in pancreatic cancer. CA19-9, cancer antigen 19-9; CCL5, C-C chemokine ligand 5; IFN γ , interferon- γ ; IQR, interquartile range; WHO PS, World Health Organisation Performance Status.

	CCL5 data (n=60)		No CCL5 data (n=14)		Total (n=74)	
	n	%	n	%	n	%
Treatment						
Gemcitabine	30	50	6	43	36	49
Capecitabine	30	50	8	57	38	51
Sex						
Male	33	55	8	57	41	55
Female	27	45	6	43	33	45
Age						
<65 years	33	55	5	36	38	51
≥65 years	27	45	9	64	36	49
WHO PS						
0	34	57	6	43	40	54
1	26	43	8	57	34	46
CA19-9 Median (IQR) U/mL	240.5 (77.0, 822.0)*		110.0 (71.0, 720.0)**		212.0 (73.0, 815.0)	
Disease diameter Median (IQR) cm	3.80 (3.00, 4.58)		4.35 (3.15, 4.95)		3.90 (3.00, 4.85)	

Table 3. Patient characteristics for all patients randomised within the SCALOP trial subdivided by the availability of data relating to serum C-C chemokine ligand 5 (CCL5) quantification. Disease diameter refers to the longest axis of the tumour. CA19-9, cancer antigen 19-9; IQR, interquartile range; n, number; WHO PS, World Health Organisation Performance Status. *n=54. **n=13

	n	Univariable		Multivariable (n=54)	
		HR (95% CI)	p-value	HR (95% CI)	p-value
Biomarker					
CCL5 (ng/ml)	60	1.01 (1.00-1.03)	0.013	1.01 (1.00-1.03)	0.011
CA19-9 (U/mL)	54	1.20 (1.11-1.31)	<0.001	1.19 (1.08-1.30)	<0.001
Age (years)					
<65	33	1.00		1.00	
≥65	27	0.83 (0.48-1.43)	0.503	0.69 (0.37-1.27)	0.234
Performance status					
0	34	1.00		1.00	
1	26	1.89 (1.09-3.27)	0.024	1.92 (0.96-3.84)	0.064
Trial arm					
Capecitabine	30	1.00		1.00	
Gemcitabine	30	1.25 (0.74-2.12)	0.409	0.98 (0.52-1.84)	0.951

Table 4. Univariable and multivariable Cox regression analysis by characteristic. Median CCL5 was 30.0 (IQR 6-48) ng/ml and median CA19-9 was 241 (IQR 77-822) U/ml. For multivariable analysis, six patients were missing cancer antigen 19-9 (CA19-9) data. Hazard ratios (HRs) for CCL5 (1.35) and CA19-9 (1.20) were calculated for each increase in CCL5 of 1ng/ml and increase in CA19-9 of 1000U/ml, respectively. 95% CI, 95% confidence interval; HR, hazard ratio; IQR, interquartile range; n, number; OS, overall survival.

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Supplementary tables

	Biomarker Data Available (n=63)		No Biomarker Data (n=11)		Total (n=74)	
	n	%	n	%	n	%
Treatment						
Gemcitabine	32	51	6	55	38	51
Capecitabine	31	49	5	45	36	49
Sex						
Male	36	57	5	45	41	55
Female	27	43	6	55	33	45
Age [years]						
<65	35	56	3	27	38	51
≥65	28	44	8	73	36	49
Performance status						
0	37	59	3	27	40	54
1	26	41	8	73	34	46
Disease diameter [cm], (IQR)	3.8 (3.0, 4.8)		4.2 (3.3, 5.3)		3.9 (3.0, 4.9)	

Supplementary Table 1. Patient characteristics for all patients randomised within the SCALOP trial by availability of biomarker data. Disease diameter refers to the longest axis of the tumour. IQR, interquartile range; WHO PS, World Health Organisation Performance Status.

