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Epidermal growth factor receptor (EGFR) and prostaglandin-endoperoxide synthase 2 (PTGS2) are prognostic biomarkers for patients with resected colorectal cancer liver metastases

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Background: Resection of colorectal cancer liver metastasis (CRCLM) with curative intent has long-term benefit in ~40% of cases. Prognostic biomarkers are needed to improve clinical management and reduce futile surgeries. Expression of epidermal growth factor receptor (EGFR) and prostaglandin-endoperoxide synthase 2 (PTGS2; also known as cyclooxygenase-2) has been associated with carcinogenesis and survival. We investigated the prognostic value of EGFR and PTGS2 expression in patients with resected CRCLM.

Methods: Formalin-fixed paraffin-embedded CRCLM tissue and corresponding primary tumour specimens from a multi-institutional cohort of patients who underwent liver resection between 1990 and 2010 were incorporated into tissue microarrays (TMAs). TMAs were stained for EGFR and PTGS2 by immunohistochemistry. The hazard rate ratio (HRR) for the association between expression in CRCLM and overall survival was calculated using a 500-fold cross-validation procedure.

Results: EGFR and PTGS2 expression could be evaluated in 323 and 351 patients, respectively. EGFR expression in CRCLM was associated with poor prognosis (HRR 1.54; $P<0.01$) with a cross-validated HRR of 1.47 ($P=0.03$). PTGS2 expression was also associated with poor prognosis (HRR 1.60; $P<0.01$) with a cross-validated HRR of 1.63 ($P<0.01$). Expression of EGFR and PTGS2 remained prognostic after multivariate analysis with standard clinicopathological variables (cross-validated HRR 1.51; $P=0.02$ and cross-validated HRR 1.59; $P=0.01$, respectively). Stratification for the commonly applied systemic therapy regimens demonstrated prognostic value for EGFR and PTGS2 only in the subgroup of patients who were not treated with systemic therapy (HRR 1.78; $P<0.01$ and HRR 1.64; $P=0.04$, respectively), with worst prognosis when both EGFR and PTGS2 were highly expressed (HRR 3.08; $P<0.01$). Expression of PTGS2 in CRCLM was correlated to expression in patient-matched primary tumours ($P=0.02$, 69.2% concordance).

Conclusions: EGFR and PTGS2 expressions are prognostic molecular biomarkers with added value to standard clinicopathological variables for patients with resectable CRCLM.

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Colorectal cancer (CRC) is the third most prevalent cancer type worldwide and the second leading cause of cancer mortality in developed countries, which is mainly due to hematogenous dissemination to the liver (Welch and Donaldson, 1979; Ferlay *et al*, 2010). Liver resection is the only intentionally curative treatment option for patients with CRC liver metastases (CRCLM). Patient eligibility relies on standard prognostic clinicopathological variables such as presence of multiple liver metastases, positive lymph nodes at the time of primary tumour resection, maximal CRCLM diameter larger than 5 cm and presentation of metastases within 12 months after diagnosis of the primary tumour (Nordlinger *et al*, 1996; Fong *et al*, 1999; Rees *et al*, 2008; Yamaguchi *et al*, 2008). However, 5-year survival rates hardly exceed 40%, indicating the need for better prognostic biomarkers to improve clinical management of CRCLM patients (Kanas *et al*, 2012).

