The differential expression of omega-3 and omega-6 fatty acid metabolising enzymes in colorectal cancer and its prognostic significance

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Running title: omega hydroxylases and colorectal cancer

Abstract

Background: Colorectal cancer is a common malignancy and one of the leading causes of cancer related deaths. The metabolism of omega fatty acids has been implicated in tumour growth and metastasis.

Methods: This study has characterised the expression of omega fatty acid metabolising enzymes CYP4A11, CYP4F11, CYP4V2 and CYP4Z1 using monoclonal antibodies we have developed. Immunohistochemistry was performed on a tissue microarray containing 650 primary colorectal cancers, 285 lymph node metastasis and 50 normal colonic mucosa.

Results: The differential expression of CYP4A11 and CYP4F11 showed a strong association with survival in both the whole patient cohort (HR=1.203, 95% CI=1.092-1.324, χ^2=14.968, p=0.001) and in mismatch repair proficient tumours (HR=1.276, 95% CI=1.095-1.488, χ^2=9.988, p=0.007). Multivariate analysis revealed that the differential expression of CYP4A11 and CYP4F11 was independently prognostic in both the whole patient cohort (p=0.019) and in mismatch repair proficient tumours (p=0.046).

Conclusions: A significant and independent association has been identified between overall survival and the differential expression of CYP4A11 and CYP4F11 in the whole patient cohort and in mismatch repair proficient tumours.

Keywords: biomarker, colorectal cancer, cytochrome P450, omega fatty acid, prognosis

Introduction

Colorectal cancer is one of the major contributors to cancer related mortality in the developed world (Siegel *et al*, 2014, Siegel *et al*, 2016). The introduction of screening programmes and the development of new drugs have improved the survival rate of colorectal cancer patients, however the average five-year survival rate remains poor at 55% (Brenner *et al*, 2014). The characterisation of novel biomarker targets can further improve the survival rate since it provides a better understanding of the complex molecular events underpinning tumour development, and if clinically validated these biomarkers have potential roles in screening, diagnosis, prognosis and monitoring disease progression (Alnabulsi and Murray, 2016, Coghlin and Murray, 2015).

The CYP4 cytochrome P450 family of enzymes metabolises omega-3 and omega-6 fatty acids to biologically active eicosanoids that are implicated in tumour initiation, development and progression (Johnson *et al*, 2015, Yu *et al*, 2011). Arachidonic acid, an omega-6 fatty acid, is converted by CYP4A11 to 20-hydroxyicosatetraenoic acid (20-HETE) which is considered a key modulator in tumours progression, angiogenesis and metastasis (Guo *et al*, 2007, Ljubimov and Grant, 2005). CYP4F11 is not an efficient metaboliser of arachidonic acid compared to CYP4A11, however it is the predominant CYP4 enzyme involved in the metabolism of omega 3-fatty acids (Dhar *et al*, 2008). The substrate specificity is not yet fully characterised for CYP4V2 and CYP4Z1 (Guengerich and Cheng, 2011). Despite the recognition of the involvement of omega fatty acids in tumourigenesis, the role of the cytochrome P450 enzymes involved in this pathway has received very limited attention in cancer biology (Panigrahy *et al*, 2010).

Using monoclonal antibodies we have developed to the cytochrome P450 enzymes CYP4A11, CYP4F11, CYP4V2 and CYP4Z1, this study has profiled the expression of these enzymes by immunohistochemistry on a tissue microarray containing a large and well-characterised cohort of colorectal cancers. The expression profile of each enzyme was established by light microscopy using a semi-quantitative scoring system. The prognostic significance of each enzyme was determined by assessing the relationship between their expression in tumours and overall survival.

Materials and methods

Monoclonal antibody development

Monoclonal antibodies to CYP4A11, CYP4V2 and CYP4Z1 were developed using short synthetic peptides (Murray *et al*, 1998). Multiple sequence alignments of the amino acid sequences were performed on these enzymes and other CYP4 family members to identify regions with the highest amino acid diversity. To avoid poorly antigenic sequences of amino acids (e.g. transmembrane region), a range of bioinformatics tools were used to predict and model hydrophilic, accessible and antigenic polypeptide sequences as well as the secondary and tertiary structures of each enzyme (Supplementary Materials and Methods S1).

The amino acid sequences of peptides used to generate the antibodies and their location on each enzyme are specified in Supplementary Table S1. All peptides (Almac Sciences Ltd, Edinburgh, UK) were conjugated to ovalbumin for immunisations and to bovine serum albumin for the enzyme-linked immunosorbent assay (ELISA) screenings (Duncan *et al*, 1992). The immunisation *via* the subcutaneous route, the production of hybridomas and the ELISA screenings were carried out as previously described (Brown *et al*, 2014, Murray *et al*, 1996, Murray *et al*, 1998). The development of the monoclonal antibody to CYP4F11 has been described previously (Kumarakulasingham *et al*, 2005).

Immunoblotting

The specificity of the monoclonal antibodies was established by immunoblotting using whole cell lysate (human embryonic kidney cells-HEK 293, Novus Biologicals, Cambridge, UK) overexpressing the relevant CYP as a positive control and lysates from cells containing empty vector as a negative control. Microsomal fractions prepared from human liver tissues (BD Gentest Human Liver Microsomes (HLM) Pooled Male Donors 20 mg/mL cat no. 452172, BD Biosciences, Bedford, USA) were also used to carry out immunoblotting

validation for each antibody. The immunoblotting was carried out as described, except that the polyvinylidene difluoride membrane was incubated overnight at 4°C with undiluted monoclonal antibody (neat hybridoma tissue culture supernatant), and the secondary antibody, horseradish-peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich, Dorset, UK), was diluted 1/3000 in phosphate buffered saline-Tween-20 (Swan *et al*, 2016). When using liver microsomes, 30 μg of samples were loaded per lane compared to 5 μg when using overexpression lysate.

Patient cohort and colorectal cancer tissue microarray

The patient cohort was retrospectively acquired from the Grampian Biorepository (www.biorepository.nhsgrampian.org). The cohort is composed of tissue samples from 650 patients who had undergone surgery for primary colorectal cancers between 1994 and 2009, at Aberdeen Royal Infirmary (Aberdeen, UK) which is the principal teaching hospital of NHS Grampian. Patients who had received neoadjuvant chemotherapy and/or radiotherapy were excluded.

Survival time was defined to be the period in whole months from the date of surgery to the date of death from any cause (i.e. all-cause mortality). Survival data on a 6-monthly basis was updated from the NHS Grampian electronic patient management system and no patients were lost to follow-up. At the time (March 2012) of the censoring of patient outcome data there had been 309 (47.5%) deaths and patients who were still alive were censored. The median survival was 103 months (95% CI=86–120 months), the mean survival was 115 months (95% CI=108–123 months) and the median follow-up time, calculated by the "reverse Kaplan-Meier" method, was 88 months (95% CI=79–97 months). The clinico-pathological characteristics of the patients and their association with survival are described in Table 1.

Histopathology reporting was in accordance with The Royal College of Pathologists UK guidelines for the histopathological reporting of resection specimens of colorectal cancer which includes guidance from version 5 of the tumour, node, metastasis (TNM) staging system (Williams *et al*, 2007).

Blocks of formalin fixed, paraffin embedded tissue specimens were used to construct the tissue microarray as previously described (Brown *et al*, 2014, O'Dwyer *et al*, 2011, Swan *et al*, 2016). The histopathological processing of tissue specimens and the construction of the tissue microarray are described in Supplementary Materials and Methods S1

Immunohistochemistry

A Dako autostainer (Dako, UK) was used to perform the immunohistochemistry for each antibody using the Dako EnVision™ system (Dako, Ely, UK) (Brown *et al*, 2014, Kumarakulasingham *et al*, 2005). Antigen retrieval (microwaving in 10mM citrate buffer pH 6.0 for 20 minutes) was performed for all antibodies except CYP4A11. The immunohistochemistry procedure and the antigen retrieval are described in Supplementary Materials and Methods S1. A semi-quantitative scoring system was used to evaluate the intensity of immunostaining of each antibody (Brown *et al*, 2016, Kumarakulasingham *et al*, 2005, O'Dwyer *et al*, 2011, Swan *et al*, 2016). The scoring was conducted independently by two observers (RS and GIM) who were unaware of the clinical data and outcome. The assessment of cores was performed using light microscopy (Olympus BX 51, Olympus, Southend-on- Sea, Essex, UK). Simultaneous re-evaluation of the cores by both investigators was used to resolve any discrepancies in the scores (< 5% of cases).

Assessment of mismatch repair protein (MMR) status

The status of MMR in the patient cohort was classified as either defective or proficient based on the immunohistochemical assessment of MLH1 and MSH2 proteins (Brown *et al*, 2014).

Data analysis and statistics

The data was entered into an Excel 2013 spreadsheet before being analysed using IBM SPSS version 24 for Windows 7™ (IBM, Portsmouth, UK). The following statistical tests were used; Mann-Whitney U test, Wilcoxon signed rank test, chi-squared test, Kaplan-Meier survival analysis, log-rank test and Cox multivariate analysis (variables entered as categorical variables) including the calculation of hazard ratios and 95% CIs. A probability value of $p \leq 0.05$ was regarded as statistically significant. The survival analysis of the different patients groups was conducted using the log rank test. The scores for each protein were dichotomised using the following cut-off points; negative staining versus positive staining, negative and weak staining versus moderate and strong staining and strong staining versus negative/weak/ moderate staining. Further details of data analysis and statistics are provided in Supplementary Materials and Methods S1.

Ethics

The use of colorectal tissue samples in this study was approved by the Grampian Biorepository scientific access group committee (Tissue request No. 0002). No written consent was required from patients for the use of formalin fixed wax embedded tissue samples in the colorectal cancer tissue microarray.

Results

Monoclonal antibodies

During the hybridoma production, sequential ELISA screenings (immunogenic peptide specific to each enzyme) were used to determine the specificity of the monoclonal antibodies towards CYP4A11, CYP4V2 and CYP4Z1 (Duncan *et al*, 1992). Furthermore, immunoblotting showed a band migrating at the expected molecular weight for each antibody while no band was detected in the negative controls (Supplementary Figure S1). The specificity of the antibody to CYP4F11 was confirmed previously (Kumarakulasingham *et al*, 2005).

Immunohistochemistry

CYP4A11, CYP4F11 and CYP4V2 antibodies showed immunoreactivity in normal colonic epithelium, primary colorectal tumours and lymph node metastasis, while CYP4Z1 showed immunoreactivity only in a very small proportion of primary tumours. The immunostaining was exclusively localised to the cytoplasm of the cells (Supplementary Figure S2). Intra-tumour heterogeneity was not observed in either primary or metastatic colorectal tumours.

There was a significant increase in the intensity of immunostaining in primary tumours compared to normal colonic mucosa for CYP4A11 ($p<0.001$), CYP4F11 ($p<0.001$) and CYP4V2 ($p<0.001$) (Table 2; Supplementary Figure S3). In contrast, a significant decrease in the expression of CYP4A11 ($p=0.007$), CYP4F11 ($p<0.001$) and CYP4V2 ($p<0.001$) was observed in lymph node metastasis compared with all primary tumours. There was also a significant decrease in the expression of CYP4A11 ($p=0.002$), CYP4F11 ($p<0.001$) and CYP4V2 ($p<0.001$) in lymph node metastasis compared to their corresponding primary Dukes C tumours.

Relationship with pathological parameters

The relationships between the main pathological parameters and the expression of CYP4A11, CYP4F11, CYP4V2 and CYP4Z1 are summarised in Supplementary Tables S2A, B and C. Both CYP4A11 (χ^2=13.148, p=0.041) and CYP4V2 (χ^2=24.474, p<0.001) showed significant associations with Dukes stage, but only CYP4V2 displayed a significant relationship with tumour stage (χ^2=17.837, p=0.037). The expression of CYP4A11 was significantly associated with tumour site (χ^2=15.703, p=0.015). CYP4F11 also showed significant associations with tumour site (χ^2=20.947, p=0.002), tumour differentiation (χ^2=8.5552, p=0.036) and MMR status (χ^2=13.441, p=0.004).

Survival analysis

Whole patient cohort

Different cut-off points of the immunostaining scores were used to investigate the association between the expression of CYP4A11, CYP4F11 and CYP4V2 and overall survival (Supplementary Table S3). The expression of CYP4A11 showed a consistent and significant association with overall survival (Figure 1). Overall, increasing intensity of CYP4A11 immunostaining was significantly associated with poorer outcome (HR=1.135, 95% CI=1.032-1.249, χ^2=9.080, p=0.028). When each level of the intensity groups of CYP4A11 expression was considered independently using one reference group (negative expression), strong CYP4A11 immunostaining was significantly associated with poorer outcome (HR=1.541, 95% CI=1.144-2.077, χ^2=8.006, p=0.005) (Supplementary Table S4).

The median survival was 137 months (95% CI undefined) and the mean was 132 months (95% CI=117-147 months) for CYP4A11 negative tumours (n=175), whereas the

median survival was 75 months (95% CI=58-91 months) and the mean was 96 months (95% CI=84-109 months) for CYP4A11 strong expression tumours (n=197).

Immunoreactivity for CYP4A11 was significantly associated with poorer prognosis (HR=1.346, 95% CI=1.032-1.756, χ^2=4.881, p=0.027) when compared with CYP4A11 negative tumours. For CYP4A11 positive tumours (n=450) the median survival was 88 months (95% CI=71-104 months) and the mean was 105 months (95% CI=96-114 months), compared to a median of 137 (95% CI undefined) and a mean of 132 months (95% CI=117-147 months) for CYP4A11 negative tumours (n=175). Comparing strong CYP4A11 expressing tumours with negative/weak/moderate expressing tumours also showed a significant association with survival (HR=1.379, 95% CI=1.089–1.746, χ^2=7.234, p=0.007). The median survival was 113 months (95% CI=89-136 months) and the mean was 124 months (95% CI=114-134 months) for negative/weak/moderate CYP4A11 immunostaining tumours (n=428), whereas the median survival was 75 months (95% CI=58-91 months) and the mean was 96 months (95% CI=84-109 months) for strong CYP4A11 immunostaining tumours (n=197).

