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Intra-tumoural stromal morphometry predicts disease recurrence but not response to 5-fluorouracil – results from the QUASAR trial of colorectal cancer

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Running title - Stromal morphometry in stage II colorectal cancer

Abstract

Introduction: The biological importance of tumour-associated stroma is increasingly apparent, yet clinical utility remains ill-defined. In stage-II / Dukes B colorectal cancer (CRC), clinical biomarkers are urgently required to direct therapeutic options. We report here prognostic/predictive analyses, and molecular associations, of stromal morphometric quantification in the Quick and Simple and Reliable (QUASAR) trial of CRC. Materials and methods: Relative proportions of tumour epithelium (PoT) or stroma (PoS) were morphometrically quantified using digitised haematoxylin and eosin sections derived from 1,800 patients enrolled in QUASAR which randomised 3,239 (91% stage II) CRC patients between adjuvant fluorouracil/folinic acid (FUFA) chemotherapy and observation. The prognostic/predictive value of PoT/PoS measures were determined by stratified log-rank analyses. Results: High tumour stroma (≥50%) was associated with increased recurrence risk: 31.3% (143/457) recurrence for ≥50% versus 21.9% (294/1,343) if <50% [Rate ratio (RR)=1.62; 95%CI 1.30-2.02, p<0.0001)]. For stromal proportions of ≥65%, 40% (46/115) of patients had recurrent disease within 10 years. The adverse prognostic effect of high stroma was independent of established prognostic variables, and maintained in stage II / Dukes B patients (RR=1.62; 95%CI=1.26-2.08; p=0.0002). *KRAS* mutation in the presence of high stroma augmented recurrence risk (RR=2.93; 95%CI=1.87-4.59; p=0.0005). Stromal morphometry did not predict response to FUFA chemotherapy. Discussion: Simple digital morphometry applied to a single representative H&E section identifies CRC patients with over 50% higher risk of disease recurrence. This technique can reliably partition patients into sub-populations with differential risks of tumour recurrence in a simple and cost-effective manner. Further prospective validation is warranted.

Keywords

Colon

Colorectal

Rectal

Cancer

Stroma

INTRODUCTION

The use of adjuvant chemotherapy in colorectal cancer (CRC) following presumptive curative resection is directed by high-quality pathological assessment.[1-3] Detection of tumour within lymph nodes (stage III disease) is generally regarded as an absolute indication for adjuvant therapy[1,4,5] because of significant clinical benefit (~10% absolute improvement in overall survival [OS].[6,7] The value of adjuvant chemotherapy in locally advanced, node-negative (stage II) disease is less clear because the more modest benefits (~4% improvement in OS),[8] may not outweigh the toxicity and patient inconvenience.[1,9,10]

Thus, adjuvant chemotherapy is generally restricted to a minority of stage II patients with 'high-risk' pathological features including extra-mural vascular and/or peritoneal invasion.[1,11,12] Such features impart a recurrence risk of similar magnitude to that associated with lymph node metastasis[2] and so serve to identify stage II patients who may derive worthwhile benefit from adjuvant regimens.[1,4,5] A limitation of this approach is that identification of such features is subjective with variability in reporting quality and reproducibility.[3,13] Also, only a minority of recurrences among these 'high-risk'

patients will be prevented by 'appropriate' chemotherapy,[8,10,14] and it is not currently possible to identify who is more or less likely to benefit.[15] Consequently, identification and clinical validation of effective prognostic and, in particular, predictive biological indicators would help facilitate therapeutic decisions.

Mismatch repair (MMR) / microsatellite instability (MSI) testing can identify CRC patients at a reduced risk of recurrence for whom adjuvant therapy is usually not indicated.[14,16-18] However, only a minority of CRC patients, 12-15% at most, demonstrate MSI and/or attenuated expression of one or more MMR proteins.[16,17] The Oncotype DX® recurrence score has been prospectively validated for assessment of recurrence risk in post-operative stage II colon cancer patients but is of only modest prognostic value and is not predictive of chemotherapy benefit.[19] Widely applicable biomarkers are thus required, particularly ones that negate the need for expensive molecular testing,[20-23] for cost-effective application in a diverse, non-specialist setting.

Associations between disease recurrence and cancer-associated stromal gene[23-27] or protein[28-35] expression has been reported in a variety of malignant conditions with some evidence of differential chemotherapeutic response defined by stromal gene expression profiles.[36-38] In CRC, the potential prognostic value of stromal gene expressional analysis is highlighted by inclusion in the Oncotype DX® colon recurrence score.[23] Whilst the majority of studies have been performed at the transcriptomic or proteomic level, several studies indicate that, using established mathematical principles,[39-42] simple, inexpensive, visual evaluation of tumour composition[43-47] or stromal phenotype[48,49] may yield equally valuable prognostic information.