Activation of the tyrosine kinase receptor epidermal growth factor receptor (EGFR) triggers RAS/RAF/MAPK signalling and promotes proliferation, angiogenesis and metastasis, rendering EGFR as a clinical target for cancer therapy (Ciardiello and Tortora, 2008). Likewise, increased expression of prostaglandin-endoperoxide synthase 2 (PTGS2) leads to increased production of PGE_2, which promotes cancer cell growth through EP2 receptor-mediated signalling (Castellone *et al*, 2005). Inhibition of PTGS2, for example, by non-steroidal anti-inflammatory drugs such as aspirin, has been shown to reduce CRC incidence as well as to improve clinical outcome following CRC surgery (Tougeron *et al*, 2013; Zoratto *et al*, 2014). Importantly, there exists a complex interplay between EGFR and PTGS2 expression. EGFR activation can induce PTGS2 expression in colon cancer cells (Coffey *et al*, 1997). In turn, upregulation of PTGS2 leads to PGE_2-mediated transactivation of EGFR (Pai *et al*, 2002; Buchanan *et al*, 2003). Both EGFR and PGE_2 can activate the PI3-kinase signalling pathway. Although activating mutations in *PIK3CA* are potential biomarkers for resistance to treatment with anti-EGFR monoclonal antibodies in metastatic CRC, some patients with *PIK3CA* mutations may benefit from treatment with the PTGS2 inhibitor aspirin (Liao *et al*, 2012; Mao *et al*, 2012; Tougeron *et al*, 2013). These data underscore the potential biological and clinical relevance of EGFR and PTGS2 expressions in metastatic CRC. In the current retrospective study, we assessed the prognostic value of EGFR and PTGS2 expressions in CRCLM of patients who underwent liver resection with curative intent.

MATERIALS AND METHODS

Patient study population. The patient study population was selected as previously described (Goos *et al*, 2013). In brief, patients were identified who underwent CRCLM resection in one of the seven Dutch hospitals affiliated with the DeCoDe PET group between 1990 and 2010. Formalin-fixed paraffin-embedded (FFPE) tissue specimens were collected from one histologically confirmed CRCLM sample and adjacent control liver tissue. When available, also the corresponding primary tumour and adjacent control colon tissue were collected. Patients with multiple primary tumours were excluded. Collection, storage and use of clinicopathological data and tissue specimens were performed in compliance with the 'Code for Proper Secondary Use of Human Tissue in The Netherlands', and approved according to local and national regulations (Stichting FMWV Rotterdam, 2011).

Tissue microarrays. Tissue microarrays (TMAs) were generated as previously described (Simon *et al*, 2004; Goos *et al*, 2013). Briefly, three tissue core biopsies of 0.6 mm diameter were punched from morphologically representative areas of all FFPE donor blocks and transferred into TMA recipient paraffin blocks using the 3DHISTECH TMA Master (v1.14, 3DHISTECH Ltd, Budapest, Hungary).

Immunohistochemistry. TMA sections (4 μm) were deparaffinised by xylene and rehydrated with decreasing alcohol series. For EGFR staining, sections were preprocessed with ER2 (Leica Biosystems, Newcastle, UK) and incubated with primary mouse monoclonal antibody directed against human EGFR (1 : 25, 15 min, Novocastra Laboratories, Newcastle, UK), followed by incubation with secondary anti-mouse antibody (8 min, Novocastra Detection System, Leica Biosystems, Newcastle, UK) using BondMax Immunostainer (Menarini Diagnostics, Firenze, Italy). For PTGS2 staining, antigen retrieval was performed by microwave heating in citric acid (10 mM, pH 6.0) and endogenous peroxidase quenching in 0.3% H_2O_2/methanol (25 min). Primary rabbit polyclonal monospecific antibody directed against human PTGS2 (1 : 200, 1 h, Atlas Antibodies, Stockholm, Sweden) was incubated at room temperature. Secondary anti-rabbit antibodies (Envision Plus, Dako, Heverlee, Belgium) were incubated for 30 min. Secondary antibodies were visualised by liquid diaminobenzidine substrate chromogen system. FFPE A431 cells were stained as positive controls for EGFR expression and FFPE Caco-2 cells as positive controls for PTGS2 expression. Incubation without primary antibody served as negative control.

Evaluation of protein expression. The Mirax slide scanner system equipped with a 20 × objective with a numerical aperture of 0.75 (Carl Zeiss B.V., Sliedrecht, The Netherlands) and a Sony DFW-X710 Fire Wire 1/3″-type progressive SCAN IT CCD (pixel size 4.65 × 4.65 μm, Tokyo, Japan) was used to digitally capture the immunohistochemical stainings, as described previously (Goos *et al*, 2013). The actual scan resolution (effective pixel size in the sample plane) at 20 × was 0.23 μm. Computer monitors used for image analysis were calibrated using the Spyder2PRO software (v1.0-16, Pantone Colorvision, Regensdorf, Switzerland). Frequencies of neoplastic epithelial cells expressing EGFR at the plasma membrane and PTGS2 at the nuclear membrane were scored for individual TMA core biopsies (categories 0, 1–25, 26–50, 51–75, 76–100%) using dedicated TMA scoring software (v1.14.25.1, 3DHISTECH Ltd). Tissue samples were independently evaluated by a second investigator without knowledge of clinicopathological information at the time of assessment ($K_{w,EGFR} = 84\%$ and $K_{w,PTGS2} = 85\%$).