Exploratory analysis of CYP4 enzyme expression showed there was a significant association between the differential (combined) expression of CYP4A11 and CYP4F11 and survival (Supplementary table S5). Therefore, a new variable, based on the differential expression of CYP4A11 and CYP4F11, was created to stratify tumours into three groups; CYP4A11 greater than CYP4F11 (CYP4A11>CYP4F11), CYP4A11 equal to CYP4F11 (CYP4A11=CYP4F11) and CYP4A11 less than CYP4F11 (CYP4A11<CYP4F11). Overall survival was significantly associated with the expression profiles of CYP4A11>CYP4F11, CYP4A11=CYP4F11 and CYP4A11<CYP4F11 groups (HR=1.311, 95% CI=1.140-1.506, χ^2=14.968, p=0.001) (Figure 2). When each level of the differential expression groups was considered independently using pairwise comparisons and one reference group

(CYP4A11<CYP4F11), both CYP4A11>CYP4F11 (HR=1.733, 95% CI=1.306-2.300, χ^2=14.405, p=<0.001) and CYP4A11=CYP4F11 (HR=1.432, 95% CI=1.064-1.928, χ^2=5.425, p=0.020) were significantly associated with poorer outcome (Supplementary Table S6). The mean survival was 137 months (95% CI= 124-151 months) (median survival undefined) for the CYP4A11<CYP4F11 group (n=214), the median survival was 95 months (95% CI=72-117 months) and the mean was 102 months (95% CI=90-114 months) for the CYP4A11=CYP4F11 group (n=185), while the median survival was 75 months (95% CI=60-89 months) and the mean survival was 94 months (95% CI=81-106 months) for CYP4A11>CYP4F11 group (n=217).

The associations between the expression of CYP4A11, CYP4F11, CYP4V2 and CYP4Z1 and overall survival in relation to different tumour sites, Dukes stage and extramural venous invasion (EMVI) are shown in Supplementary Tables S7, S8, S9 and S10.

MMR proficient cohort

There was a significant association between the expression of CYP4A11 and overall survival in MMR proficient tumours (HR=1.156, 95% CI=1.040-1.286, χ^2=11.221, p=0.011) (Figure 3; Supplementary Table S11). When each level of the intensity groups of CYP4A11 expression was considered separately using pairwise comparisons and one reference group (negative expression), strong CYP4A11 immunoreactivity was significantly associated with poorer prognosis (HR=1.644, 95% CI=1.183-2.284, χ^2=8.626, p=0.003) (Supplementary Table S4). When comparing strong CYP4A11 expressing tumours with negative/weak/moderate expressing tumours the strong expression of CYP4A11 showed a significant association with worse survival (HR=1.491, 95% CI=1.152-1.929, χ^2=9.404, p=0.002). The positive expression of CYP4A11 was also significantly associated with poorer

outcome when positive CYP4A11 expressing tumours were compared with negative CYP4A11 expressing tumours (HR=1.375, 95% CI=1.022-1.851, χ^2=4.485, p=0.034).

There was also a significant association between the differential expression of CYP4A11 and CYP4F11 and survival in MMR proficient tumours (HR=1.276, 95% CI=1.05-1.488, χ^2=9.988, p=0.007) (Figure 2). When each level of the intensity groups was considered independently using pairwise comparisons and one reference group (CYP4A11<CYP4F11), CYP4A11>CYP4F11 expressing tumours were significantly associated with poorer outcome (HR=1.629, 95% CI=1.199-2.214, χ^2=9.261, p=0.002) (Supplementary Table S6). The median survival was 75 months (95% CI=85-121 months) and the mean was 97 months (95% CI=83-111 months) for CYP4A11>CYP4F11 expressing tumours (n=181). While, the mean survival was 137 months (95% CI=123-151 months) (median survival undefined) for CYP4A11<CYP4F11 expressing tumours (n=186).

MMR deficient cohort

The lack of expression of CYP4F11 was significantly associated with worse overall survival compared with CYP4F11 positive tumours (HR=0.479, 95% CI=0.241-0.952, χ^2=4.682, p=0.03) (Supplementary Table S11; Supplementary Figure S4). The median survival was 28 (95% CI=21-34 months) and the mean was 49 months (95% CI=28–70 months) for CYP4F11 negative tumours (n=16) compared with a median of 114 (95% CI=78-149 months) and a mean of 104 months (95% CI= 84-123 months) for CYP4F11 positive tumours (n=77).

Overall, the association between survival and the differential expression of CYP4A11 and CYP4F11 just failed to reach the threshold for statistical significance in MMR deficient cohort (HR=1.433, 95% CI=0.993-2.067, χ^2=5.676, p=0.059) (Figure 2). When each level of the intensity groups was considered independently using pairwise comparisons and one

reference group (CYP4A11<CYP4F11), both CYP4A11>CYP4F11 expressing tumours (HR=1.733, 95% CI=1.306-2.300, χ^2=14.405, p=<0.001) and CYP4A11=CYP4F11 expressing tumours (HR=1.432, 95% CI=1.064-1.928, χ^2=5.425, p=0.020) were significantly associated with poorer outcome (Supplementary Table S6).

Multivariate analysis

To evaluate the prognostic value of the differential expression of CYP4A11 and CYP4F11 (as a single variable) in relation to established prognostic parameters multivariate analysis was performed using "Forward Stepwise: Conditional LR" Cox regression method. The model showed there was a significant and independent prognostic value of using the differential expression of CYP4A11 and CYP4F11 in the whole patient cohort (p=0.019) and in MMR proficient tumours (p=0.046) (Table 3; Supplementary Tables S12 and S13). The differential expression was also a significant independent prognostic indicator in a multivariate analysis using only parameters that would be available at the time of biopsy in both the whole patient cohort (p=0.001) and in MMR proficient tumours (p=0.006) (Supplementary Table S14).

Discussion

The rise in incidence and the poor survival rate makes colorectal cancer a major health burden in the developed world (Brenner *et al*, 2014, Siegel *et al*, 2014, Siegel *et al*, 2016). There is still urgent need to identify and validate biomarkers of colorectal cancer that can play a role in clinical practice (Alnabulsi and Murray, 2016).

In this study, we have produced monoclonal antibodies to P450 enzymes CYP4A11, CYP4V2 and CYP4Z1 using short synthetic peptides that are specific to the targets of interest. The antibody for CYP4F11 was generated in a previous study (Kumarakulasingham *et al*, 2005). The antibodies were used to profile the expression of each enzyme by immunohistochemistry which was performed on a well-characterised colorectal cancer tissue microarray.

The cytochrome P450 superfamily is classified into families, subfamilies and individual forms according to sequence homology and substrate specificity (Almira Correia *et al*, 2011, Fleming, 2011, Spector, 2009). Members of CYP1, CYP2 and CYP3 families are the major xenobiotic metabolising enzymes whose roles in cancer have been extensively studied (Murray *et al*, 1991, Murray *et al*, 1993, Murray *et al*, 1999, Murray *et al*, 2001, Murray *et al*, 2010, Rodriguez-Antona *et al*, 2010, Stenstedt *et al*, 2012, Xu *et al*, 2012). The CYP4 and higher numbered families are involved in the metabolism of a diverse range of endogenous compounds including eicosanoids, fatty acids, steroids and vitamins (Arnold *et al*, 2010, Fleming, 2011, Guengerich and Cheng, 2011, Niwa *et al*, 2011, Panigrahy *et al*, 2010, Spector, 2009). The role of CYP4 family and higher numbered families is not well studied in tumour biology with the exception of those CYPs involved in sex hormone metabolism in relation to breast and prostate cancer (Brueggemeier *et al*, 2005, Leroux, 2005, Stein *et al*, 2012). Therefore, this study aimed to examine the role of the main CYP4 family

enzymes in colorectal cancer by characterising the expression of these enzymes using a large and well-characterised patient cohort.

This study revealed there was a significant increase in the expression of CYP4A11 in primary colorectal tumours compared to normal colonic mucosa and the increased expression was significantly associated with poorer prognosis. Consistent with our finding, an upregulation of CYP4A11 was demonstrated by a cDNA microarray-bioinformatics analysis of 10 colorectal tumours and their corresponding normal tissues (Yeh *et al*, 2006). Furthermore, the overexpression of CYP4A11 has been linked to rise in 20-HETE levels and upregulation of vascular endothelial growth factor (VEGF) and matrix metalloproteinases-9 (MMP-9) in non-small cell lung cancer (Yu *et al*, 2011). Both VEGF and MMP-9 are strong promoters of tumour invasion and metastasis (Brown and Murray, 2015, Goel and Mercurio, 2013, Yu *et al*, 2011). Previous research also showed that using selective inhibitors to downregulate the expression of CYP4A11 in cell lines and animal models resulted in a decrease in tumour growth, angiogenesis and metastasis of non-small cell lung cancer, renal adenocarcinoma and glioma (Alexanian *et al*, 2009, Guo *et al*, 2008, Yu *et al*, 2011). Our data has shown CYP4A11 is overexpressed in colorectal cancer, therefore CYP4A11 may be a relevant therapeutic target in this type of cancer.

Comparing primary colorectal tumours to normal colonic mucosa also showed there was a significant increase in the expression of CYP4F11 which is a novel finding. In recent research, CYP4F11 expressed in cell lines (non-small cell lung cancer) converted oxalamides and benzothiazoles into stearoyl CoA desaturase (SCD) inhibitors (Theodoropoulos *et al*, 2016). SCD is emerging as a therapeutic target in cancer and therefore, colorectal tumours with high CYP4F11 expression may be a valid target for SCD targeted therapy.

The differential expression of CYP4A11 and CYP4F11 emerged as the best prognostic marker in this study. The distinct prognostic impact of the differential expression

of CYP4A11 and CYP4F11 may be explained by differences in the enzymes substrates (Supplementary Figure S5). CYP4A11 converts arachidonic acid to metabolites that promote tumour growth and metastasis, while CYP4F11 metabolises omega 3-fatty acids to eicosanoids that inhibit tumour development and progression (Barone *et al*, 2014, Dhar *et al*, 2008, Gelsomino *et al*, 2013, Kalsotra and Strobel, 2006, Larsson *et al*, 2004). The differential expression of CYP4A11 and CYP4F11 was independently prognostic in multivariate analysis using the main prognostic parameters and also when only using information available at the time of biopsy diagnosis of colorectal cancer. Therefore, this biomarker combination could be a useful risk stratification tool especially if only tumour biopsies are available at the time of initial treatment decisions, which is a likely scenario considering colorectal cancer, especially rectal cancer, is moving towards neoadjuvant therapy followed by either observational follow-up or salvage surgery (Garcia-Aguilar *et al*, 2015).

The expression of each enzyme based on MMR status was also evaluated in this study since this represents a major pathway in colorectal cancer (Boland and Goel, 2010, Geiersbach and Samowitz, 2011, Kim and Kim, 2014). Tumours lacking MMR proteins are already considered a distinct subgroup when dealing with prognosis and treatment of colorectal cancer (Hewish *et al*, 2010). MMR proficient tumours represent the majority of colorectal cancer cases with a significantly worse prognosis than MMR deficient tumours. Furthermore, novel promising treatments such as those targeting immune checkpoints have shown that MMR proficient tumours are less responsive compared to MMR deficient tumours (Le *et al*, 2015). Therefore, it is of particular interest to identify biomarkers for MMR proficient tumours. In this study, the differential expression of CYP4A11 and CYP4F11 was significantly associated with prognosis in MMR proficient tumours, and more importantly both enzymes are actionable targets.

This study also found the expression of CYP4A11, CYP4F11 and CYP4V2 were significantly reduced in lymph node metastasis compared with their corresponding primary tumours. This provides further evidence to the concept that the phenotype of cancer cells is defined by their exposure to/ and interaction with different microenvironment factors during their migration and within the metastatic site (Brown and Murray, 2015, Klein *et al*, 2012, Maman and Witz, 2013, Witz, 2008). The interrelationship between cancer cells and non-cancer cells within the microenvironment is increasingly acknowledged as a major factor in determining and understanding metastasis (Coghlin and Murray, 2010, Coghlin and Murray, 2014, McKay *et al*, 2000). The variation in the phenotypic expression between primary and metastatic tumours raises further doubts over the effectiveness of existing metastatic treatment models that is based on phenotypic assessment of primary tumour specimens.

In summary, CYP4A11, CYP4F11 and CYP4V2 are overexpressed in colorectal cancer, the increased expression of CYP4A11 is associated with poorer prognosis in both the total patient cohort and in MMR proficient tumours, while the expression of CYP4F11 is associated with better outcome in MMR deficient tumours. The differential expression of CYP4A11 and CYP4F11, which was independently prognostic in both the whole patient cohort and in MMR proficient tumours, could provide the basis for a risk stratification tool in colorectal cancer. Furthermore, both enzymes are actionable drug targets and therefore could have therapeutic applications in colorectal cancer.

Conflict of interest

Abdo Alnabulsi is a PhD student supported by Vertebrate Antibodies, Beatriz Cash and Ayham Alnabulsi are employees of Vertebrate Antibodies and Graeme Murray is a scientific advisor to Vertebrate Antibodies. Rebecca Swan has no conflict of interest to declare.

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Figure legends

Figure 1.
The overall relationship between the expression of CYP4A11 and survival in the whole patient cohort using different cut-off points: negative *versus* weak *versus* moderate *versus* strong (A, further details of median survival times of individual groups, p-values and hazard ratios are found in Table S4), strong *versus* negative/weak/moderate (B), positive expression *versus* negative expression (C) and negative and weak *versus* moderate and strong (D).

Figure 2.
The overall relationship between the differential expression of CYP4A11 and CYP4F11 and survival in the whole patient cohort (A), in MMR proficient tumours (B) and in MMR deficient tumours (C). Further details of median survival times of individual groups, p-values and hazard ratios are found in Table S6

Figure 3.
The relationship between the expression of CYP4A11 and survival in MMR proficient tumours using different cut-off points: negative *versus* weak *versus* moderate *versus* strong (A, further details of median survival times of individual groups, p-values and hazard ratios are found in Table S4), strong *versus* negative/weak/moderate (B) and positive expression *versus* negative expression (C).

Table 1. Clinico-pathological characteristics of all patients, their tumours and the relationship of each variable with overall survival.