Studies by Mesker *et al*,[50,51], West *et al*,[52] and Huijbers *et al*,[47] suggest that simple visual[47,50,51] or morphometric[52] assessment of CRCs provides independent prognostic information, a premise recently tested in the VICTOR CRC trial.[47] To improve on the reproducibility of these visual estimation methods described by Huijbers *et al* (2013),[47] we have developed a quantitative compositional analysis technique utilising digital pathology.[53] This methodology, however, has so far only been tested in a small cohort of 145 all-stage CRC patients.[52]

Given biological plausibility, existing literature, and the recent recognition of CRCs with mesenchymal/stroma rich gene signatures as biologically distinct and clinically significant tumour sub-populations[54], we hypothesise that quantitative tumour-stromal compositional analysis might represent a simple yet powerful methodology to determine risk of disease recurrence and differential response to adjuvant chemotherapy in CRC. We report here results of a test of this hypothesis utilising digitised material and clinicopathological data from the QUASAR trial, which randomised 3,239 predominantly stage II (91%) CRC patients between 6 months of fluorouracil and folinic acid (FUFA) chemotherapy and observation.[8]

MATERIALS AND METHODS

Ethics

Ethical approval was obtained from both the West Midlands Multi-Centre Research Ethics Committee (JR/MT/MREC/02/7/56a) and the Northern and Yorkshire Research Ethics Committee (08/H0903/62).

Patients

The design and details of QUASAR (ISRCTN82375386) are reported elsewhere.[8] Briefly, 3,239 patients (post-curative resection for colon or rectal cancer, 91% stage II disease) were randomized to FUFA chemotherapy (n=1622) or observation only (n=1617) with chemotherapy considered in the event of recurrence.

Study design

Datasets were randomly partitioned into exploratory and validatory groups (figure 1). For initial analyses, an exploratory dataset (n=399), representative of the entire study cohort, was used to develop *a priori* prognostic[50-52] and predictive hypotheses using data derived from 3 specific analytical tumour regions (figure 2). Independent validation was performed using a separate validatory patient cohort dataset (n=1,800). All pathological and laboratory assessments were undertaken blind to the patients' treatment allocation and clinical outcomes.

Clinicopathological data

Pathological data (TNM 5) were abstracted by central review of anonymised histopathological reports collected from local units.

Morphometric analysis of tumour components

One tumour block per patient was selected for analyses. When more than one block was available, blocks were chosen to optimally represent the area of maximal tumour infiltration of the bowel wall. Histological sections (5µm thick) were cut from selected tumour blocks and stained with haematoxylin and eosin (H&E) using standard methodologies.

H&E sections were digitised at x20 magnification (0.46 µm per pixel) using an automated scanning system (Aperio XT, Aperio Technologies, Vista, CA, USA). Slide viewing for quality control (QC) and all subsequent quantification processes were performed using open source slide viewing software (Aperio ImageScope v10.2.2.2352, Aperio Technologies, Vista, CA, USA). Following image QC, and methodological and statistical validation using existing datasets, a systematic random sample of 50 points was superimposed on selected areas (figure 2) using web-based virtual graticule software (RandomSpot, University of Leeds, Leeds, UK).

For exploratory analyses (figure 1), three areas from each selected H&E section were identified for random point grid application (figure 2). The luminal tumour area (LT) was defined as a $9mm^2$ area of greatest tumour epithelial cell density at the luminal surface of the tumour (figure 2A). The highest tumour density (HT) area was defined as a $9mm^2$ area of highest tumour cell density (if not located at the luminal surface); figure 2B. The whole tumour (WT) area included the total area of tumour infiltration extending from the luminal surface and encompassing the lateral and deep invasive fronts of tumour (figure 2C). For $9mm^2$ regions, large areas of necrosis and mucin were avoided if possible.

Tumour morphometry was determined by counting the frequency of occurrence of specific phenotypic categories underlying each of the 50 points. The following categories were used; 1: tumour epithelium, 2: tumour-associated stroma, 3: necrosis, 4: vessel, 5: inflammation, 6: tumour lumen, 7: mucin, 8: muscle and 0: non-informative / unclassifiable. Tumour morphometry was performed by technical staff under direct supervision of experienced pathologists (GH / PQ).

For final outcome analyses, relative tumour composition was expressed as a percentage of total informative points for tumour epithelium (PoT – phenotype category 1 only) or stroma (PoS – phenotype category 2 only) or other categories (phenotype categories 3-8).

Determination of morphometric stratification cut-off points

For prognostic / predictive analyses, we applied the 'pool adjacent violators' algorithm to the continuous variables PoT or PoS to determine points where the relationship between the measured variable (e.g. stroma) and the recurrence risk demonstrated a distinct change[55-59]. Analyses were performed (by KH/RG) across all tumour / stromal measurements derived from each area within the training set (n=399) indicating the most appropriate cut-off values to stratify scores into two (<50%, ≥50%) or four groups (<35%, 35-49%, 50-64%, ≥65%).

Statistics and bioinformatics

The prognostic/predictive value of PoT/PoS measures were determined by stratified log-rank analyses.[60] For analyses of the prognostic value of PoT or PoS, recurrence was used as outcome measure. Recurrence is a reliable marker of the natural history of the cancer and correlates well with the effects of adjuvant chemotherapy on survival.[61] Recurrence was calculated as the time elapsed from randomisation to recurrence with censoring at last contact with patient or death without recurrence. For assessment of the prognostic value of variables, recurrence rates over the whole follow-up period were analysed. Because adjuvant FU/FA reduces the risk of recurrence only in the first two years following randomisation with no further benefit, or loss of benefit, thereafter,[8] investigations of differential treatment efficacy within subgroups included recurrences over the first two years only. Analyses were generally performed using SAS version 9.2 (SAS Institute Inc., Cary, NC) by biostatisticians within the Birmingham Clinical Trials Unit, University of Birmingham, UK (KH)

and The Nuffield Department of Population Health, University of Oxford, Oxford, UK (RG).

