Circulating biomarkers and outcomes from a randomised phase 2 trial of gemcitabine versus capecitabine-based chemoradiotherapy for pancreatic cancer

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BACKGROUND: The Phase 2 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.

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 (CCL5low) 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 CCL5high; 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.

CLINICAL TRIAL REGISTRATION: The SCALOP trial was registered with ISRCTN, number 96169987 (registered 29 May 2008).

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outcomes, assessing a panel of 35 of these systematically identified cytokines in samples from the SCALOP trial cohort.

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 7 cm 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 6 cm or less were eligible for randomisation to a further cycle of GEMCAP followed by gemcitabine (300 mg/m² weekly) or capecitabine (830 mg/m² twice daily on days of radiotherapy) concurrent with radiotherapy (50.4 Gy in 28 fractions) as illustrated in Supplementary Fig. 1.

Sample collection and analysis
Peripheral venous blood was collected in ethylenediaminetetraacetic acid (EDTA) anti-coagulant 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 centralised 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 limited to two freeze-thaw cycles. Supernatants were thawed on ice and centrifuged at 10,000×g for 5 min at 4 °C to remove precipitate. Raw biomarker levels at both baseline and, where measured, at week 17 are shown in Table 1, as is an analysis of the association between baseline cytokine levels and progression. Of the biomarkers measured in triplicate across all patients, 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 ≤ 0.7 shows independence) and Wilcoxon rank sum tests (p ≥ 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, IFNy) measured at baseline (prior to commencement of GEMCAP chemotherapy) had significant associations with OS at the q < 0.2 FDR level. IFNy 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
Circulating biomarkers and outcomes from a randomised phase 2 trial of...
F Willenbrock et al.

Table 1. Association of each biomarker with overall survival.

| Biomarker                                      | n   | Value (pg/ml) | Mean  | SD      | 95% CI          | Median | IQR        | p    | FDR     |
|-----------------------------------------------|-----|---------------|-------|---------|-----------------|--------|------------|------|---------|
| **Beta-nerve growth factor (BNGF)**          | 54  | 15.0          | 10.7  | 3.6     | 4.0, 24.0       | 10.7   | 3.6        | 0.01 | 0.177   |
| **C-C motif chemokine ligand 1 (CCL1)**      | 60  | 217.4         | 178.7 | 35.8    | 132.8, 567.5    | 159.2  | 252.7      | 0.39 | 0.885   |
| **C-C motif chemokine ligand 2 (CCL2)**      | 60  | 240.8         | 135.4 | 24.6    | 96.0, 506.0     | 209.0  | 154.9      | 0.32 | 0.829   |
| **C-C motif chemokine ligand 27 (CCL27)**    | 60  | 629.4         | 212.6 | 21.7    | 104.0, 460.0    | 611.8  | 517.3      | 0.83 | 0.941   |
| **C-C motif chemokine ligand 4 (CCL4)**      | 60  | 596.5         | 121.3 | 35.8    | 934.0, 599.8    | 532.5  | 746.7      | 0.36 | 0.829   |
| **C-C motif chemokine ligand 5 (CCL5)**      | 47  | 37205.9       | 24871.1 | -11541.3 | 85953.2        | 30108.5 | 20941.1 | 0.01 | 0.177   |
| **C-C motif chemokine ligand 7 (CCL7)**      | 60  | 1178          | 39.9  | 49.2    | 195.9, 114.3    | 114.3  | 86.9       | 0.70 | 0.885   |
| **Interleukin-2 receptor alpha chain (CD25/IL2RA)** | 44  | 177.4         | 90.3  | 0.3     | 34.5, 167.1     | 167.1  | 127.3      | 0.89 | 0.944   |
| **Interleukin-3 (IL-3)**                     | 60  | 63.4          | 50.5  | -35.5   | 162.3, 50.8     | 50.8   | 58.7       | 0.43 | 0.822   |
| **Interleukin-4 (IL-4)**                     | 60  | 152.6         | 403.2 | -637.5  | 942.8, 17.8     | 17.8   | 9.7        | 0.26 | 0.821   |
| **Interleukin-10 (IL-10)**                   | 50  | 712.5         | 174.6 | 370.4   | 1054.5, 609.7   | 670.9  | 617.6      | 0.64 | 0.886   |
| **Granulocyte colony stimulating factor (G-CSF)** | 50  | 17.8          | 11.7  | -5.2    | 40.7, 16.2      | 16.2   | 12.6       | 0.86 | 0.941   |
| **Hepatocyte growth factor (HGF)**           | 60  | 442.8         | 364.7 | -21.9   | 1157.5, 381.4   | 60.1   | 54.8       | 0.43 | 0.829   |

Relative change in CCL5 levels between baseline (prior to start of CRT, data available for n = 47) did not demonstrate any association with OS or any other biomarker.