Statistical analysis. Statistical analysis was performed using IBM SPSS Statistics 20.0 software (SPSS Inc., Chicago, IL, USA) and R Statistics 3.0.1 software (RStudio Inc., Boston, MA, USA). Excluded from the analyses were patients who died within 2 months after liver resection, when no data was available on survival status or time of survival, or when tissue cores were non-evaluable due to technical reasons (Figure 1). Overall survival (OS) was defined as the time in months after surgery until death in a follow-up period of 10 years. The prognostic value of EGFR and PTGS2 expressions was assessed using a 500-fold cross-validation procedure (Goos *et al*, 2013). Per cross-validation cycle the study population was randomly subdivided in a training and a validation set (50% : 50%). In each training set, the optimal cutoff for dichotomizing frequency scores into 'low' and 'high' expressions was calculated using receiver operating characteristic curve analysis for survival data with 3-year OS as the outcome of interest (Heagerty and Zheng, 2005; Zlobec *et al*, 2007). Frequency scores of the corresponding validation sets were dichotomised using this cutoff and a crude hazard rate ratio (HRR) was calculated in a Cox regression analysis with OS as outcome. Established clinicopathological prognostic variables were included in a multivariate Cox regression analysis (Fong *et al*, 1999). The average cross-validated HRR (HRR_{av}) of the validation sets was calculated and the *P*-value of the cross-validation analysis was defined as the percentage of

cross-validated HRRs smaller than 1 ($HRR_{av}<1$). The relation between protein expression and OS in the total study population and a number of population subgroups was visualised by Kaplan–Meier curves using the most frequently selected cutoff in the cross-validation procedure as the optimal cutoff. The potential interaction between protein expression and systemic therapy or primary tumour localisation was investigated using Cox regression. Combined prognostic values of EGFR and PTGS2 expressions were assessed by stepwise backward regression analysis with $P>0.1$ as exclusion criterion. Correlations were calculated using Pearson's correlation test. All statistical tests were two sided with *P*-values considered significant when <0.05. All data reported were REMARK compliant (McShane *et al*, 2005).

RESULTS

Demographic characteristics. Current study population consisted of 507 patients with CRCLM who were treated with liver surgery with curative intent. Cumulative 5-year OS of the study population was 41.2% (Supplementary Figure S1). Tissue specimens were available of all 507 patients, and corresponding primary tumour tissue was obtained for 234 patients. Characteristics of the patient study population are summarised in Supplementary Table S1 and described previously (Goos *et al*, 2013).

EGFR and PTGS2 expressions are associated with poor prognosis. Tissue specimens were immunohistochemically stained for EGFR and PTGS2 (Figure 2). EGFR was scored based on its CRCLM epithelial plasma membrane expression in 323 patients (Figure 1 and Supplementary Table S2). Patients with high EGFR expression had a significantly lower OS than patients with low EGFR expression (HRR_{av} 1.47; $P=0.03$; Supplementary Figure S2A). The total study population was dichotomised based on EGFR expression (Supplementary Figure S3A). Median OS for patients with low EGFR expression was 58 months and for patients with high EGFR expression 40 months (HRR 1.54, 95% CI 1.12–2.11, $P<0.01$; Figure 3A). To evaluate whether the prognostic value of EGFR expression was independent of established prognostic clinicopathological variables, a multivariate analysis was performed including EGFR protein expression, primary tumour-to-liver metastasis interval <12 months, number of liver metastases >1, maximal tumour diameter >5.0 cm, serum CEA level >200 ng ml^{-1} and lymph node positivity at the time of diagnosis of the primary tumour. Also on inclusion of these variables, EGFR expression was associated with poor OS (HRR_{av} 1.54; $P=0.02$; Supplementary Figure S2B).