Integrity of scoring data was validated by inter-observer comparison analysis of scores generated by 2 independent observers using kappa statistics on 2,975 individual data points. Inter-observer variability analyses were discontinued after 10 cases because of extremely high inter-observer agreement (see below). Subsequent to inter-observer analyses, all generated data were visually screened by experienced pathologists (GH/PQ).

RESULTS

Tumour tissue was obtained for 75% (2,439/3,239) of QUASAR participants with material from 2,199 patients suitable for analysis (figure 1). Comparisons of morphometry scores generated by two independent observers on 2,975 informative data points confirmed high agreement of 99.1%, Cohen's kappa=0.986 (95%CI=0.985-0.990; p<0.001).

Exploratory dataset analyses

Analyses of the 399-patient exploratory dataset provided no indication of any significant association between tumour or stroma density and chemotherapeutic efficacy (Supporting information, figure S1); prognostic analyses of the exploratory dataset therefore included both treated and control patients. A higher percentage of stroma cells was associated with worse prognosis in the exploratory data set, particularly in the 'highest' and 'whole' stroma categorisations (Supporting information, figures S2-S7). A lower percentage of tumour cells was also associated with worse prognosis in the exploratory data set, particularly in the 'highest' and 'lumenal' tumour categorisation.

Validatory dataset analyses

Analyses of the 1800-patient validatory dataset also provided little evidence for any association between tumour or stroma density and chemotherapy efficacy (see below). Therefore, prognostic analyses of the validatory data again included both chemotherapy treated and control patients.

There was a highly significant trend of increasing 10-year recurrence risk with increasing stromal proportions in the whole tumour area in the test data set (log-rank p<0.0001; figure 3). By contrast, other stromal and tumour scores were only weakly associated with worse prognosis (Supporting information, figures S8-S12). This highly significant association in the whole stroma categorisation is consistent with the exploratory data analyses where the association was strongest in the whole and highest stroma categories, whereas the lack of association in the highest stroma category is not.

For stromal proportions of ≥65% in the whole tumour region, the 10-year risk of recurrent disease was 43% compared to 25% for tumours with <50% scores. The adverse prognostic impact of higher whole tumour PoS scores remained highly significant (p=0.0002) in analyses restricted to stage II / Dukes B patients only (figure 4). Notably, there was no association between the pattern of recurrence (local versus distant) and increasing stromal proportions within the whole tumour region (p=0.105).

The distribution of PoS risk categories derived from the WT region differed significantly by tumour site, stage, histological subtype and MMR phenotype (table 1). There were significantly more tumours classified as PoS ≥65% in the rectum than colon: 11.4% (43/377) compared to 5.1% (72/1,423), p<0.0001. There were more low (<35%) PoS scores in Dukes' B than Dukes' C lesions 41.1% (658/1,603) versus 26.7% (43/161), p=0.002. Predictably, there was a lower frequency of PoS ≥65% lesions in mucinous tumours when compared to adenocarcinomas 1.9% (3/162) versus 6.4%

(95/1,473) respectively, p<0.0001). The weak (p=0.012) association of lower scores in the dMMR phenotype is also unsurprising given the previously reported interactions between dMMR, anatomical location and mucinous and medullary histology.

To investigate whether confounding with other pathological prognostic variables might at least partly explain the association of high stroma counts with recurrence, we undertook analyses stratified by these other variables using a binary dichotomisation (<50% low, ≥50% high) of PoS scores. The risk of recurrence was over 50% higher in the 25% (457/1,800) of patients with high (≥50%) PoS scores than in patients with low (<50%) PoS scores: [Rate ratio (RR)=1.61; 95%CI 1.30-2.00, p<0.0001)]; figure 5. Analyses stratified by chemotherapy allocation, tumour site and stage did not indicate any significant variability in the prognostic importance of stroma. Nor did any other of the variables tested except for the analyses stratified by *KRAS* mutation status where significant (p=0.001) heterogeneity was seen with a stronger prognostic association seen in *KRAS* mutant tumours (RR=2.93; 95%CI=1.85-2.55) than in *KRAS* wild-type tumours (RR=1.14; 95%CI=0.60-1.62), (figure 5).

We investigated any differential chemotherapeutic response by PoS measurements using 2 year disease recurrence as outcome (figure 6). There were one third fewer recurrences with chemotherapy than control within the 2 year post-randomisation period [unstratified analyses; RR=0.64; 95%CI=0.49-0.84; p=0.001]. Analyses sub-stratified by increasing WT PoS measures, failed to demonstrate a significant interaction between chemotherapy benefit and PoS measurements (p_{trend}=0.22; figure 6).