P values and FDRs are corrected for multiple comparisons using the FDR method. Significant associations (p < 0.05) are highlighted by asterisks.

**Results**

CCL5 (Supplementary Fig. 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 CCL5low of 11.2 months (95% CI: 8.25–21.3) and 11.3 months (95% CI: 9.86–16.1) in patients with CCL5high (39/60); as demonstrated in Fig. 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 Fig. 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 CCL5low and 37 as CCL5high. Using this approach, patients classified as CCL5low had a median OS of 19.68 months (95% CI 16.3–27.7) and CCL5high 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 found to be a poor prognostic factor independent of concurrent chemotherapy received during radiotherapy (capetitabine 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.
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.

| Biomarker | CCLS Median (IQR) μg/ml | p-value | IFNγ Median (IQR) pg/ml | p-value |
|-----------|--------------------------|---------|------------------------|---------|
| Age (years) |
| <65 (n = 33) | 34.0 (19.2, 49.8) | 0.859 | 59 (51.5, 79) | 0.019 |
| ≥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 | 0.516 |

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-1β; IL-6, IL-8, VEGF, TGFβ, 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 CCLS and IFNγ as being significantly associated with OS, of which CCLS remained significant in multivariable analysis. CCLS can be secreted by a variety of tumour cells (including pancreatic cancer) as well as non-malignant stromal cells including T regulatory cells (Treg) and macrophages. Engagement of CCLS with its receptor, C-C chemokine receptor type 5 (CCR5), can favour tumour growth and metastasis through modulating the activity of the PI3K/Akt, MAPK/ERK and NF-κB pathways, and via induction of matrix metalloproteinases.24

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 (CCLS) quantification.

| CCLS data | No CCLS data | Total |
|-----------|--------------|-------|
| n (60) | n (14) | n (74) |
| Treatment |
| Gemcitabine | 30 | 20 | 50 |
| Capecitabine | 30 | 5 | 35 |
| Sex |
| Male | 33 | 5 | 38 |
| Female | 27 | 9 | 36 |
| Age (years) |
| <65 | 33 | 5 | 38 |
| ≥65 | 27 | 9 | 36 |
| WHO PS |
| 0 | 34 | 6 | 40 |
| 1 | 26 | 3 | 29 |
| CA19–9 Median (IQR) U/mL |
| 240.5 (77.0, 482.0) | 110.0 (71.0, 270.0)** | 212.0 (73.0, 815.0) |
| Disease diameter |
| 3.60 (3.00, 4.58) | 4.35 (3.15, 4.95) | 3.90 (3.00, 4.85) |

Table 4. Univariable and multivariable Cox regression analysis by characteristic.

| n | Univariable | Multivariable (n = 54) |
|---|-------------|-----------------------|
| HR (95% CI) | p-value | HR (95% CI) | p-value |
| Biomarker |
| CCLS (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 |

Median CCLS 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 CCLS (1.35) and CA19–9 (1.20) were calculated for each increase in CCLS of 1 mg/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.
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 and 5 higher than in some previous reports. Levels of IL-1β 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 CCLS 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 CCLS-CCRS axis in the pathogenesis of pancreatic cancer.

CONCLUSION

Circulating CCLS 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 CCLS as a tumour marker in LAPC. Blockade of the CCLS-CCRS axis may provide opportunities to modulate the efficacy of immunotherapy in pancreatic cancer.

AUTHOR CONTRIBUTIONS

C.N.H., E.O.N. and S.M. devised and led the translational component SCALOP trial. F.W. performed the in vitro measurement of biomarkers. C.M.C., C.S.W-B. and C.N.H. led the analysis of data, with contributions from E.E.P., A.G.A., R.O., A.S., C.M.J., D.L.I.H. and T.M. All authors contributed to the interpretation of the results. F.W., C.C., C.M.J. and S.M. authored the initial drafts of the paper. All authors agree to be accountable for all aspects of the work.

ADDITIONAL INFORMATION

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.
Circulating biomarkers and outcomes from a randomised phase 2 trial of... F Willenbrock et al.

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 competing interests.

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