Characteristic	Number	Percentage	Relationship with survival
Sex			
Male	340	52.3	$\chi2$= 0.027, p=0.870
Female	310	47.7	
Age			
<70	305	46.9	$\chi2$=29.213, **p<0.001**
≥70	345	53.1	
Screen detected			
Yes	52	8	$\chi2$=16.381, **p<0.001**
No	598	92	
Tumour site			
Proximal colon	261	40.2	Proximal v distal, $\chi2$= 8.418, **p=0.004**
Distal colon	245	37.7	Distal v rectal, $\chi2$= 0.906, p=0.341
Rectum	144	22.2	Colon v rectum, $\chi2$=0.098, p=0.754
Tumour differentiation			
Well/moderate	600	92.3	$\chi2$=0.976, p=0.323
Poor	50	7.7	
Extra-mural venous invasion			
Present	140	21.5	$\chi2$=100.946, **p<0.001**
Absent	510	78.5	
Microsatellite instability status			
Defective	96	15.2	$\chi2$=2.848, p=0.091
Intact	536	84.8	
pT stage			
T1	30	4.6	T1 v T2, $\chi2$=0.382, p=0.536
T2	114	17.5	T2 v T3, $\chi2$=24.739, **p<0.001**
T3	411	63.2	T3 v T4, $\chi2$=30.159, **p<0.001**
T4	95	14.6	
pN stage			
N0	364	56	N0 v N1, $\chi2$=54.071, **p<0.001**
N1	177	27.2	N1 v N2, $\chi2$=17.636, **p<0.001**
N2	109	16.8	
Dukes stage			
A	120	18.5	A v B, $\chi2$=5.059, **p=0.025**
B	244	37.5	B v C, $\chi2$=65.510, **p<0.001**
C	286	44	

Significant values are highlighted in bold.

Table 2. Comparison of the expression of CYP4's in normal colonic mucosa, primary colorectal cancer and lymph node metastasis.

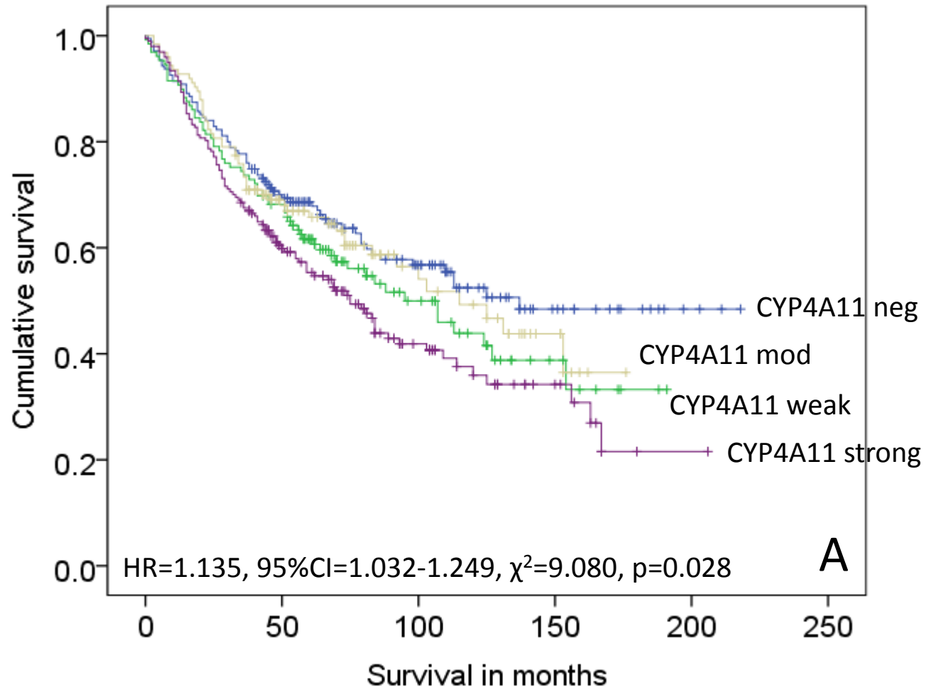
	Immunoreactivity (p value, normal versus primary tumour)	Change in expression in tumour	Immunoreactivity (p value, primary tumour versus lymph node metastasis)	Change in expression in lymph node	Immunoreactivity (p value, paired primary Dukes C tumour versus lymph node metastasis)	Change in expression in lymph node
CYP4A11	**p<0.001**	↑	**p=0.007**	↓	**p=0.002**	↓
CYP4F11	**p<0.001**	↑	**p<0.001**	↓	**p<0.001**	↓
CYP4V2	**p<0.001**	↑	**p<0.001**	↓	**p<0.001**	↓
CYP4Z1	p=0.303	-	**p=0.028**	↓	p=0.083	-

Evaluation of normal colonic epithelium versus primary tumour samples for immunoreactivity (Mann-Whitney U test, ↑=increased in tumour, ↓=decreased in tumour, =no change between tumour and normal) and evaluation of primary Dukes C colorectal tumour samples and their corresponding metastasis samples for immunoreactivity (Wilcoxon signed rank sum test, ↑=increased in lymph node metastasis, ↓=decreased in lymph node metastasis, -=no change between primary and metastatic tumour). Significant values are highlighted in bold.

Table 3. The final multivariate model showing the significance of the differential expression of CYP4A11 and CYP4F11 in multivariate analysis for the whole patient cohort and MMR proficient tumours.

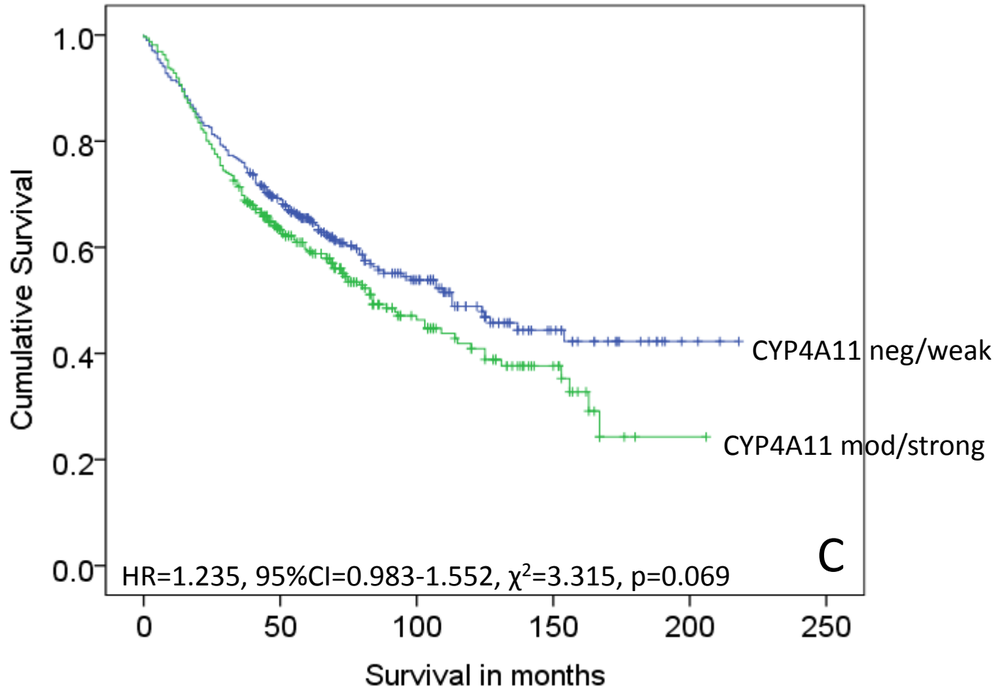
	Whole patient cohort			MMR proficient tumours		
	Wald value	p-value	Hazard ratio (95% CI)	Wald value	p-value	Hazard ratio (95% CI)
Age (< 70 v ≥ 70)	31.115	**<0.001**	1.982 (1.554-2.529)	25.568	**<0.001**	1.993 (1.526-2.604)
EMVI (present v absent)	38.825	**<0.001**	2.278 (1.758-2.951)	29.637	**<0.001**	2.245 (1.678-3.004)
Dukes stage (Av B v C)	53.435	**<0.001**	2.826 (0.762- 4.191)	35.144	**<0.001**	2.622 (0.785-3.961)
Differential expression of CYP4A11 and CYP4F11 (CYP4A11>CYP4F11 v CYP4A11=CYP4F11 v CYP4A11<CYP4F11)	5.515	**0.019**	1.186 (1.029-1.368)	3.983	**0.046**	1.173 (1.003-1.371)

Significant values are highlighted in bold. Details of the intermediate steps and omnibus tests of model coefficients are shown in Supplementary Tables S12 and S13.



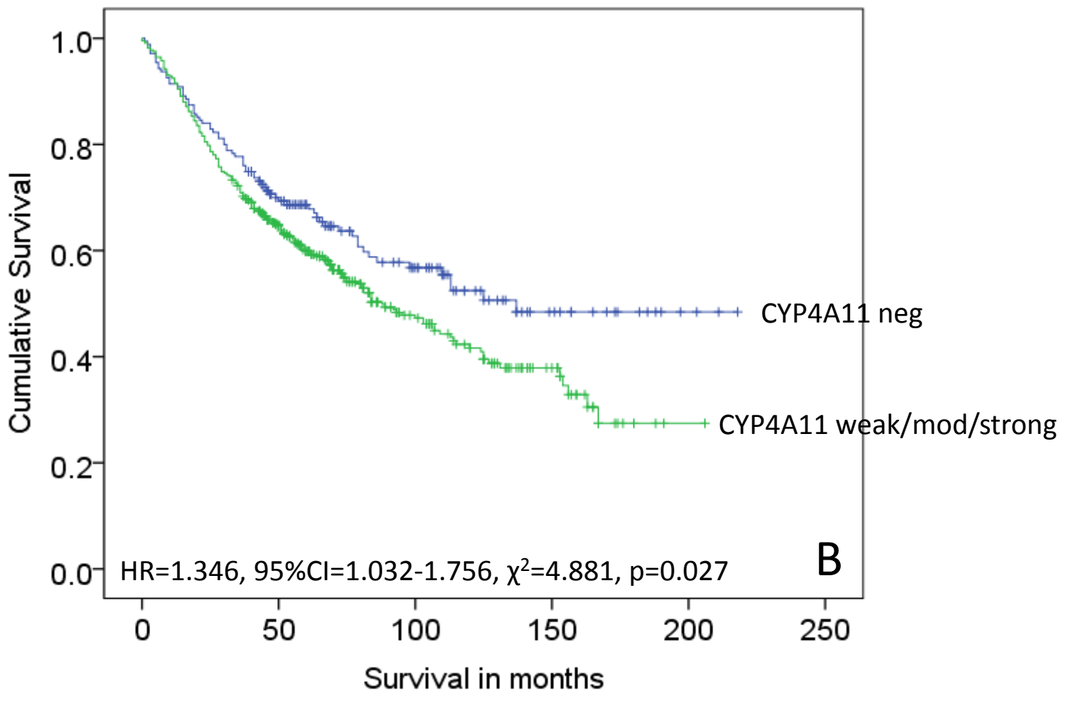
Number at risk

CYP4A11 neg	175	105	75	25	0	0
CYP4A11 weak	129	79	28	7	0	0
CYP4A11 mod	124	65	23	7	0	0
CYP4A11 strong	197	97	35	11	0	0