PTGS2 immunohistochemical staining was scored based on its nuclear membrane expression in neoplastic CRCLM epithelium in 351 patients (Figure 1 and Supplementary Table S2). High expression of PTGS2 was associated with decreased OS (HRR_{av} 1.63; $P<0.01$; Supplementary Figure S2C). On the basis of dichotomisation of PTGS2 expression (Supplementary Figure S3B), median OS was 53 months for patients with PTGS2-negative CRCLM and 32 months for patients with PTGS2-positive CRCLM (HRR 1.60, 95% CI 1.14–2.26, $P<0.01$; Figure 3B). Multivariate analysis including clinicopathological variables showed that PTGS2 expression remained associated with poor prognosis (HRR_{av} 1.59; $P=0.01$; Supplementary Figure S2D).

When EGFR and PTGS2 expressions were combined with standard clinicopathological variables in a multivariate analysis, both EGFR (HRR 1.91, 95% CI 1.31–2.81, $P<0.01$) and PTGS2 (HRR 1.50, 95% CI 1.00–2.26, $P=0.05$) were retained, indicating that they function as independent prognostic variables. Patients with high CRCLM expression of both EGFR and PTGS2 had a significantly lower OS than patients without elevated EGFR and/or PTGS2 expression (HRR 1.88, 95% CI 1.18–2.98, $P<0.01$; Figure 3C), with a median OS difference of 43 months between patients with and without elevated levels of both EGFR and PTGS2.

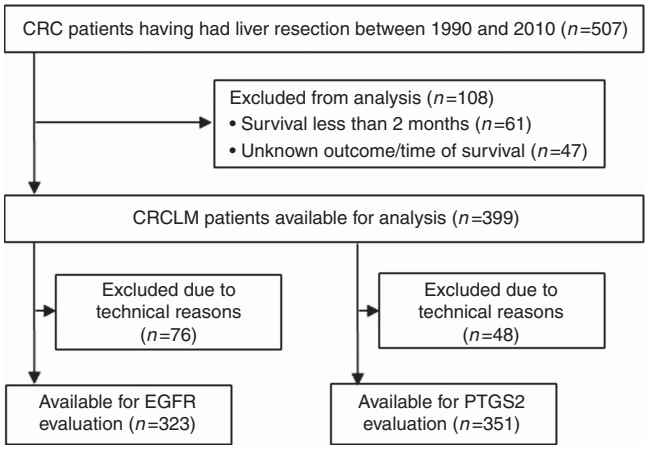

Figure 1. Schematic overview of the study cohort.

Prognostic value of EGFR and PTGS2 expressions in patient subgroups. Administration of systemic therapy, pre-, peri- or postoperative to liver resection, may affect clinical outcome.

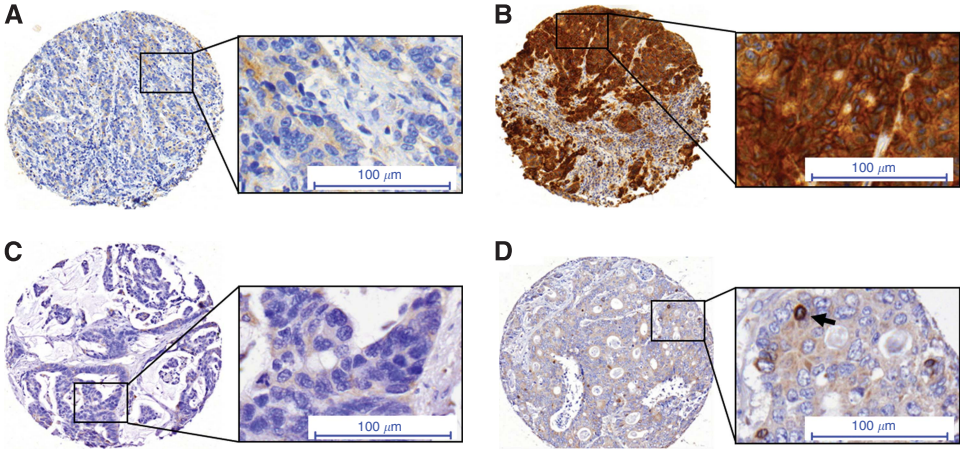

Figure 2. Staining examples of (**A**) low EGFR expression, (**B**) high EGFR expression, (**C**) low PTGS2 expression and (**D**) high PTGS2 expression (arrow) in epithelium of CRCLM.