Unexpectedly, the beneficial effect of chemotherapy was as strong in analyses of all recurrences as in analyses of 2-year recurrence (Supporting information, figures S13-S16): 20.8% (183/880) of adjuvant treated patients recurred at 10 years compared to 27.6% (254/920) of untreated patients [RR=0.71; 95%CI=0.59-0.85; p=0.0003]. There was a suggestion (p_{trend}=0.039) that the reduction in 10-year recurrence with chemotherapy increased with increasing stromal proportions (PoS) in the WT region (Supporting information, figures S13-S16).

Analysis of other prognostic variables were performed to compare the prognostic strength of these variables with that of stroma count, and to determine if their prognostic value was independent of stromal count. (Supporting information, figures S17-S23) A borderline significant interaction between stroma and Dukes stage (p_{trend}=0.032) was seen with no apparent prognostic effect of nodal status in tumours with ≥65% PoS values. No interactions between TNM T-category (TNM 5) and increasing PoS values were identified with the favourable prognostic effect of T3 versus T4 disease appearing similar across PoS subgroups (Supporting information, figure S18). Small subgroup numbers (2 recurrences in 5 patients with dMMR tumours with ≥65% PoS measurements) precluded any meaningful investigation of potential interactions (Supporting information, figure S20). *KRAS* mutation was associated with a higher disease recurrence rate when compared to *KRAS* wild-type tumours [115/401; 28.7% versus 170/780; 21.8% recurred (RR=1.39; 95%CI=1.09-1.78, p=0.009); (Supporting information, figure S21) with the adverse prognostic effect of tumour PoS appearing greater in *KRAS*-mutant than *KRAS* wild-type tumours (Supporting information, figure S21). These observations parallel the analysis of dichotomised stroma stratified by *KRAS* mutation status (figure 5). *BRAF* status was non-prognostic (Supporting information, figure S22).

DISCUSSION

By applying simple digital morphometry to tumour blocks from 2,199 QUASAR CRC trial patients,[8] we have demonstrated the powerful prognostic value of tumour-stromal counts; our division of tumours into four prognostic groups based on cut-offs in the validatory cohort was confirmed in the test data with high intra-tumoural stroma (>65%) being associated with a disease recurrence risk approximately twice that of patients whose tumours contained low intra-tumoural stroma (<50%).

Our primary analysis with 2-year disease recurrence as outcome failed to substantiate any predictive chemotherapeutic effect of stroma count (figure 6). However, benefits of chemotherapy appeared to increase with increasing stroma count and this trend reached statistical significance in analyses of all recurrence stratified by intra-tumoural stromal proportion, suggesting that the benefits of chemotherapy are at least as good in the higher risk stroma groups. This contrasts with previous reports suggesting that tumour associated stroma may attenuate the efficacy of chemotherapeutic agents.[62-70] Although some propose pharmacological targeting of tumour associated stroma as a mechanism of countering chemoresistance (and thus enhancing chemotherapeutic efficacy) in cancer [70-73] the present study provides no support for this approach, at least in CRC.

The mechanism by which varying proportions of intra-tumoural stroma influence clinical outcome (also reported in breast,[43,46] lung,[44,74] ovarian,[75] cutaneous,[45] and prostatic[76] neoplasia) is unknown. Postulated theories commonly relate to stroma-associated cellular phenotypes and include enhanced pro-invasive signalling by intra-stromal myofibroblasts[77] or growth factor/cytokine production by cancer associated fibroblasts (CAF) inducing angiogenesis, increased tumour growth and invasion.[71,78-81] Other possible explanations may involve mechanisms relating to tumour hypoxia[82] and/or tumour associated inflammation.[71,83] The possibility that differential proportions of

tumour and stroma may simply indirectly reflect the stage of disease has been previously suggested.[51,52] We found no evidence for this potential explanation of the prognostic effect of stroma with similar findings in stratified and unstratified analyses and no interaction between stromal proportions, Dukes or TNM T categories.

Stromal TGFβ,[84,85] a critical regulator of epithelial-mesenchymal transition,[86] is required to support metastatic dissemination when mutational inactivation of TGFβ has occurred, as is common in CRC.[87] In view of the possible role of stromal-derived TGFβ in the promotion of metastases in CRC,[87] the observation that *KRAS* mutant, high stromal tumours had a significantly more elevated recurrence risk than *KRAS* wild-type high stromal lesions is noteworthy. There is abundant evidence indicating that RAS signalling operates in unison with TGFβ to enhance tumour cell invasiveness, either directly[88,89] or by suppressing TGFβ mediated growth inhibitory signals.[90,91] A note of caution, however, is that the association between stroma and *KRAS* mutation was unanticipated, so our findings need confirming, but should encourage further investigation.

In summary, our analyses of the largest reported validation dataset confirm the prognostic value of simple morphometric analyses and reciprocate similar findings of morphological / morphometric prognostic studies in CRC reported by Mesker *et al*[50,51], Huijbers *et al*[92] and West *et al*.[52] Collectively, our data and that of others[50-52,92] adds substance to the concept that simple morphometric appraisal of the quantitative relationship of tumour and / or tumour stroma can reliably identify patient sub-populations with differential risks of tumour recurrence in a simple and cost-effective manner. Our group (led by AW/DT) are now in the process of using the described dataset to develop an automated computational analytical platform for CRC prognostication.