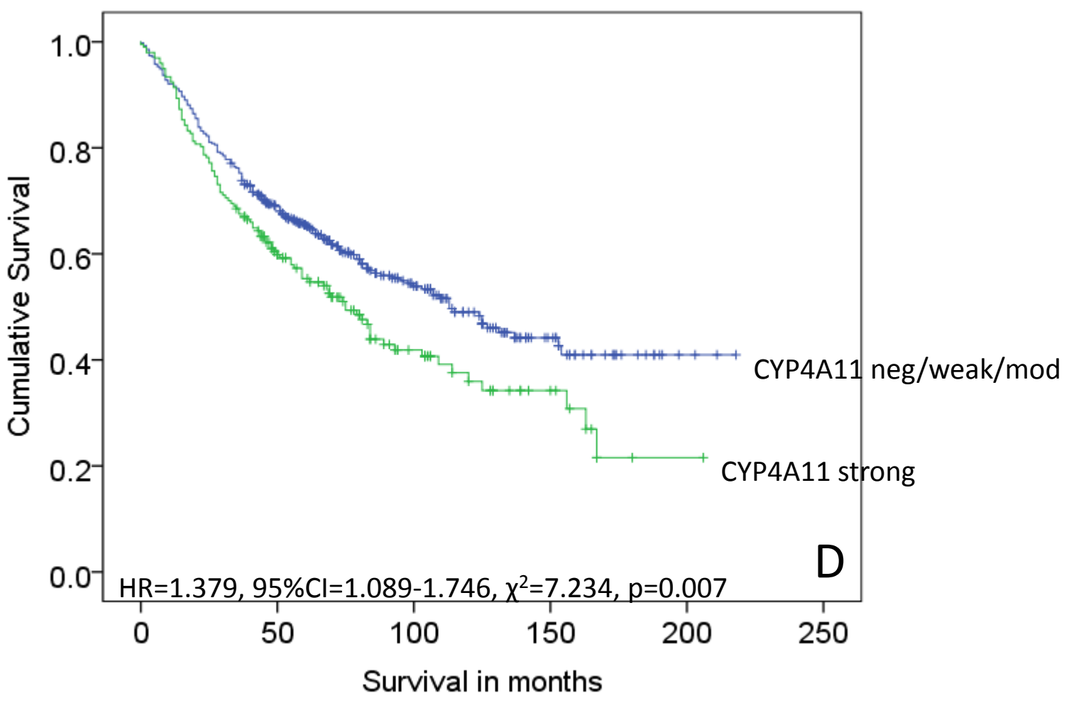
Number at risk

CYP4A11 neg/weak	304	191	80	23	2	0
CYP4A11 mod/strong	321	163	59	19	0	0



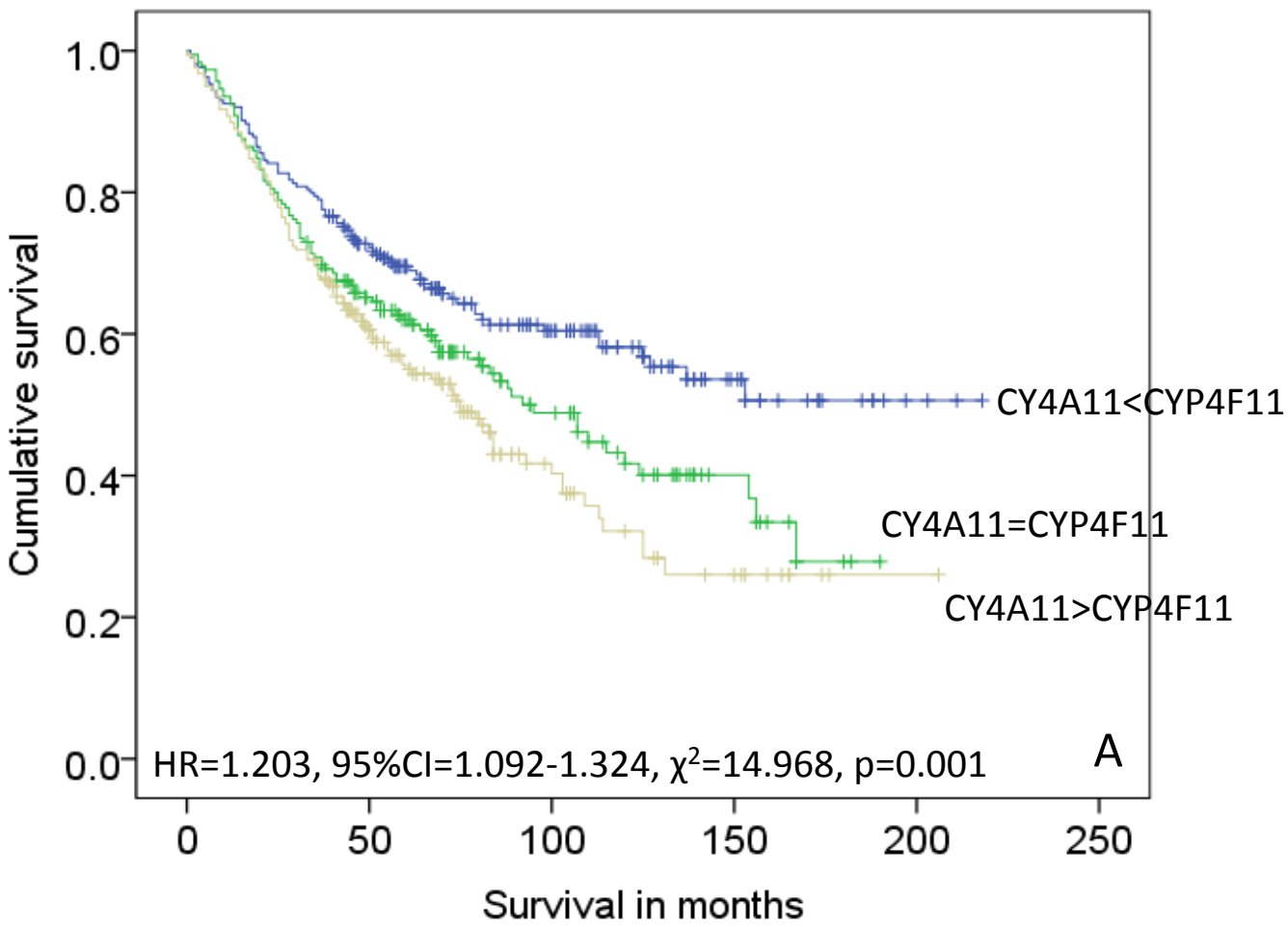
Number at risk

CYP4A11 neg	175	105	51	15	2	0
CYP4A11 weak/mod/strong	450	249	88	27	0	0



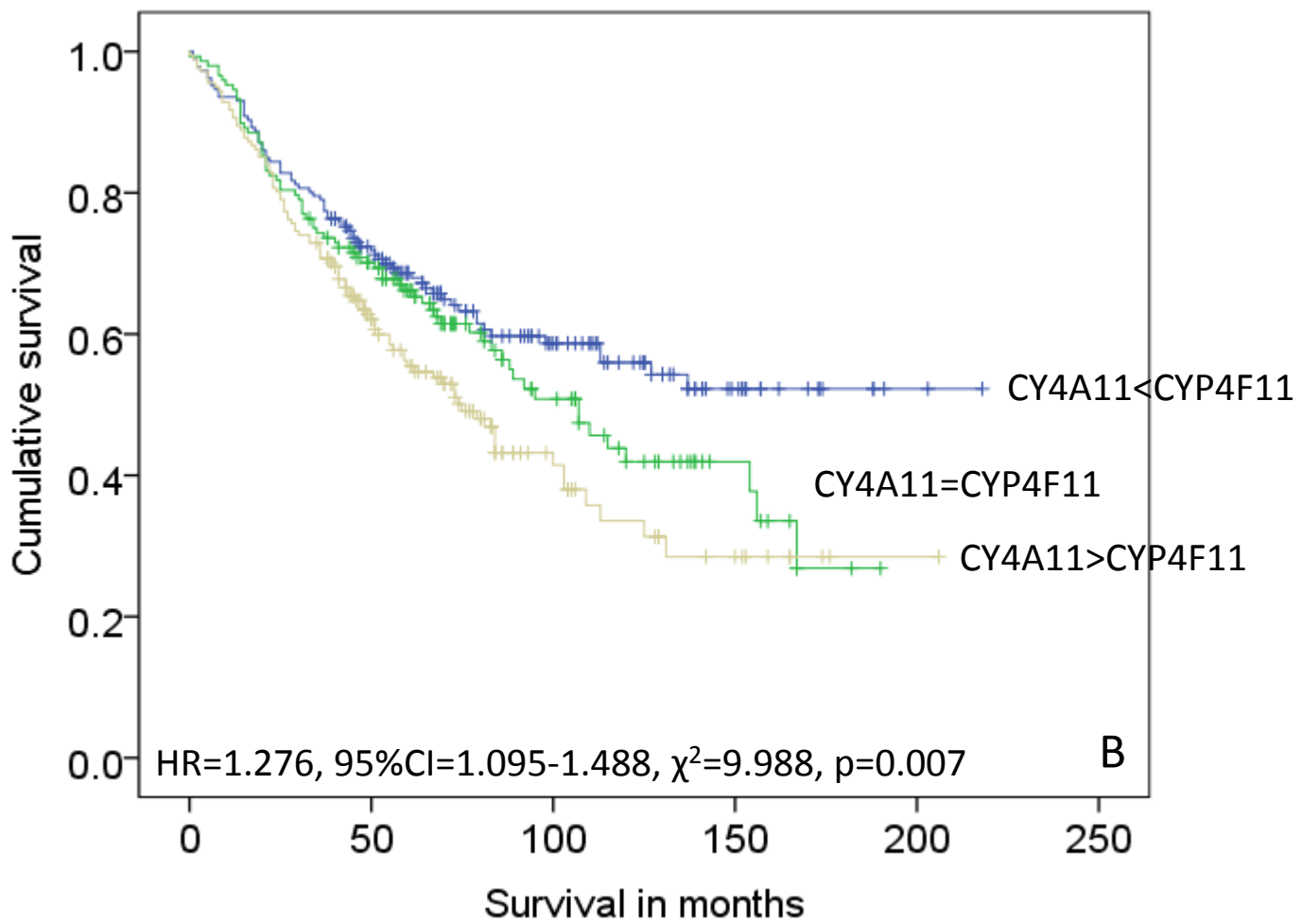
Number at risk

CYP4A11 neg/weak/mod	428	257	103	31	2	0
CYP4A11 strong	197	97	35	11	0	0



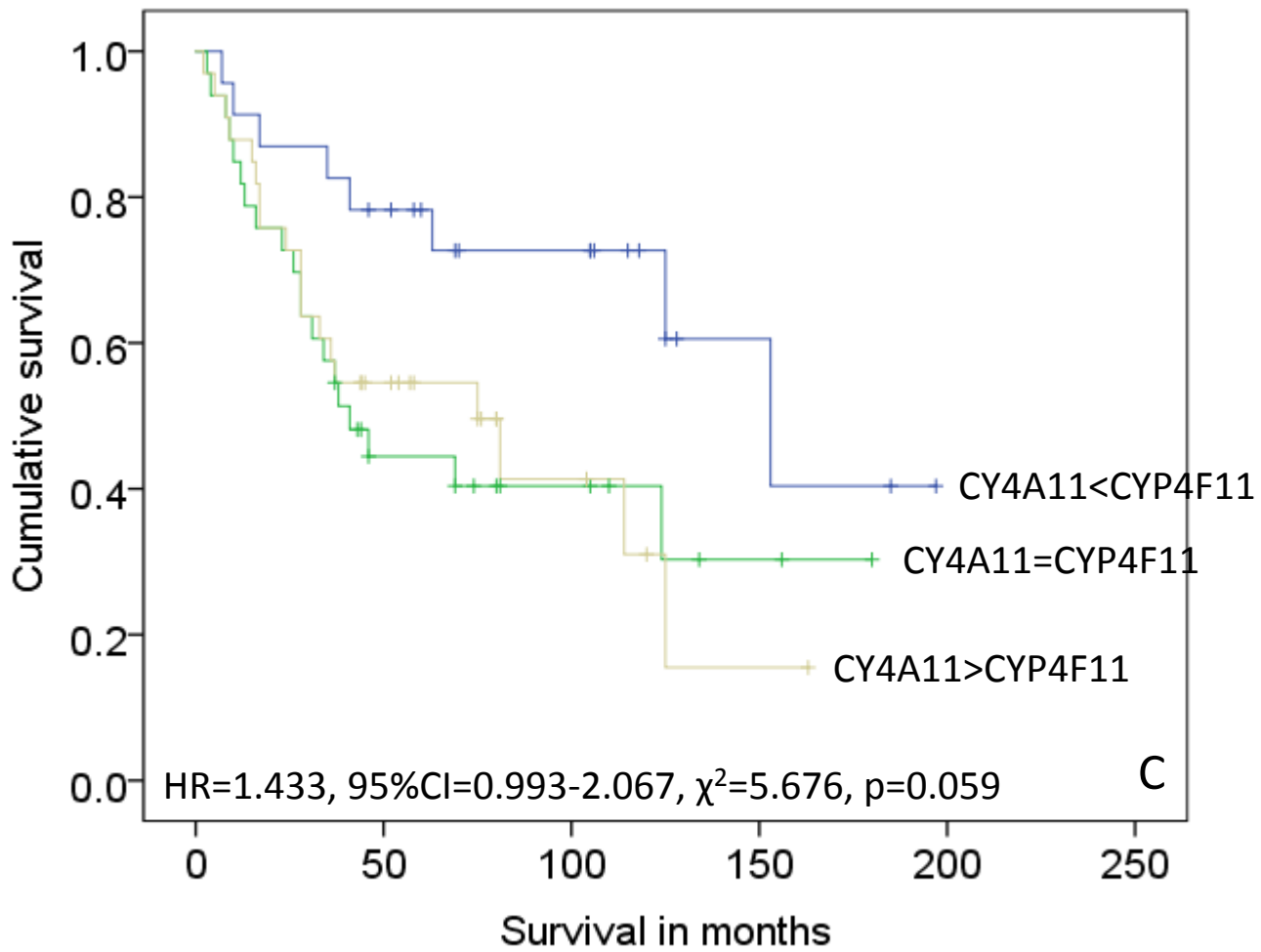
Number at risk

CYP4A11<CYP4F11	214	140	67	21	3	0
CYP4A11=CYP4F11	185	107	42	12	0	0
CYP4A11>CYP4F11	217	103	29	9	1	0



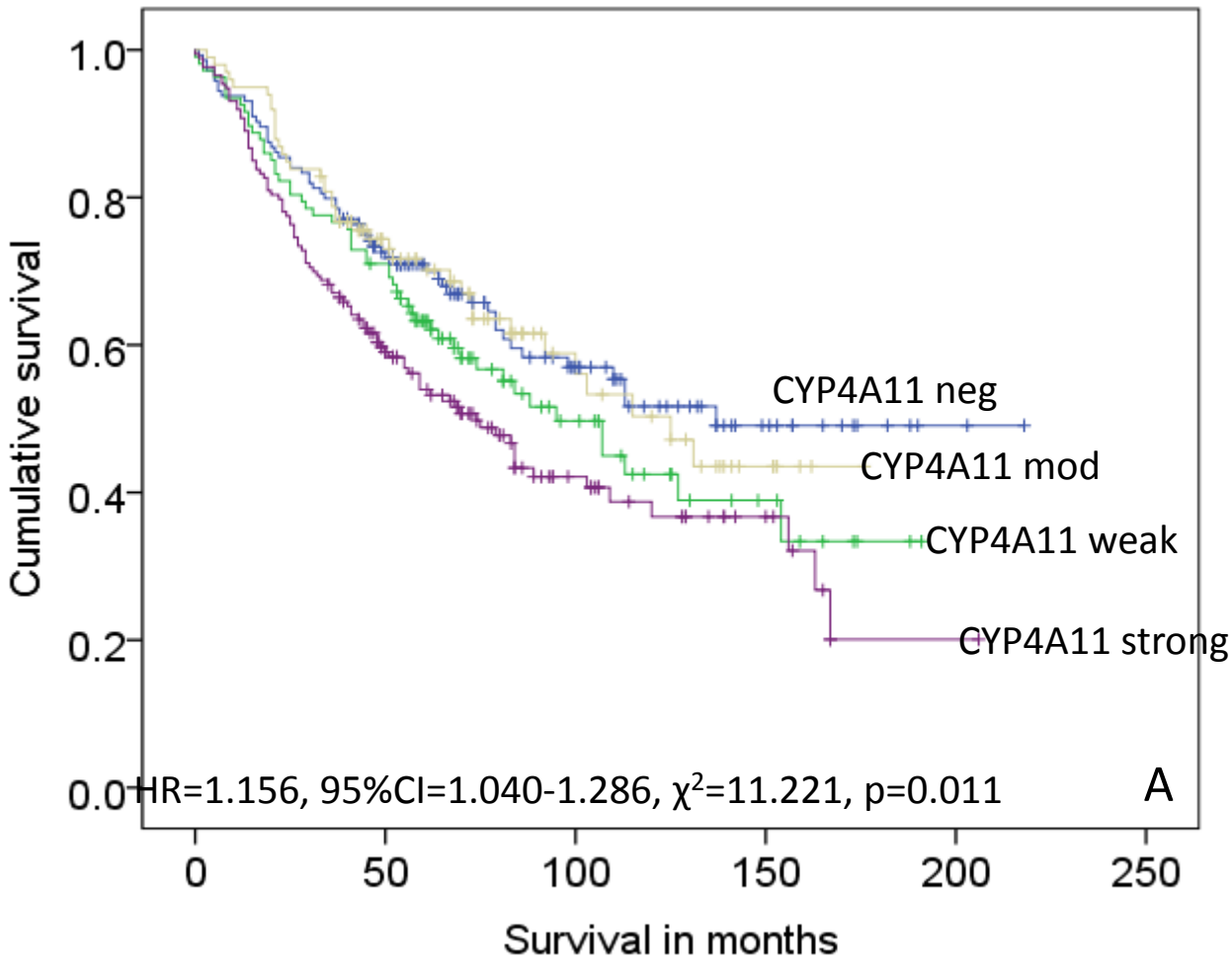
Number at risk

CYP4A11<CYP4F11	186	120	53	17	2	0
CYP4A11=CYP4F11	148	94	35	9	0	0
CYP4A11>CYP4F11	181	87	25	9	1	0



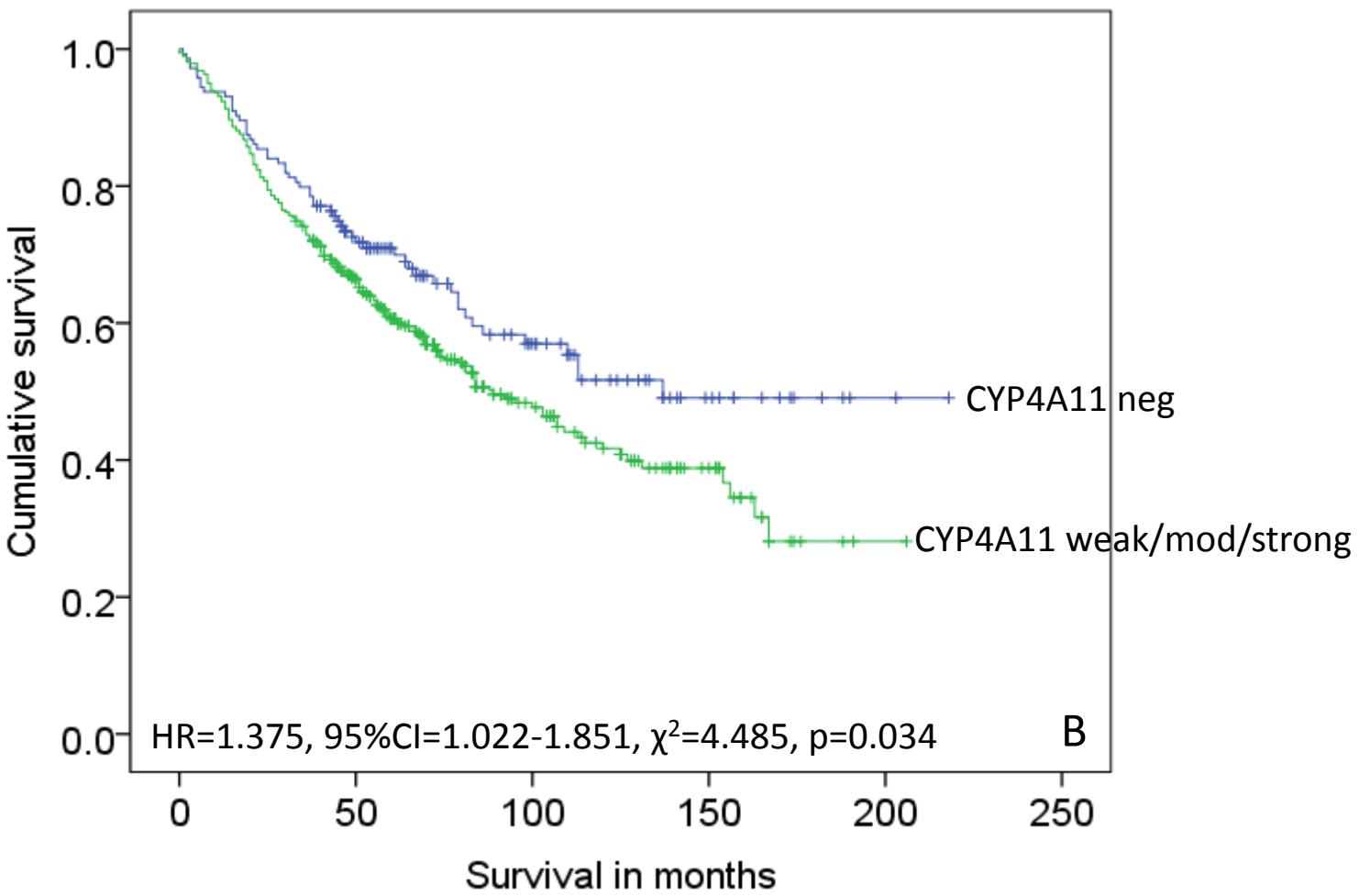
Number at risk

CYP4A11<CYP4F11	23	17	11	3	0	0
CYP4A11=CYP4F11	33	11	6	2	0	0
CYP4A11>CYP4F11	33	15	5	1	0	0



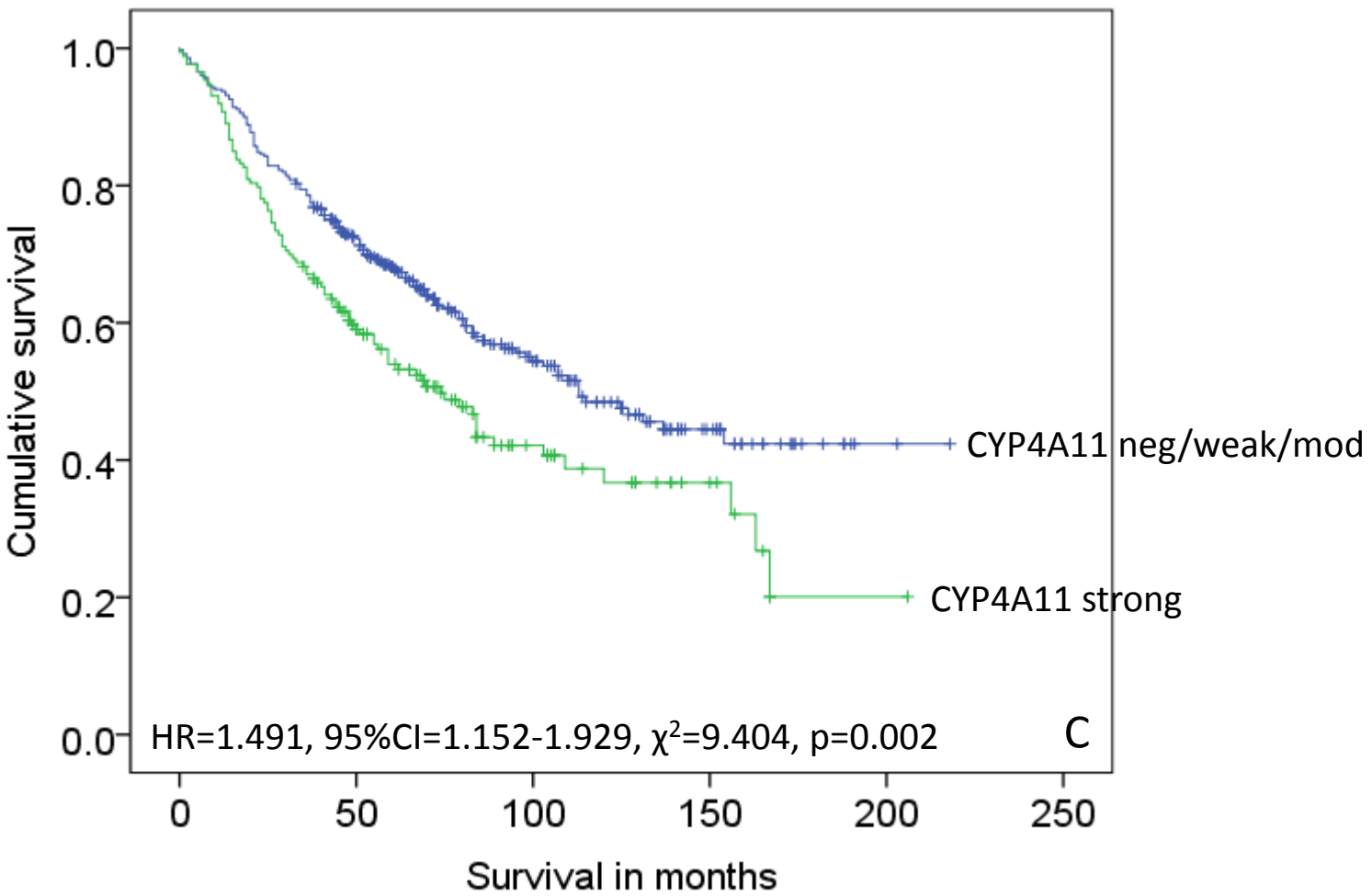
Number at risk

CYP4A11 neg	144	89	39	12	1	0
CYP4A11 weak	107	74	24	7	0	0
CYP4A11 mod	99	55	20	5	0	0
CYP4A11 strong	173	85	28	9	0	0



Number at risk

CYP4A11 neg	144	89	39	12	1	0
CYP4A11 weak/mod/strong	379	216	74	23	0	0



Number at risk

CYP4A11 neg/weak/mod	350	220	84	26	1	0
CYP4A11 strong	173	85	28	9	0	0

Supplementary information

Materials and methods S1

Monoclonal antibodies

Multiple sequence alignments were performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). To avoid undesirable regions such as transmembrane regions and signal peptides, the secondary and tertiary structures of proteins were predicted using tools such as http://wlab.ethz.ch/protter/start/ and http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index. The B cell epitope prediction software available at (http://tools.immuneepitope.org/bcell/) predicts polypeptide stretches of amino acids that are accessible, flexible, and hydrophilic. Furthermore, BLAST against UniProtKB 'Complete database' (http://web.expasy.org/blast/) was performed to ensure that the selected peptides are unique to the targets of interest. Finally, Vertebrate Antibodies Ltd utilised its own optimized computer algorithm to select the ideal peptides to ensure antigenicity.

The histopathological processing of tissues specimens

All specimens were received fresh in the diagnostic histopathology laboratory. The specimens were fixed in 10% neutral buffered formalin for at least 48 hours at room temperature after they were opened along the anti-mesenteric border proximal (distal to the tumour when appropriate) and washed in cold water. Representative tissue blocks were embedded in wax and sections were then stained with haematoxylin and eosin for histopathological diagnosis. The sections were also stained with elastic haematoxylin and eosin to permit further assessment of extramural venous invasion when required. The mean lymph node yield for all tumours in this study was 14.29 lymph nodes per tumour and for node negative tumours the mean lymph node yield was 15.07.

Construction of colorectal cancer tissue microarray

The tissue microarray was constructed using 50 normal colon mucosal samples which were acquired from at least 10 cm distant from the tumour, 650 primary and 285 metastatic colorectal cancer samples. Tumours were from patients had undergone elective surgery for primary colorectal cancer in the following periods; 1994-1998 (n=99), 1999-2003 (n=198) and 2004-2009 (n=353). The metastases were all from tumour involved lymph nodes of the