Therefore, the prognostic value of EGFR and PTGS2 was stratified for treatment with systemic therapy (Supplementary Table S3). High EGFR and high PTGS2 expressions were significantly associated with poor survival in patients who did not receive systemic therapy within 6 months before or following surgery (HRR 1.78, 95% CI 1.19–2.67, $P<0.01$; Figure 4A and HRR 1.64, 95% CI 1.04–2.59, $P=0.04$; Figure 4B, respectively). In this subgroup, patients with high CRCLM expression of both EGFR and PTGS2 had particularly poor prognosis (HRR 3.08, 95% CI 1.66–5.73, $P<0.01$; Figure 4C). However, in the subgroup of patients who did receive systemic therapy, no significant associations between OS and EGFR or PTGS2 expression were observed (HRR 1.08, 95% CI 0.63–1.84, $P=0.78$; Figure 4D and HRR 1.27, 95% CI 0.74–2.17, $P=0.40$; Figure 4E, respectively). Similarly, in this subgroup, combined expression of EGFR and PTGS2 lacked prognostic value (HRR 0.81, 95% CI 0.37–1.77, $P=0.59$; Figure 4F). Notwithstanding these results in the subgroups, the interaction terms for treatment with systemic therapy and either EGFR expression ($P=0.12$) or PTGS2 expression ($P=0.33$) were not significant.

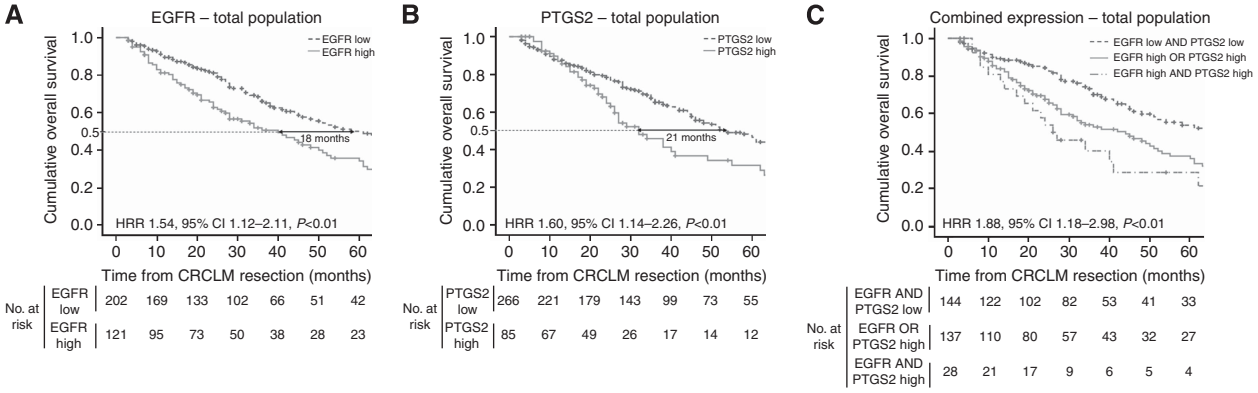

Figure 3. Kaplan–Meier graphs depicting OS in months stratified by (**A**) EGFR expression, (**B**) PTGS2 expression and (**C**) combined EGFR and PTGS2 expression. (**A**, **B**) HRR compares patients with high and low expression. (**C**) HRR compares patients in whom both EGFR and PTGS2 were highly expressed with patients in whom neither or either EGFR or PTGS2 levels were elevated.