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Administrative support: GH, RG, PQ

Provision of study materials or patients: LM, MS, DK, DT, AW, RG, PQ

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Data analysis and interpretation: GH, KH, MS, DK, RG, PQ

Manuscript writing: GH, DT, AW, KH, LM, ETH, KS, MS, DK, RG, PQ

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Disclaimer

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Figure legends

Figure 1 - Study schema.

Figure 2 - Tumour regions for morphometric analysis. (A) Selected tumor block representing maximal lateral and deep tumor infiltration; (B) “Luminal” 9mm2 area; (C) “Highest” 9mm2 area of maximal tumor epithelial density (if away from luminal surface; (D) “Whole” tumor area encompassing lateral and deep invasive fronts. Scale bars=5mm

Figure 3 - Recurrence risk stratified by proportion of stroma (PoS) in the whole tumour (WT) region – validatory dataset, stage II / III patients (n=1,800).

Figure 4 - Recurrence risk stratified by proportion of stroma (PoS) in the whole tumour (WT) region – validatory dataset, stage II patients only (n=1,603)

Figure 5 - Recurrence risk by dichotomised proportion of stroma (PoS) stratified by clinicopathological variables in the whole tumour (WT) region. Validatory dataset (n=1,800); (O-E)=observed minus expected; Var=variance

Figure 6 - Recurrence risk by chemotherapy (2 years) sub-stratified by tumour morphometric subgroups. Validatory dataset (n=1,800); (O-E)=observed minus expected; Var=variance.

List of online supporting information

Figure S1 - Recurrence risk (2 years) by chemotherapy, stratified by sub-stratified by tumour morphometric subgroups

Figure S2 - Recurrence risk by proportion of stroma (PoS) in highest tumour (HT) area

Figure S3 - Recurrence risk by proportion of stroma (PoS) in the luminal tumour (LT) area

Figure S4 - Recurrence risk by proportion of stroma (PoS) in whole tumour (WT) area

Figure S5 – Recurrence risk by proportion of tumour (PoT) in the highest tumour (HT) area

Figure S6 – Recurrence risk by proportion of tumour (PoT) in the luminal tumour (LT) area

Figure S7 - Recurrence risk by proportion of tumour (PoT) in whole tumour (WT) area

Figure S8 - Recurrence risk by proportion of stroma (PoS) in highest tumour (HT) area

Figure S9 - Recurrence risk by proportion of stroma (PoS) in luminal tumour (LT) area

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Figure S13 - Recurrence risk by chemotherapy stratified by proportion of stroma (PoS) in the whole tumour (WT) region across clinicopathological sub-groups

Figure S14 - Recurrence risk by chemotherapy stratified by proportion of stroma (PoS) in the whole tumour (WT) region across clinicopathological sub-groups

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Figure S16 - Recurrence risk by chemotherapy stratified by proportion of stroma (PoS) in the whole tumour (WT) region across molecular sub-groups

Figure S17 – Recurrence risk by AJCC stage stratified by proportion of stroma (PoS) in the whole tumour (WT) region

Figure S18 – Recurrence risk by T-stage (TNM 5) stratified by proportion of stroma (PoS) in the whole tumour (WT) region

Figure S19 – Recurrence risk by lymph node yield stratified by proportion of stroma (PoS) in the whole tumour (WT) region

Figure S20 – Recurrence risk by extra-mural vascular invasion stratified by proportion of stroma (PoS) in the whole tumour (WT) region

Figure S21 – Recurrence risk by mismatch repair status stratified by proportion of stroma (PoS) in the whole tumour (WT) region

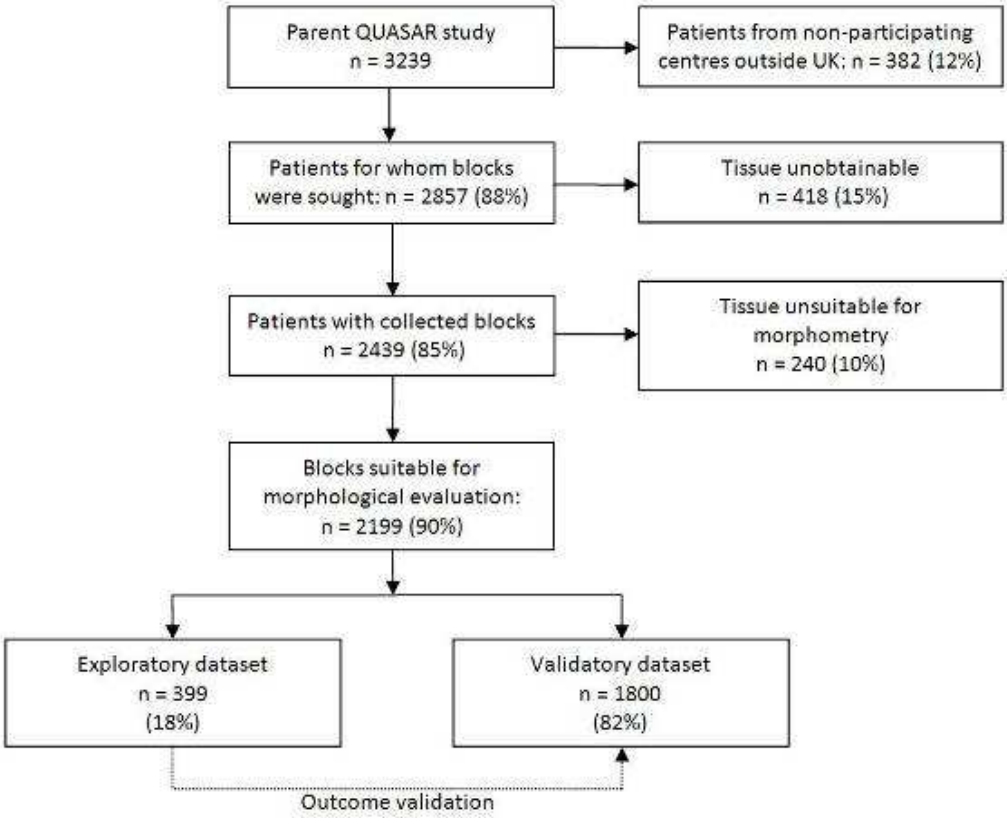
Figure S22 – Recurrence risk by KRAS mutation status stratified by whole tumour PoS