Dukes C cases. All the cases were reviewed and areas of tissue to be sampled were first identified and marked on the appropriate haematoxylin and eosin stained slide by an expert consultant gastro-intestinal pathologist (GIM). Two cores measuring 1mm in diameter were taken from these areas of the corresponding wax embedded block and placed in a recipient paraffin block.

Immunohistochemistry procedure

The tissue microarray sections were dewaxed in xylene for a minimum of 10 minutes and rehydrated by immersion in decreasing ethanol concentrations. Antigen retrieval was performed when required and it consisted of heating the sections by microwaving (800W) for 20 minutes. During the microwaving, the sections were fully immersed in citrate buffer (pH 6.0). The slides were incubated with undiluted primary antibody for 60 minutes at room temperature. After being washed twice with buffer (Dako) the sections were blocked with peroxidase for 7 minutes which was followed by two buffer washes. Thereafter peroxidase-polymer labelled goat anti-mouse secondary antibody (Envision, Dako) was applied for 30 minutes at room temperature and followed by further two washes with buffer. Then the diaminobenzidine substrate was applied for 7 minutes to reveal sites of peroxidase activity. The sections were washed in water, immersed in copper sulphate for 2 minutes counterstained with haematoxylin for 10 seconds, and placed in Scott's tap water substitute for 2 minutes. Finally dehydrated in increasing ethanol concentrations, immersed in a xylene and mounted. As a negative control antibody diluent was used to replace the primary monoclonal antibody. Normal liver tissue known to express all the enzymes was used as a positive control.

Data analysis and statistics

Biomarkers were first assessed separately using Kaplan-Meier (log rank test) and Cox regression univariate analysis to determine the best risk classifier among individual CYP4 markers. Duke's stage and extramural venous are the main prognostic parameters currently used in CRC to risk stratify patients to different subgroups and therefore new prognostic biomarkers need to be examined in relation to these parameters to determine if the relationship is specific to one subgroup. The anatomical site of primary colorectal cancer is also an important factor which affects the initial assessment, treatment and prognosis. Furthermore, colon (proximal and distal) and rectum differ in terms of their embryological origin, anatomy and may have distinct molecular profiles.

Furthermore, Kaplan-Meier survival analysis (stratified by other CYP4) was used to examine the overall relationship of the expression of CYP4 enzymes with outcome. The aim of this analysis was to determine the prognostic value of using a combination of CYP4 markers.

Key measurements used to determine the best prognostic marker include; the ability to distinguish between low and high risk groups (Kaplan-Meier plot), variations between risk groups (mean or median survival), chi-square value, p-value and hazard ratio with 95% confidence intervals.

A multivariate Cox's proportional hazard model "Forward Stepwise: Conditional LR" was used to determine the prognostic significance of CYP4 markers. The model included only established prognostic parameters and biomarkers with the best risk classification.