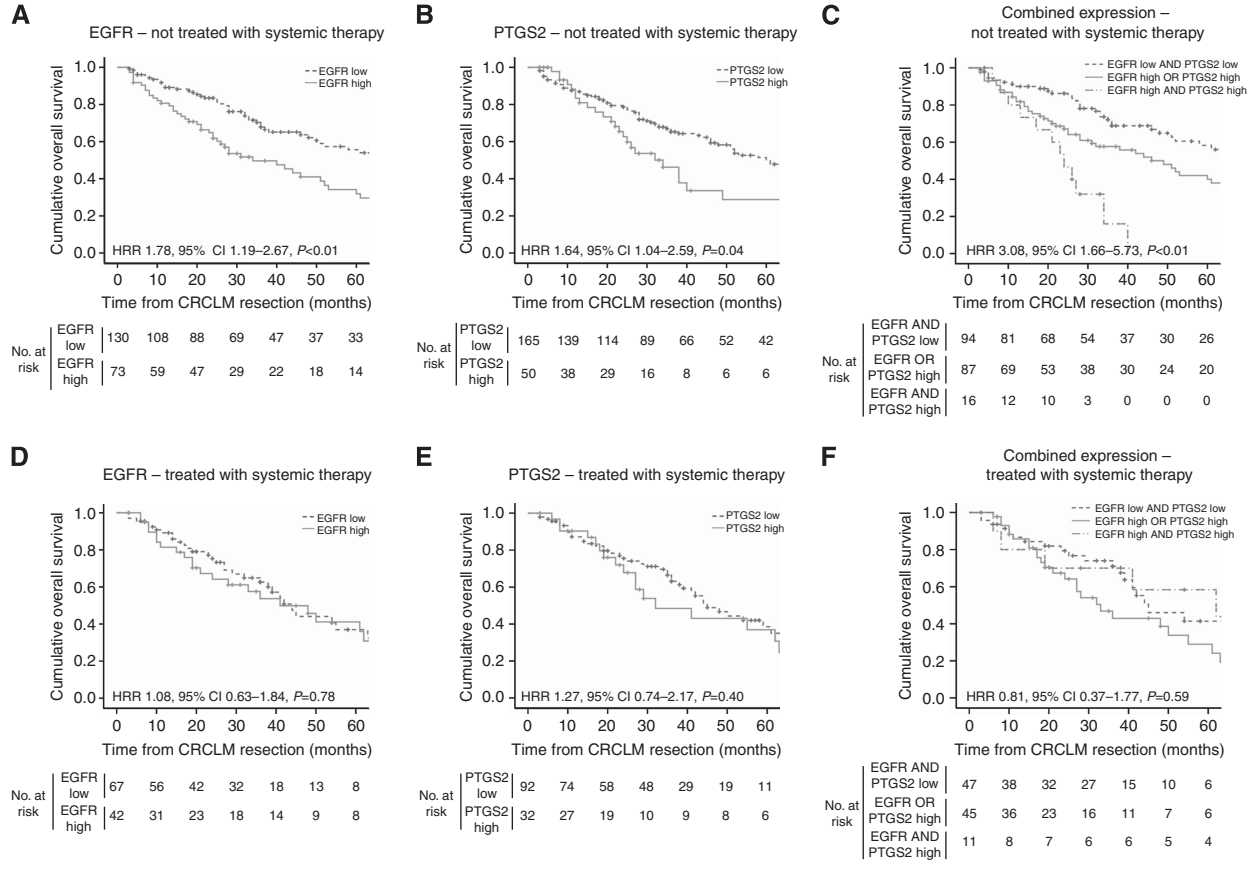

Figure 4. Kaplan–Meier graphs depicting OS in months for (**A**–**C**) patients in which CRCLM were not treated with systemic therapy and (**D**–**F**) patients in whom CRCLM were treated with systemic therapy. OS was stratified by (**A**, **D**) EGFR expression, (**B**, **E**) PTGS2 expression and (**C**, **F**) combined EGFR and PTGS2 expression. (**A**, **B**, **D**, **E**) HRR compares patients with high and low expression and (**C**,**F**) HRR compares patients in whom both EGFR and PTGS2 were highly expressed with patients in whom neither or either EGFR or PTGS2 levels were elevated. Information of systemic therapy was unavailable for $n=11$ and $n=12$ patients of which tissue samples were evaluated for EGFR and PTGS2 expression, respectively.