Biomarkers	Progressed	Not Progressed	p-value	FDR
	Median (IQR) pg/ml	Median (IQR) pg/ml		
bNGF	-	6 (3, 14)	-	-
CCL11	153 (96, 189)	161 (100, 252)	0.5942	0.7475
CCL2	178 (128, 282)	195 (150, 298)	0.4990	0.7475
CCL27	706 (483, 869)	621 (517, 751)	0.3024	0.7475
CCL5	29690 (15444, 39089)	29402 (19493, 48992)	0.5165	0.7475
CCL7	105 (77, 145)	114 (87, 142)	0.6222	0.7475
CD25	1216 (934, 1564)	1105 (850, 1378)	0.3288	0.7475
CXCL1	139 (116, 162)	161 (128, 214)	0.4083	0.7475
CXCL8	9 (8, 16)	18 (10, 71)	0.0348	0.6521
CXCL9	666 (607, 729)	681 (616, 767)	0.5800	0.7475
CXCL10	70 (43, 82)	51 (38, 74)	0.1574	0.7475
G-CSF	14 (10, 17)	16 (12, 19)	0.4085	0.7475
HGF	452 (265, 491)	375 (171, 531)	0.7276	0.8396
ICAM	861999 (797902, 1217948)	859438 (710587, 1062396)	0.5708	0.7475
IFN	66 (52, 79)	71 (57, 84)	0.5031	0.7475
IGF-1	265 (180, 335)	156 (84, 279)	0.8164	0.8747
IL-3	49 (41, 72)	73 (34, 71)	0.9782	0.9782
IL-6	5.5 (5, 9.5)	5.5 (4.5, 8)	0.9075	0.9388
IL-7	7 (5.5, 11.5)	7.5 (6, 9)	0.8004	0.8747
IL-10	3.5 (3.5, 4.5)	4 (3, 4.5)	0.5894	0.7475
IL-16	428 (148, 917)	1059 (145, 3405)	0.1420	0.7475
IL-18	896 (310, 1402)	992 (362, 3155)	0.3933	0.7475
IL-1BETA	23 (17, 31)	29 (19, 56)	0.2085	0.7475
IL-1RA	2694 (970, 3197)	4344 (969, 11352)	0.1921	0.7475
M-CSF	492 (448, 752)	561 (418, 1006)	0.6229	0.7475
MIF	14985 (7374, 22596)	23541 (6894, 44987)	0.5895	0.7475
PDGF	2191 (1540, 4350)	3188 (1963, 5300)	0.4403	0.7475
SCF	108 (71, 117)	91 (73, 106)	0.2064	0.7475
TRAIL	69 (45, 88)	57 (39, 86)	0.3709	0.7475
VCAM	1445101 (1252282, 1665204)	1289097 (1010007, 1595178)	0.2114	0.7475
VEGF-A	34 (19, 51)	62 (32, 117)	0.0435	0.6521

Supplementary Table 2: Association of each biomarker when quantified at baseline, prior to treatment, with progression-free survival. Association with progression free survival was determined using univariable Cox regression. FDR, false discovery rate; n, number.

Dichotomised CCL5 Signature	Overall survival (months)		Univariate	
	n	Median (95% CI)	HR (95% CI)	<i>p-value</i>
<0.398	17	19.68 (16.30 - 27.79)	-	-
≥0.398	37	11.20 (8.25 - 13.24)	2.69 (1.40 - 5.17)	0.003

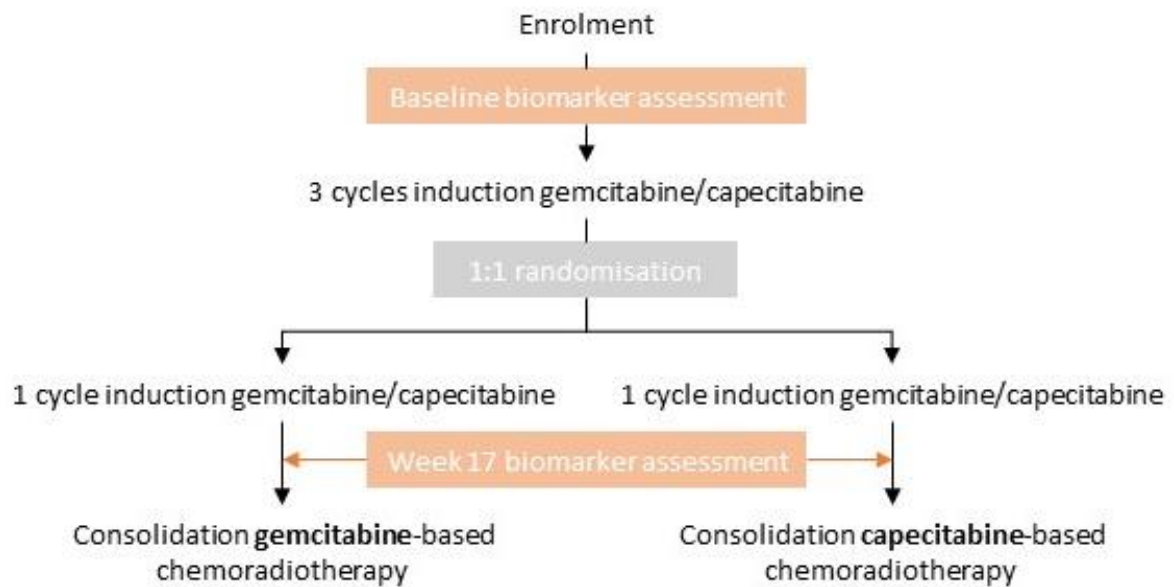
Supplementary Table 3: Overall survival and univariate analysis of dichotomised C-C chemokine ligand 5 (CCL5) signature. 95% CI, 95% confidence interval; HR, hazard ratio.