Results S1

Survival analysis in colon cancers

There was a significant association between the intensity of CYP4A11 immunostaining and overall survival in colon cancers (HR=1.153, 95% CI=1.033-1.287, χ^2=10.084, p=0.018) (Supplementary Figure S6). When each level of the intensity groups of CYP4A11 expression was considered separately using pairwise comparisons and one reference group (negative expression), strong intensity of CYP4A11 immunostaining was associated with poorer outcome (HR=1.640, 95% CI=1.168-2.302, χ^2=7.953, p=0.005) (Supplementary Table S15). Comparing strong CYP4A11 expressing tumours with negative/weak/moderate CYP4A11 expressing tumours also showed a significant association with survival (HR=1.494, 95% CI=1.135-1.967, χ^2=8.354, p=0.004). Similarly, the immunoreactivity of CYP4A11 was significantly associated with survival when CYP4A11 negative tumours were compared with CYP4A11 positive tumours (HR=1.354, 95% CI=1.005-1.824, χ^2=4.045, p=0.044). The expression of CYP4A11 was independently prognostic (p=0.017) when using only parameters available at time of biopsy (Supplementary Table S16C).

Table S1. Peptide sequences used as immunogens to generate monoclonal antibodies.

Enzyme	Hybridoma clone	Peptide sequence	Amino acid location
CYP4A11	M25-P2A10	KNGIHLRLR	499 – 507
CYP4F11	F21 P6 F5	RVEPLGANSQ	514 – 524
CYP4V2	M29P3B10	KREELGLEGQ	495 – 504
CYP4Z1	N7P2G5*D8	KLAPDHSRPP	473 – 483

Table S2. The relationship between the expression of each cytochrome P450 and individual pathological parameter.

A. CYP4A11

Pathological parameter	Number (percent) of patients in each group	Chi-square	p-value
Bowel screening programme detected	Yes=49 (7.8%) No=576 (92.2%)	3.673	0.299
Colon or rectum	Colon=485 (77.6%) Rectum=140 (22.4%)	13.487	**0.004**
Tumour site	Proximal colon=246 (39.4%) Distal colon=239 (38.2%) Rectum=140 (22.4%)	15.703	**0.015**
Tumour differentiation	Well/moderate=577 (92.3%) Poor=48 (7.7%)	3.816	0.282
EMVI	Absent=489 (78.2%) Present=136 (21.8%)	5.911	0.116
MMR status	Intact=523 (85.3%) Defective=90 (14.7%)	2.303	0.512
Tumour stage	T1=29 (4.6%) T2=112 (18%) T3=390 (62.4%) T4=94 (15%)	15.585	0.076
Nodal stage	N0=348 (55.7%) N1=169 (27%) N2=108 (17.3%)	9.852	0.131
Dukes stage	A=117 (18.7%) B=231 (37%) C=277 (44.3%)	13.148	**0.041**

Number (percent) of patients classified by the level of CYP4A11 expression; negative= 175 (28%), weak=129 (20.7%), moderate=124 (19.8%) and strong=197 (31.5%). Significant values are highlighted in bold.

B. CYP4F11

Pathological parameter	Number (percent) of patients in each group	Chi-square	p-value
Bowel screening programme detected	Yes=580 (92.1%) No=50 (7.9%)	0.486	0.922
Colon or rectum	Colon=490 (77.8%) Rectum=140 (22.2%)	17.026	**0.001**
Tumour site	Proximal colon=254 (40.3%) Distal colon=236 (37.5%) Rectum=140 (22.2%)	20.947	**0.002**
Tumour differentiation	Well/moderate=580 (92.1%) Poor=50 (7.9%)	8.552	**0.036**
EMVI	Absent=493 (78.3%) Present=137 (21.7%)	7.563	0.056
MMR status	Intact=523 (84.9%) Defective=93 (15.1%)	13.441	**0.004**
Tumour stage	T1=30 (4.8%) T2=113 (17.9%) T3=392 (62.2%) T4=95 (15.1%)	11.008	0.275
Nodal stage	N0=355 (56.3%) N1=168 (26.7%) N2=107 (17%)	10.656	0.100
Dukes stage	A=119 (18.9%) B=236 (37.5%) C=275 (43.6%)	10.517	0.104

Number (percent) of patients classified by the level of CYP4F11 expression; negative=53 (8.4%), weak=247 (39.2%), moderate=236 (37.5%) and strong=94 (14.9%). Significant values are highlighted in bold.

C. CYP4V2

Pathological parameter	Number (percent) of patients in each group	Chi-square	p-value
Bowel screening programme detected	Yes=49 (7.8%) No=576 (92.2%)	4.644	0.200
Colon or rectum	Colon=486 (77.8%) Rectum=139 (22.2%)	0.975	0.807
Tumour site	Proximal colon=250 (40%) Distal colon=236 (37.8%) Rectum=139 (22.2%)	11.965	0.063
Tumour differentiation	Well/moderate=575 (92%) Poor=50 (8%)	1.759	0.616
EMVI	Absent=490 (78.4%) Present=135 (21.6%)	3.174	0.365
MMR status	Intact=519 (84.8%) Defective=93 (15.2%)	7.231	0.065
Tumour stage	T1=30 (4.8%) T2=113 (18.1%) T3=389 (62.2%) T4=93 (14.9%)	17.837	**0.037**
Nodal stage	N0=353 (56.5%) N1=165 (26.4%) N2=107 (17.1%)	2.205	0.900
Dukes stage	A=119 (19.1%) B=234 (37.4%) C=272 (43.5%)	24.474	<**0.001**

Number (percent) of patients classified by the level of CYP4V2 expression; negative=336 (59.5%), weak=219 (35%), moderate=28 (4.5%) and strong=6 (1%). Significant values are highlighted in bold.

Table S3. The relationship between the expression of each cytochrome P450 and survival using different cut-off points for the intensity of the immunostaining.

CYP4A11	Negative=175 (28%) Weak=129 (20.7%) Moderate=124 (19.8%) Strong=197 (31.5%)	Negative=175 (28%) Weak/moderate /strong=450 (71.2%)	Negative/weak=304 (48.7%) Moderate/strong=321 (51.3%)	Strong=197 (31.5%) Negative/weak/moderate=428 (68.5%)
	χ^2=9.080, p=**0.028**	χ^2=4.881, p=**0.027**	χ^2=3.315, p=0.069	χ^2=7.234, p=**0.007**
CYP4F11	Negative=53 (8.4%) Weak=247 (39.2%), Moderate=236 (37.5%) Strong=94 (14.9%)	Negative=53 (8.4%) Weak/moderate/strong=577 (91.6%)	Negative/weak=300 (47.6%) Moderate/strong=330 (52.4%)	Strong=94 (14.9%) Negative/weak/moderate=536 (85.1%)
	χ^2=3.411, p=0.333	χ^2=2.054, p=0.152	χ^2=1.376, p=0.241	χ^2=1.697, p=0.193
CYP4V2	Negative=372 (59.5%) Weak=219 (35%) Moderate=28 (4.5%) Strong=6 (1%)	Negative=372 (59.5%) Weak/moderate/strong=253 (40.5%)	Negative/weak=591 (94.5%) Moderate/strong=34 (5.5%)	Strong=6 (1%) Negative/weak/moderate=619 (99%)
	χ^2=2.339, p=0.505	χ^2=0.093, p=0.761	χ^2=1.656, p=0.198	χ^2=0.014, p=0.907
CYP4Z1	-	-	-	-

Significant values are highlighted in bold.

Table S4. The association between the expression of CYP4A11 and survival in the whole patient cohort and in MMR proficient tumours.

CYP4A11 categories	Number (percent) of patients in each group	Mean and median survival in months		Pairwise comparisons: negative expression as a reference group		
		Mean (95% CI)	Median (95% CI)	Chi-square	p-value	Hazard ratio (95% CI)
Whole cohort						
Negative	175 (28%)	132 (117-147)	137 (undefined)	-	-	-
Weak	129 (20.7%)	104 (90-119)	95 (63-126)	1.892	0.169	1.277 (0.912-1.789)
Moderate	124 (19.8%)	106 (93-119)	115 (79-151)	0.305	0.581	1.127 (0.790-1.608)
Strong	197 (31.5%)	96 (83-109)	75 (58-91)	8.006	**0.005**	1.541 (1.144-2.077)
MMR proficient tumours						
Negative	144 (27.5%)	134 (118-151)	137 (undefined)	-	-	-
Weak	107 (20.5%)	105 (90-121)	95 (67-122)	1.823	0.177	1.298 (0.893-1.887)
Moderate	99 (18.9%)	111 (97-126)	125 (90-159)	0.014	0.905	1.045 (0.695-1.571)
Strong	173 (33.1%)	96 (82-109)	74 (57-91)	8.626	**0.003**	1.644 (1.183-2.284)

Significant values are highlighted in bold. When the cumulative survival proportion of patients was more than half the group, the median survival and/or its 95% confidence interval were undefined by SPSS.

Table S5. The relationship between the expression of CYP4A11 and CYP4F11 and survival in the whole cohort.

A. The relationship between the expression of CYP4A11 and survival in the whole patient cohort stratified by CYP4F11.

CYP4F11	CYP4A11	Number (percent) of patients in each group	Mean and median survival in months		Chi-square	p-value	Hazard ratio (95% CI)
			Mean (95% CI)	Median (95% CI)			
Negative	Negative	28 (55%)	86 957-115)	53 (9-97)	5.668	**0.017**	1.385 (1.057-1.815)
	Positive	23 (45%)	84 (57-111)	93 (26-160)			
Positive	Negative	147 (26%)	139 (123-155)	Undefined			
	Positive	418 (74%)	107 (97-116)	89 (71-107)			

Significant values are highlighted in bold. When the cumulative survival proportion of patients was more than half the group, the median survival and/or its 95% confidence interval were undefined by SPSS.

B. The relationship between the expression of CYP4F11 and survival in the whole patient cohort stratified by CYP4A11.

CYP4A11	CYP4F11	Number (percent) of patients in each group	Mean and median survival in months		Chi-square	p-value	Hazard ratio (95% CI)
			Mean (95% CI)	Median (95% CI)			
Negative	Negative	28 (16%)	86 (57-115)	53 (9-97)	4.844	**0.028**	0.657 (0.450-0.959)
	Positive	147 (84%)	139 (123-155)	Undefined			
Positive	Negative	23 (5.2%)	84 (57-111)	93 (26-160)			
	Positive	418 (84.8%)	107 (97-116)	89 (71-107)			

Significant values are highlighted in bold. When the cumulative survival proportion of patients was more than half the group, the median survival and/or its 95% confidence interval were undefined by SPSS.

Table S6. The relationship between the differential expression of CYP4A11 and CYP4F11 and survival in the whole patient cohort, in MMR proficient tumours and in MMR deficient tumours.

Differential expression of CYPA11 and CYP4F11	Number (percent) of patients in each group	Mean and median survival in months		Pairwise comparisons: CYP4F11>CYP4A11 as a reference group		
		Mean (95% CI)	Median (95% CI)	Chi-square	p-value	Hazard ratio (95% CI)
Whole cohort						
CYP4A11< CYP4F11	214 (34.8%)	137 (124-151)	Undefined)	-	-	-
CYP4A11=CYP4F11	185 (30%)	102 (91-114)	95 (72-117)	5.425	**0.020**	1.432 (1.064-1.928)
CYP4A11>CYP4F11	217 (35.2%)	94 (82-107)	75 (60-89)	14.405	<**0.001**	1.733 (1.306-2.300)
MMR proficient tumours						
CYP4A11< CYP4F11	186 (36.1%)	137 (123-152)	Undefined)	-	-	-
CYP4A11=CYP4F11	148 (28.7%)	106 (84-112)	107 (83-131)	2.070	0.150	1.275 (0.918-1.770)
CYP4A11>CYP4F11	181 (35.2%)	97 (110-128)	75 (85-121)	9.261	**0.002**	1.629 (1.199-2.214)
MMR deficient tumours						
CYP4A11< CYP4F11	23 (25.8%)	133 (101-166)	153 (102-204)	-	-	-
CYP4A11=CYP4F11	33 (37.1%)	82 (56-109)	41 (25-56)	4.782	**0.029**	2.507 (1.098-5.725)
CYP4A11>CYP4F11	33 (37.1%)	76 (54-98)	75 (25-124)	4.973	**0.026**	2.390 (1.036-5.511)

Significant values are highlighted in bold. When the cumulative survival proportion of patients was more than half the group, the median survival and/or its 95% confidence interval were undefined by SPSS.

Table S7. The relationship of the expression of each cytochrome P450 and survival using individual cut-off points for immunostaining intensity with groups stratified by proximal and distal colon cancers.

	Number (percent) of patients in each group	Negative *versus* weak *versus* moderate *versus* strong		Negative *versus* weak, moderate and strong		Negative and weak *versus* moderate and strong		Strong *versus* negative, weak and moderate	
		Chi-square	p-value	Chi-square	p-value	Chi-square	p-value	Chi-square	p-value
CYP4A11									
Proximal	246 (50.7%)	3.455	0.327	1.598	0.206	0.180	0.671	1.456	0.228
Distal	239 (49.3%)	7.764	0.051	2.128	0.145	3.299	0.069	7.545	**0.006**
CYP4F11									
Proximal	254 (51.8%)	1.983	0.576	1.752	0.186	0.826	0.363	0.090	0.764
Distal	236 (48.2%)	1.241	0.743	0.119	0.730	0.093	0.761	1.137	0.286
CYP4V2									
Proximal	250 (51.5%)	1.209	0.751	0.100	0.752	1.167	0.280	0.054	0.817
Distal	236 (48.5%)	1.154	0.764	0.153	0.696	1.055	0.304	0.425	0.514
CYP4Z1									
Proximal	-	-	-	-	-	-	-	-	-
Distal	-	-	-	-	-	-	-	-	-

Significant values are highlighted in bold. Numbers (percent) of patients classified by the level of each CYP4 expression are given in Table S3.

Table S8. The relationship of the expression of each cytochrome P450 and survival using individual cut-off points for immunostaining intensity with groups stratified by individual Dukes stage.