The prognostic value of EGFR and PTGS2 expressions in CRCLM was also separately evaluated in colon and rectal cancer patients. High EGFR expression was associated with poor survival of colon cancer patients (HRR 1.71, 95% CI 1.16–2.51, $P<0.01$; Supplementary Figure S4A), which was not observed for rectal cancer patients (HRR 1.19, 95% CI 0.66–2.15, $P=0.57$; Supplementary Figure S4C). PTGS2 expression was associated with decreased OS in both colon cancer patients (HRR 1.60, 95% CI 1.06–2.42, $P=0.03$; Supplementary Figure S4B) and rectal cancer patients (HRR 1.89, 95% CI 1.01–3.51, $P=0.05$; Supplementary Figure S4D). The interaction terms for primary tumour location and either EGFR expression ($P=0.55$) or PTGS2 expression ($P=0.62$) were not significant.

EGFR and PTGS2 expressions in primary CRC and corresponding CRCLM. As primary CRC tissue material is more readily available for pathological examination than CRCLM surgical specimens, we investigated whether EGFR and PTGS2 expressions in the primary tumour were correlated to their expression in patient-matched CRCLM. EGFR and PTGS2 expressions could be evaluated for 141 and 166 CRC-CRCLM pairs, respectively (Supplementary Figure S5). Expression of EGFR by primary tumours and corresponding CRCLM was inconsistent ($P=0.51$), whereas PTGS2 expression was concordant for 69.2% of CRC-CRCLM pairs ($P=0.02$; Supplementary Table S4). When patients who received systemic therapy preoperatively to primary CRC resection and/or liver resection were excluded from analysis, EGFR expression remained uncorrelated ($P=0.61$), whereas for PTGS2 expression the proportion of concordant pairs was 70.4% ($P<0.01$; Supplementary Table S5).

DISCUSSION

We investigated the prognostic value of EGFR and PTGS2 protein expressions in a large cohort of patients who underwent CRCLM resection with curative intent. High expression was associated with poor prognosis, with a difference in median OS between high and low expression of 18 months for EGFR and of 21 months for PTGS2. These differences are substantial, considering the median OS of the total study population of only 27 months, and correspond with the prognostic value of EGFR and PTGS2 expressions observed in earlier stages of CRC (Yamauchi *et al*, 2002; Soumaoro *et al*, 2004; Azria *et al*, 2005; Spano *et al*, 2005; Galizia *et al*, 2006; Ogino *et al*, 2008). Moreover, the difference in median OS was 43 months between patients with high expression of both EGFR and PTGS2 in CRCLM and patients with no elevated levels of EGFR and PTGS in CRCLM. Multivariate analysis demonstrated that EGFR and PTGS2 were prognostic biomarkers independent from each other and from well-known clinicopathological prognostic variables, such as positive lymph nodes at the time of primary tumour resection, presentation of metastases within 12 months after diagnosis of the primary tumour, presence of more than a single liver metastasis, high serum CEA level and maximal CRCLM diameter larger than 5.0 cm. Microsatellite instability (MSI) is a molecular variable with prognostic value that has previously been associated with EGFR and PTGS2 expressions in primary CRC and, as such, qualifies as a potential confounding factor (Karnes *et al*, 1998; Ogino *et al*, 2006; Yuan *et al*, 2009; Colussi *et al*, 2013). The MSI status of the patients in our cohort was not known; however, as only 3% of CRCLM is estimated to be microsatellite instable (Haddad *et al*, 2004), the confounding effects of MSI status are presumed to have a minor role in our study population.

Approximately one-third of the patients in our study population were treated with systemic therapy during the course of their disease. Stratification for treatment revealed that the prognostic value of EGFR and PTGS2 expressions was restricted to the subgroup of patients who did not receive systemic therapy. Especially patients with CRCLM that highly expressed both EGFR and PTGS had a dismal prognosis. In contrast, EGFR and PTGS2 expressions were not associated with OS in patients who did receive systemic therapy, suggesting that EGFR- and/or PTGS2-expressing tumours respond well to the commonly used 5FU-based chemotherapeutic regimens. Several inhibitors that specifically target EGFR (for example, cetuximab, panitumumab) or PTGS2 (for example, celecoxib) are being applied in clinical practice and significantly improved clinical outcome (Ciardiello and Tortora, 2008; Wang and Dubois, 2010). Dual blockade of EGFR and PTGS2 by specific inhibitors has proven effective in preclinical setting (Buchanan *et al*, 2007). In current study population, only a limited number of patients were treated with such inhibitors. That is, only two patients were treated with cetuximab and two patients with panitumumab. Therefore, whether these targeted drugs would be beneficial to EGFR- and/or PTGS2-expressing patients with resectable CRCLM remains to be established.