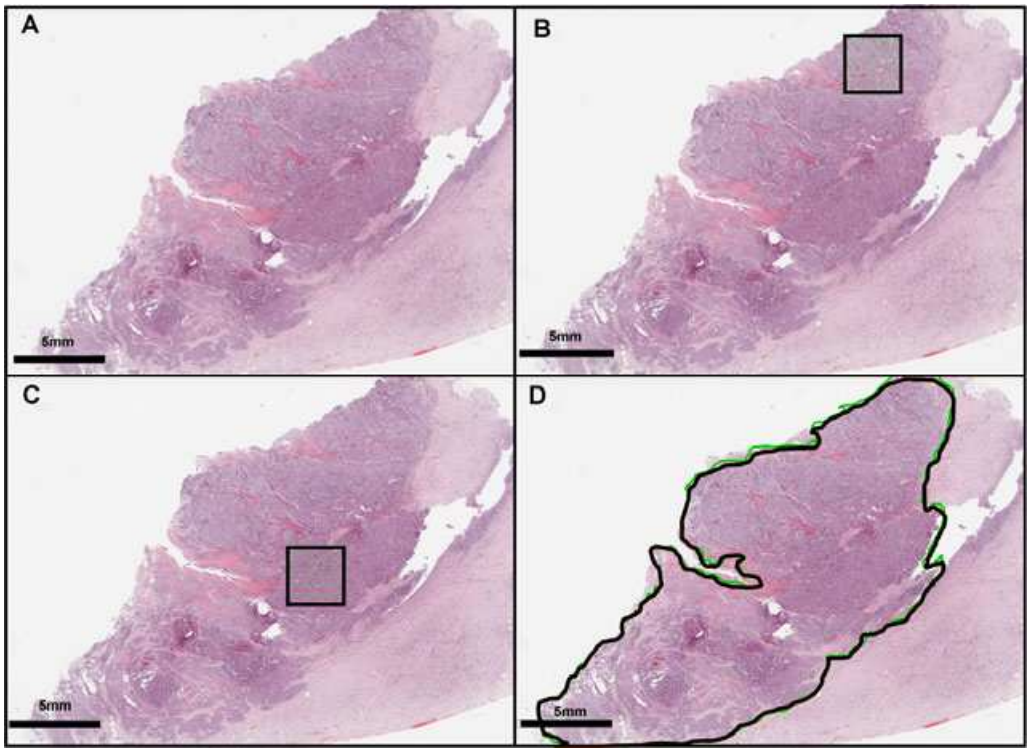
Figure S23 – Recurrence risk by BRAF mutation status stratified by whole tumour PoS

Table 1 – Patient characteristics by proportion of stroma (whole tumour area)