	Number (percent) of patients in each group	Negative *versus* weak *versus* moderate *versus* strong		Negative *versus* weak, moderate and strong		Negative and weak *versus* moderate and strong		Strong *versus* negative, weak and moderate	
		Chi-square	p-value	Chi-square	p-value	Chi-square	p-value	Chi-square	p-value
CYP4A11									
Dukes A	117 (18.7%)	4.358	0.225	3.884	**0.049**	3.239	0.072	1.179	0.278
Dukes B	231 (37%)	2.369	0.499	0.574	0.448	0.011	0.918	1.152	0.283
Dukes C	277 (44.3%)	1.448	0.694	0.001	0.977	0.220	0.639	1.175	0.278
CYP4F11									
Dukes A	119 (18.9%)	0.591	0.899	0.132	0.717	0.077	0.782	0.503	0.478
Dukes B	236 (37.5%)	1.434	0.698	0.463	0.496	0.132	0.717	0.580	0.446
Dukes C	275 (43.6%)	2.478	0.479	0.956	0.328	1.796	0.180	1.361	0.243
CYP4V2									
Dukes A	119 (19.1%)	17.752	**<0.001**	6.895	**0.009**	4.966	0.026	0.113	0.737
Dukes B	234 (37.4%)	0.772	0.856	0.511	0.475	0.406	0.524	0.066	0.797
Dukes C	272 (43.5%)	2.257	0.521	0.536	0.464	1.495	0.221	0.000	0.992
CYP4Z1									
Dukes A	-	-	-	-	-	-	-	-	-
Dukes B	-	-	-	-	-	-	-	-	-
Dukes C	-	-	-	-	-	-	-	-	-

Significant values are highlighted in bold. Numbers (percent) of patients classified by the level of each CYP4 expression are given in Table S3.

Table S9. The relationship of the expression of each cytochrome P450 and survival using individual cut-off points for immunostaining intensity with groups stratified by EMVI status.

	Number (percent) of patients in each group	Negative *versus* weak *versus* moderate *versus* strong		Negative *versus* weak, moderate and strong		Negative and weak *versus* moderate and strong		Strong *versus* negative, weak and moderate	
		Chi-square	p-value	Chi-square	p-value	Chi-square	p-value	Chi-square	p-value
CYP4A11									
Present	136 (21.8%)	2.474	0.480	0.113	0.737	0.006	0.937	1.207	0.272
Absent	489 (78.2%)	3.983	0.263	1.863	0.175	2.572	0.109	3.609	0.057
CYP4F11									
Present	137 (21.7%)	1.983	0.576	1.752	0.186	0.826	0.363	0.090	0.764
Absent	493 (78.3%)	1.241	0.743	0.119	0.730	0.093	0.761	1.137	0.286
CYP4V2									
Present	135 (21.6%)	4.226	0.238	0.071	0.790	1.239	0.266	0.081	0.776
Absent	490 (78.4%)	3.609	0.307	1.533	0.216	1.182	0.277	0.050	0.823
CYP4Z1									
Present	-	-	-	-	-	-	-	-	-
Absent	-	-	-	-	-	-	-	-	-

Significant values are highlighted in bold. Numbers (percent) of patients classified by the level of each CYP4 expression are given in Table S3.

Table S10. The relationship between the expression of each cytochrome P450 and survival using individual cut-off points for immunostaining intensity with groups stratified by tumour site (colon *versus* rectum).

	Number (percent) of patients in each group	Negative *versus* weak *versus* moderate *versus* strong		Negative *versus* weak, moderate and strong		Negative and weak *versus* moderate and strong		Strong *versus* negative, weak and moderate	
		Chi-square	p-value	Chi-square	p-value	Chi-square	p-value	Chi-square	p-value
CYP4A11									
Colon	485 (77.6%)	10.084	**0.018**	4.045	**0.044**	2.689	0.101	8.354	**0.004**
Rectum	140 (22.4%)	1.093	0.779	0.918	0.338	0.863	0.353	0.204	0.651
CYP4F11									
Colon	490 (77.8%)	2.677	0.444	2.083	0.149	0.693	0.405	0.917	0.338
Rectum	140 (22.2%)	1.061	0.787	0.001	0.978	0.631	0.427	0.756	0.384
CYP4V2									
Colon	486 (77.6%)	0.913	0.822	0.081	0.776	0.855	0.355	0.018	0.895
Rectum	140 (22.4%)	3.507	0.320	1.292	0.256	0.850	0.356	0.030	0.862
CYP4Z1									
Colon	-	-	-	-	-	-	-	-	-
Rectum	-	-	-	-	-	-	-	-	-

Significant values are highlighted in bold. Numbers (percent) of patients classified by the level of each CYP4 expression are given in Table S3.

Table S11. The relationship between the expression of each cytochrome P450 and survival using different cut-off points for immunostaining intensity with groups stratified by MMR protein status.

	Number (percent) of patients in each group	Negative *versus* weak *versus* moderate *versus* strong		Negative *versus* weak, moderate and strong		Negative and weak *versus* moderate and strong		Strong *versus* negative, weak and moderate	
		Chi-square	p-value	Chi-square	p-value	Chi-square	p-value	Chi-square	p-value
CYP4A11									
Defective	90 (14.7%)	0.512	0.916	0.397	0.529	0.054	0.817	0.000	0.948
Proficient	523 (85.3%)	11.221	**0.011**	4.485	**0.034**	3.085	0.079	9.404	**0.002**
CYP4F11									
Defective	93 (15.1%)	5.232	0.156	4.682	**0.030**	1.463	0.226	0.005	0.944
Proficient	523 (84.9%)	1.493	0.684	0.051	0.822	0.168	0.682	1.475	0.225
CYP4V2									
Defective	93 (15.2%)	0.711	0.871	0.103	0.749	0.160	0.689	0.410	0.522
Proficient	519 (84.8%)	2.261	0.520	0.000	0.997	1.539	0.215	0.041	0.839
CYP4Z1									
Defective	-	-	-	-	-	-	-	-	-
Proficient	-	-	-	-	-	-	-	-	-

Significant values are highlighted in bold. Numbers (percent) of patients classified by the level of each CYP expression are given in Table S3

Table S12. Details of the intermediate calculations and omnibus tests of model coefficients leading to the final multivariate model in the whole patient cohort (Cox regression, method: “Forward Stepwise: Conditional LR”).

Step	-2 Log Likelihood	Overall (score)		Change from previous step		Change from previous block	
		Chi-square	p-value	Chi-square	p-value	Chi-square	p-value
1[a]	3359.217	105.103	**<0.001**	99.401	**<0.001**	99.401	**<0.001**
2[b]	3326.576	154.375	**<0.001**	32.642	**<0.001**	132.043	**<0.001**
3[c]	3291.934	187.809	**<0.001**	34.642	**<0.001**	166.684	**<0.001**
4[d]	3286.356	194.243	**<0.001**	5.578	**0.018**	172.262	**<0.001**

a. Variable entered at step number 1: Dukes stage (A v B v C).
b. Variable entered at step number 2: EMVI (present v absent).
c. Variable entered at step number 3: age at Surgery (< 70 v ≥ 70).
d. Variable entered at step number 4: differential expression of CY4A11 and CYP4F11 (CYP4A11>CYP4F11 v CYP4A11=CYP4F11 v CYP4A11<CYP4F11).
The summary of the final multivariate model is shown in Table 3.
Significant values are highlighted in bold.

Table S13. The intermediate steps and omnibus tests of model coefficients leading to the final multivariate prognostic model in mismatch repair proficient tumours (Cox regression, method: “Forward Stepwise: Conditional LR”).

Step	-2 Log Likelihood	Overall (score)		Change from previous step		Change from previous block	
		Chi-square	p-value	Chi-square	p-value	Chi-square	P-value
1[a]	2672.051	79.187	**<0.001**	60.993	**<0.001**	60.993	**<0.001**
2[b]	2638.138	113.667	**<0.001**	33.913	**<0.001**	94.906	**<0.001**
3[c]	2609.881	140.816	**<0.001**	28.258	**<0.001**	123.164	**<0.001**
4[d]	2605.872	145.222	**<0.001**	4.008	**0.045**	127.172	**<0.001**

a. Variable entered at step number 1: Dukes stage (A v B v C).
b. Variable entered at step number 2: EMVI (present v absent).
c. Variable entered at step number 3: age at Surgery (< 70 v ≥ 70).
d. Variable entered at step number 4: differential expression of CY4A11 and CYP4F11 (CYP4A11>CYP4F11 v CYP4A11=CYP4F11 v CYP4A11<CYP4F11).
The summary of the final multivariate model is shown in Table 3.
Significant values are highlighted in bold.

Table S14. The significance of the differential expression of CYP4A11 and CYP4F11 in multivariate analysis for the whole patient cohort and MMR proficient tumours including only parameters that would be available at biopsy.

	Whole patient cohort			Mismatch repair proficient tumours		
Variable	Wald value	p-value	Hazard ratio (95% CI)	Wald value	p-value	Hazard ratio (95% CI)
Age (< 70 v ≥ 70)	25.585	**<0.001**	1.881 (1.472-2.403)	22.787	**<0.001**	1.926 (1.472-2.521)
Gender (male v Female)	0.364	0.546	0.931 (0.738-1.174)	0.234	0.629	0.939 (0.726-1.213)
Tumour site (colon v rectum)	0.114	0.735	0.954 (0.726-1.253)	0.262	0.609	0.926 (0.692-1.241)
Tumour differentiation (well/moderate v poor)	0.017	0.895	1.029 (0.674-1.572)	2.023	0.155	0.653 (0.362-1.175)
Differential expression of CYP4A11 and CYP4F11 **(**CYP4A11>CYP4F11 v CYP4A11=CYP4F11 v CYP4A11<CYP4F11)	12.039	**0.001**	1.281 (1.114-1.474)	7.454	**0.006**	1.240 (1.063-1.448)

Significant values are highlighted in bold.

Table S15.The association between the expression of CYP4A11 and survival in colon cancer.

CYP4A11 categories	Number (percent) of patients	Mean and median survival in months		Pairwise comparisons: negative expression as a reference group		
		Mean (95% CI)	Median (95% CI)	Chi-square	p-value	Hazard ratio (95% CI)
Negative	146 (30.1%)	128 (113-144)	137 (undefined)	-	-	-
Weak	109 (22.5%)	103 (87-119)	95 (65-125)	1.710	0.191	1.285 (0.886-1.863)
Moderate	89 (18.3%)	108 (92-125)	125 (82-167)	0.015	0.904	1.038 (0.683-1.578)
Strong	141 (29.1%)	95 (79-110)	69 (52-85)	7.953	**0.005**	1.640 (1.168-2.302)

Significant values are highlighted in bold. When the cumulative survival proportion of patients was more than half the group, the median survival and/or its 95% confidence interval were undefined by SPSS.

Table S16. Multi-variate analysis of CYP4A11 using only parameters that would be available at biopsy in the whole patient cohort, in MMR proficient tumours and in colon cancers.

A. whole patient cohort

Variable	Wald value	p-value	Hazard ratio (95% CI)
Age at Surgery (< 70 v ≥ 70)	23.422	**<0.001**	1.830 (1.433-2.337)
Gender (male v female)	0.042	0.837	0.976 (0.773-1.232)
Tumour site (Colon vs rectum)	0.066	0.798	0.965 (0.732-1.271)
Tumour differentiation (Well/moderate v poor)	0.225	0.635	0.896 (0.571-1.408)
MSI status (proficient v deficient)	1.320	0.251	1.202 (0.878-1.647)
CYP4A11 (strong v negative/weak/moderate)	6.306	**0.012**	1.361 (1.070-1.730)

Significant values are highlighted in bold.

B. MMR proficient tumours

Variable	Wald value	p-value	Hazard ratio (95% CI)
Age at Surgery (< 70 v ≥ 70)	22.711	**<0.001**	1.909 (1.463-2.490)
Gender (male v female)	0.072	0.789	0.966 (0.749-1.245)
Tumour site (Colon v rectum)	0.147	0.701	0.945 (0.707-1.263)
Tumour differentiation (Well/moderate v poor)	2.120	0.145	0.646 (0.359-1.163)
CYP4A11 (strong v negative/weak/moderate)	7.168	**0.007**	1.427 (1.100-1.852)

Significant values are highlighted in bold.

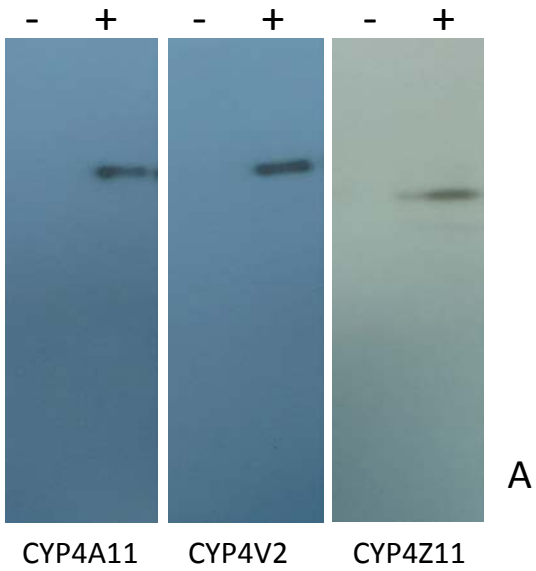
C. Colon cancer cases

Variable	Wald value	p-value	Hazard ratio (95% CI)
Age at Surgery (< 70 v ≥ 70)	18.479	**<0.001**	1.881 (1.410-2.509)
Gender (male v female)	0.066	0.797	0.966 (0.741-1.259)
Tumour differentiation (Well/moderate v poor)	0.417	0.518	0.851 (0.522-1.388)
MSI status (proficient v deficient)	2.131	0.144	1.286 (0.917-1.802)
CYP4A11 (strong v negative/weak/moderate)	5.668	**0.017**	1.403 (1.062-1.853)

Significant values are highlighted in bold.

Figure S1

Immunoblots of (59.3 kDa), CYP4V2 (60.7 kDa) and CYP4Z1 (59 kDa) monoclonal antibodies. A. The left hand lane (-) of each panel contains empty vector cell lysate while the right hand lane (+) of each panel contains lysate prepared from cells overexpressing the relevant protein. Five micrograms of each lysate was loaded per lane.



Immunoblots of CYP4A11 (59.3 kDa), CYP4V2 (60.7 kDa) and CYP4Z1 (59 kDa) monoclonal antibodies. B. microsomal fractions prepared from human liver tissues were used. Thirty micrograms of microsomes was loaded per lane.