Colon and rectal cancers are frequently combined in clinical and experimental setting, as these cancer types appeared to be very similar on genomic level (Frattini *et al*, 2004; Cancer Genome Atlas Network, 2012). However, whether colon and rectal cancers should be considered as a single entity has often been debated. In the present study, high EGFR expression was associated with poor OS of colon cancer patients but not rectal cancer patients. This lack of prognostic value in rectal cancer patients may be explained by alteration differences in rectal cancer of genes influencing the *EGFR/PI3K* axis, such as *PTEN*, of which it has been shown that these affect survival of rectal cancer patients, but are not associated with survival in colon cancer patients (Bohn *et al*, 2013). PTGS2 expression was associated with poor survival in both colon and rectal cancer patients.

We also examined the correlation of protein expression between primary CRC and corresponding CRCLM, as alterations present in the primary tumour are frequently also present in the corresponding metastases (Stange *et al*, 2010; Knijn *et al*, 2011). Although PTGS2 expression in CRCLM was correlated to its expression in patient-matched primary tumours ($P=0.02$), this was not the case for EGFR expression ($P=0.51$). Similar differences in EGFR expression between primary CRC and CRCLM have been reported by others and could be explained by an increase of genetic alterations commonly detected during tumour progression and metastasis (Diep *et al*, 2006; Yarom *et al*, 2010). The correlation between PTGS2 expression in primary CRC and CRCLM may indicate that protein levels in CRCLM are to a certain extent predetermined by molecular alterations present in the primary CRC. Although predicting PTGS2 expression in CRCLM from its expression in the corresponding primary tumour may seem appealing, one should be cautious as still 30.8% of PTGS2 expression scores of CRC-CRCLM pairs were discordant. The primary CRC-CRCLM correlation observed for PTGS2 expression, and the lack of it for EGFR expression, suggests that the role of PTGS2 expression in early colorectal carcinogenesis is more prominent than that of EGFR expression. This is in accordance with earlier findings that identify alterations of PTGS2 expression as early phase events and relate changes in EGFR expression to later stages of cancer development (Charalambous *et al*, 2003; Attolini *et al*, 2010).

In conclusion, EGFR and PTGS2 expressions are prognostic biomarkers for patients with resectable CRCLM, predominantly in patients not treated with systemic therapy. Further research is required to fully characterise the impact of these and other molecular alterations, such as mutation status of the predictive biomarkers *KRAS*, *BRAF* and *PIK3CA* (Tougeron *et al*, 2013), and establish optimal treatment for individual patients with CRCLM.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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All collaborators of the DeCoDe PET group have contributed to data acquisition and approved the final paper after critical revision. Collaborators of the DeCoDe PET group are listed in alphabetical order per medical centre: NCT van Grieken, LR Perk, MP van den Tol, EA te Velde, AD Windhorst (VU University/VU University Medical Center – Amsterdam); J Baas, AM Rijken (Amphia Medical Center – Breda); MW van Beek, HJ Pijpers (Catharina Medical Center/PAMM Foundation – Eindhoven); H Bril, HBAC Stockmann, A Zwijnenburg (Kennemer Gasthuis/Spaarne Medical Center – Haarlem); K Bosscha, AJ van den Brule, CJ Hoekstra, JC van der Linden (Jeroen Bosch Medical Center – 's-Hertogenbosch); IH Borel Rinkes, PJ van Diest, R van Hillegersberg, O Kranenburg, MG Lam, N Snoeren (UMCU – Utrecht); IH Liem, RM Roumen, W Vening (MMC – Veldhoven).

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