% Change in CCL5	Overall survival (months)		Univariate	
	n	Median (95% CI)	HR (95% CI)	<i>p-value</i>
	47	-6.7 (-354.6, 264.1)	1.00 (1.00, 1.00)	0.42-

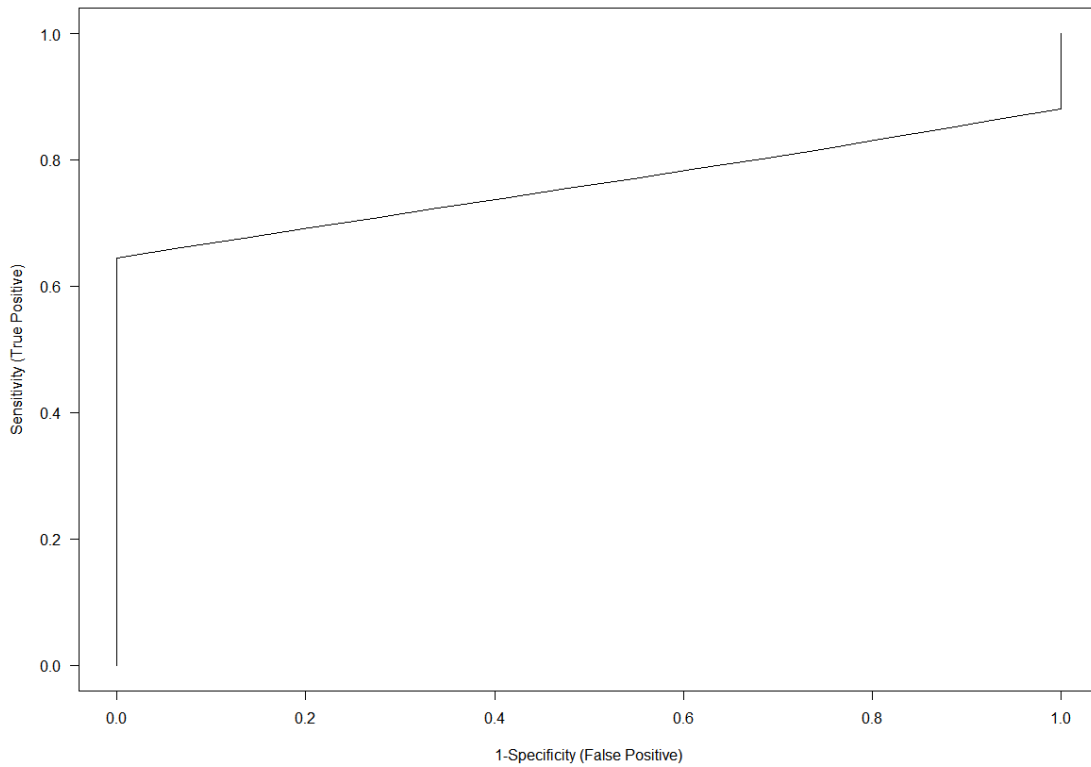
Supplementary Table 4: Overall survival and univariate analysis of percentage change in C-C chemokine ligand 5 (CCL5) signature from pre-treatment baseline to week 17 (prior to consolidation chemoradiotherapy). 95% CI, 95% confidence interval; HR, hazard ratio.