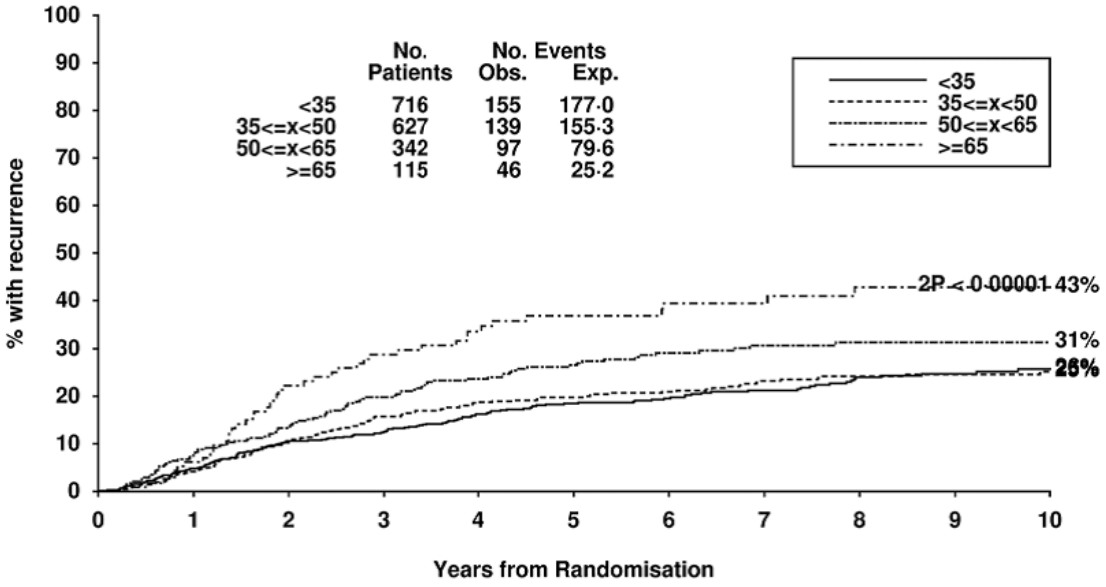
% PoS categories	<35% n, (%)	35%-49% n, (%)]	50%<65% [n, (%)]	≥65% [n, (%)]	p-value
All patients (n=1,800)	716 (39.78)	627 (34.83)	342 (19.0)	115 (6.39)	n/a
Colon (n=1,423)	597 (41.95)	497 (34.93)	257 (18.06)	72 (5.06)	**<0.0001**
Rectum (n=377)	119 (31.56)	130 (34.48)	85 (22.55)	43 (11.41)	
Dukes B / Stage II (n=1,603)	658 (41.05)	553 (34.50)	295 (18.40)	97 (6.05)	**0.0019**
Dukes C/Stage III (n=161)	43 (26.71)	62 (38.51)	40 (24.84)	16 (9.94)	
T3 (n=1,362)	545 (40.01)	481 (35.32)	259 (19.02)	77 (5.65)	0.865
T4 (n=217)	82 (37.79)	76 (35.02)	45 (20.74)	14 (6.45)	
EMVI (n=161)	51 (31.68)	58 (36.02)	41 (25.47)	11 (6.83)	0.078
No EMVI (n=1468)	594 (40.76)	517 (35.22)	271 (18.46)	86 (5.86)	
Well diff. (n=151)	70 (46.36)	51 (33.77)	26 (17.22)	4 (2.65)	0.159
Moderately diff. (n=1,343)	521 (38.79)	485 (36.11)	256 (19.06)	81 (6.03)	
Poor diff. (n=139)	57 (41.01)	40 (28.78)	30 (21.58)	12 (8.63)	

Mucinous (n=162)	100 (61.73)	47 (29.01)	12 (7.41)	3 (1.85)	**<0.0001**
AdenoCa, NOS (n=1,473)	548 (37.20)	530 (35.98)	300 (20.37)	95 (6.45)	
<12 lymph nodes (n=999)	398 (39.84)	346 (34.63)	193 (19.32)	62 (6.21)	0.699
12+ lymph nodes (n=544)	217 (39.89)	199 (36.58)	101 (18.57)	27 (4.96)	
Female (n=705)	289 (40.99)	248 (35.18)	130 (18.44)	38 (5.39)	0.481
Male (n=1,113)	427 (39.0)	379 (34.61)	212 (19.36)	77 (7.03)	
Chemotherapy (n=880)	353 (40.11)	290 (32.95)	178 (20.23)	59 (6.70)	0.330
Observation (n=920)	363 (39.46)	337 (36.63)	164 (17.83)	56 (6.09)	
MMR deficient (n=162)	85 (52.47)	48 (29.63)	24 (14.81)	5 (3.09)	**0.012**
MMR proficient (n=1,290)	508 (39.38)	468 (36.28)	241 (18.68)	73 (5.66)	
BRAF-mutant (n=92)	41 (44.57)	29 (31.52)	17 (18.48)	5 (5.42)	0.821
BRAF wild-type (n=1,091)	434 (39.78)	389 (35.66)	205 (18.79)	63 (5.77)	
KRAS mutant (n=401)	167 (41.65)	134 (33.42)	76 (18.95)	24 (5.99)	0.816
KRAS wild-type (n=780)	307 (39.36)	282 (36.15)	146 (18.72)	45 (5.77)	