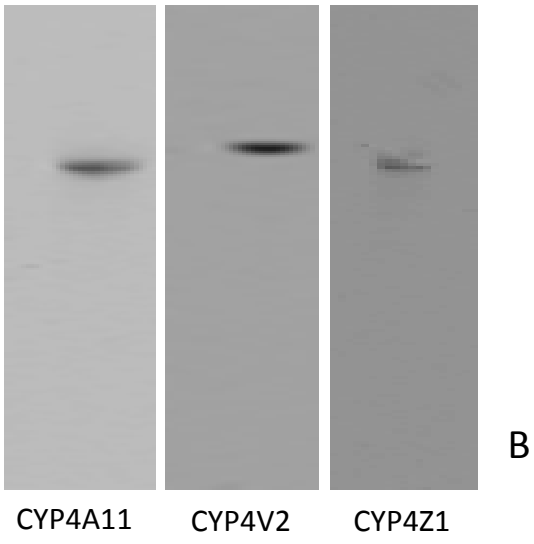


Figure S2.

Photomicrographs of CYP4A11, CYP4F11, CYP4V2 and CYP4Z1 in normal colonic mucosa, primary colorectal cancer and metastatic colorectal cancer.

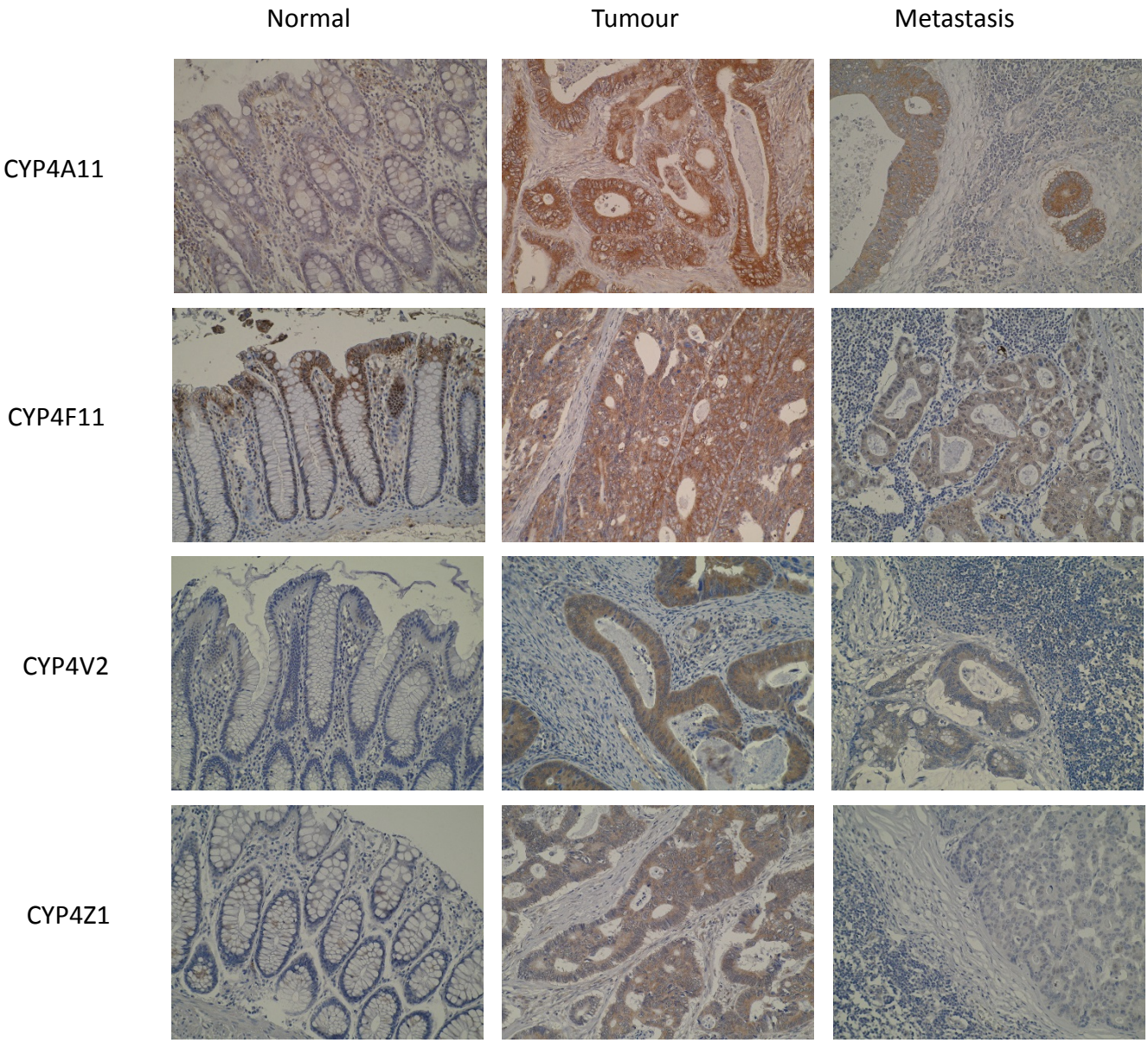


Figure S3.

The frequency distribution of the intensity of expression of CYP4A11, CYP4F11, CYP4V2 and CYP4Z1 in normal colonic mucosa, primary colorectal cancer and lymph node metastasis.

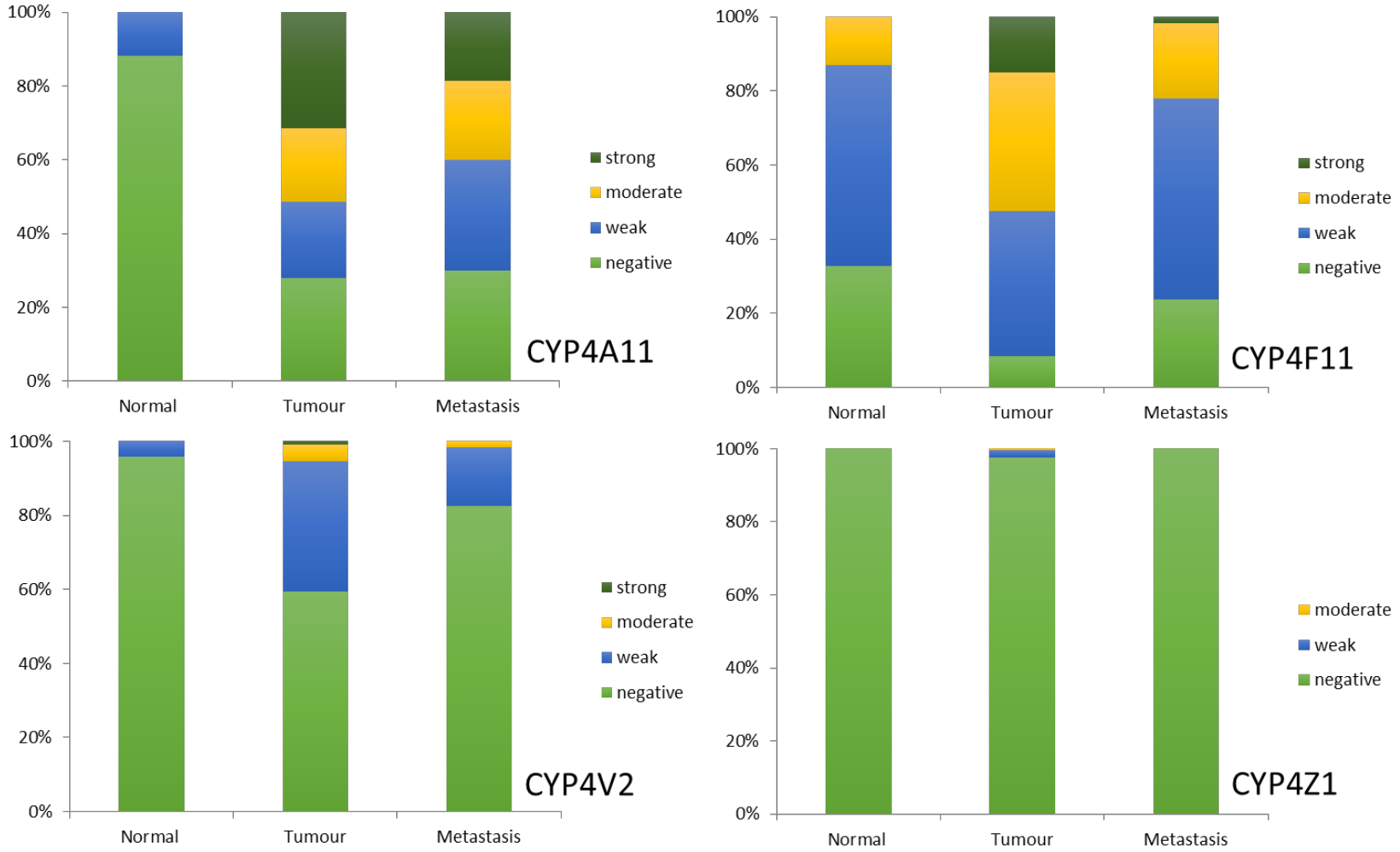
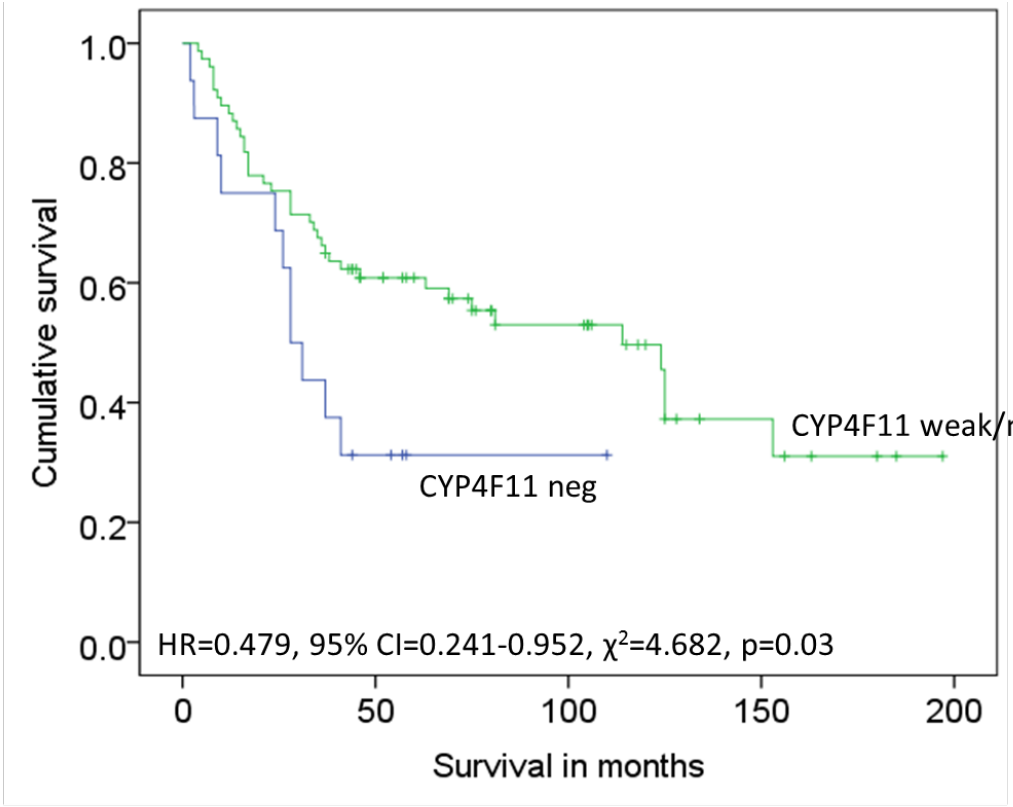


Figure S4.

The relationship between the expression of CYP4F11 and survival in MMR defective tumours.



Number at risk

CYP4A11 weak/mod/strong	77	39	20	5	0
CYP4A11 neg	16	3	0	0	0

Figure S5.

The prognostic impact of the differential expression of CYP4A11 and CYP4F11 in colorectal cancer. A. The metabolism of arachidonic acid by CYP4A11 is the dominant pathway in tumours with CYP4A11 > CYP4F11 expression ratio. These tumours will have worse prognosis since the metabolism of 20-HETE promotes the production of VEGF and MMP9. B. The metabolism of omega-3 fatty acids is the dominant pathway in tumours with the CYP4A11 < CYP4F11 expression ratio. These tumours will have better prognosis since the production of VEGF and MMPs is inhibited by the metabolism of omega-3 fatty acids.

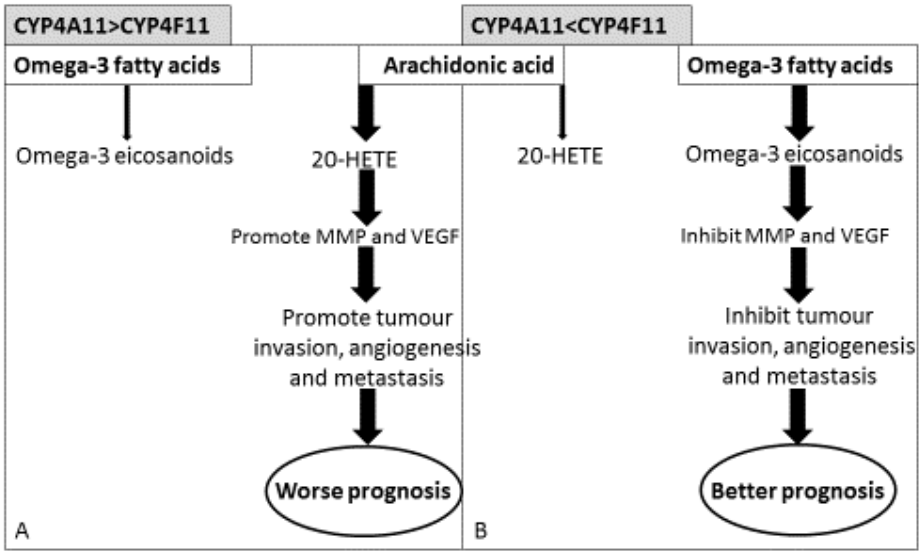


Figure S6.

The relationship between the expression of CYP4A11 and survival in colon cancers using different cut-off points: negative *versus* weak *versus* moderate *versus* strong (A), strong *versus* negative/weak/moderate (B) and positive expression *versus* negative expression (C).