SUPPLEMENTARY FILES

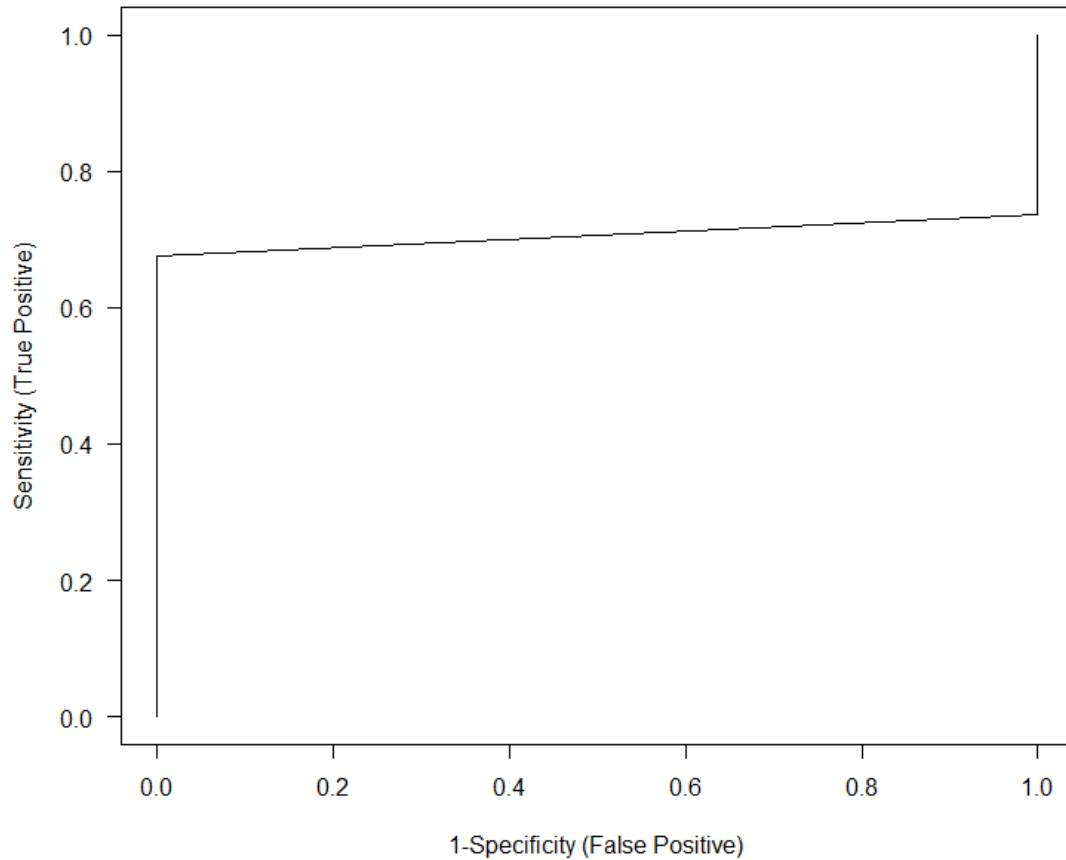
Supplementary figures



Supplementary Figure 1: A schematic overview of the SCALOP trial, including key treatment timepoints. Sample collection timepoints are highlighted in orange. Induction chemotherapy consisted of intravenous gemcitabine ($1000\text{mg}/\text{m}^2$) on days 1, 8 and 15 of each 28 day cycle and oral capecitabine ($830\text{mg}/\text{m}^2$ twice daily) on days 1-21 of each 28 day cycle. In total, four cycles of induction chemotherapy were administered prior to consolidation chemoradiotherapy at a total dose of 50.4Gy in 28 weekday fractions over 5.5 weeks with either intravenous gemcitabine ($300\text{mg}/\text{m}^2$) weekly for six doses or oral capecitabine ($830\text{mg}/\text{m}^2$) on each day radiotherapy was administered. Samples for biomarker analysis were collected prior to induction chemotherapy and prior to consolidation chemoradiotherapy.



Supplementary Figure 2: Time dependent receiver operating characteristic (ROC) curve estimation for circulating C-C chemokine ligand 5 (CCL5) association with overall survival. Area under the curve (AUC): 0.761. Optimal cut point: 25.4 ng/ml. Sensitivity: 64%. Specificity: 100%. True positive: 64%. False positive: 0%.



Supplementary Figure 3: Time-dependent receive operating characteristic (ROC) curve estimation for a signature score including a dichotomised C-C chemokine ligand 5 (CCL5) association with overall survival. Dichotomised CCL5 score was 0 if CCL5 < 25.4 ng/ml and 1 if CCL5 ≥ 25.4 ng/ml. Area under the curve (AUC): 0.707. Optimal cut point: 0.398. Sensitivity: 68%. Specificity: 100%. True positive: 68%. False positive: 0%.