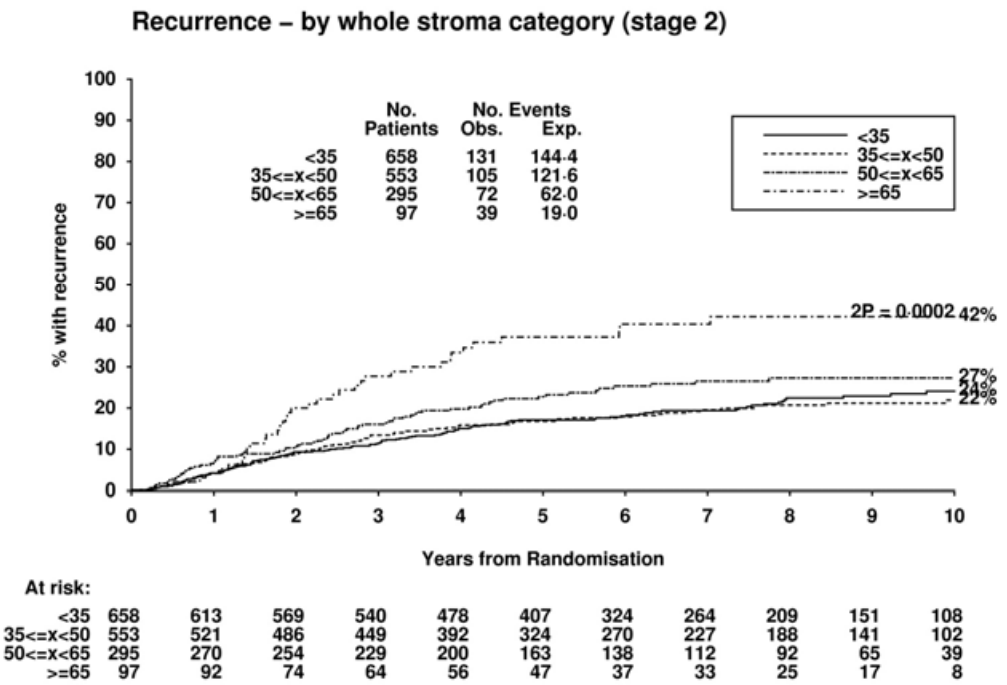


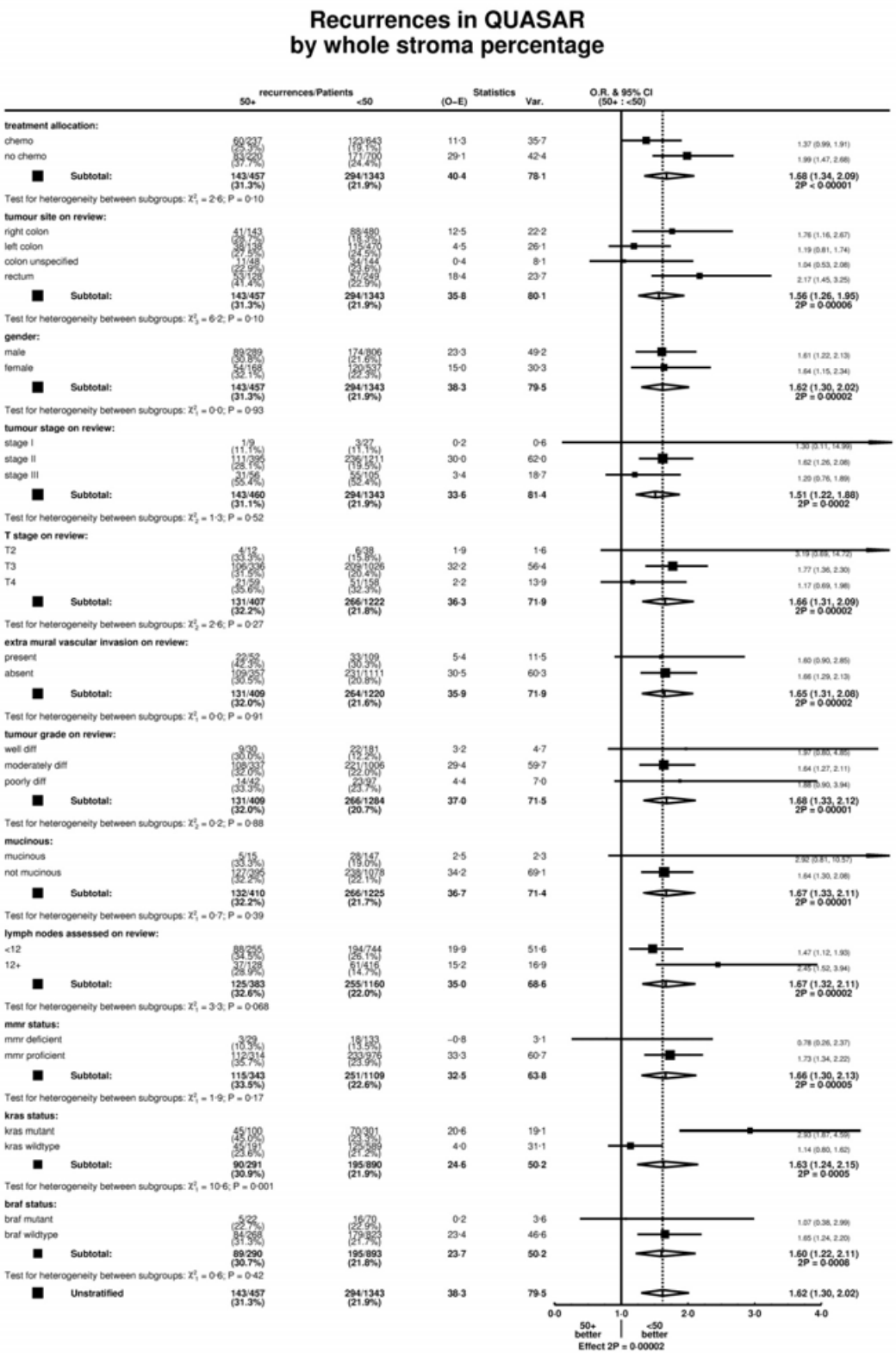
Recurrence – by whole stroma category



At risk:

<35	716	664	612	583	515	440	350	287	231	169	121
<=x<50	627	592	543	498	436	364	304	257	214	159	117
<=x<65	342	311	286	255	223	182	152	124	101	74	41
>=65	115	106	85	75	66	56	45	40	30	21	10





2 Year Recurrences in QUASAR by treatment