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Introduction

The post-operative management of stage 2 colon cancer (CC) patients remains controversial[1, 2]. Traditional clinico-pathological prognostic markers define a group of high-risk patients who *may* benefit from adjuvant chemotherapy[3-5]. Recently gene expression signatures such as Oncotype *DX* Colon Cancer[6], detection of circulating cell-free tumour DNA[7, 8] and assessment of intratumoural immune infiltrate[9, 10] have all been associated with risk of recurrence. CDX2 expression is the one marker that appears to predict benefit from adjuvant chemotherapy[11].

Recently, there has been increased interest in examining the role of adjuvant aspirin therapy in early stage colorectal cancer (CRC) [12-14]. Aspirin reduces the risk of CRC in Lynch syndrome patients and is now considered a standard primary chemoprevention in this population[15-17]. For non-Lynch syndrome patients, several retrospective studies have suggested that regular aspirin use improves survival in CRC [18-20], although this benefit may be limited to CRC patients harbouring *PIK3CA* mutations (*PIK3CA*m). Liao and colleagues reported that regular aspirin use resulted in superior cancer-specific and overall survival (OS) in CRC patients with *PIK3CA*m, but not *PIK3CA* wildtype (*PIK3CA*wt) tumours[21]. This survival benefit was reproduced by Domingo and colleagues, who conducted a post-hoc analysis of the VICTOR trial (a large randomised phase III clinical trial comparing adjuvant rofecoxib versus placebo in stage 2 and 3 CC); their study demonstrated regular aspirin use was associated with improved recurrence free survival (RFS) in patients with *PIK3CA*m stage 2 or 3 CC, but not *PIK3CA*wt patients[22]. Other studies, using real world populations, have not been able to reproduce these data: both our group and a study by Reimers and colleagues demonstrated a trend towards a survival advantage, but no statistically significant survival benefit for regular aspirin use in *PIK3CAm* CRC [23, 24]. Further, a recent meta-analysis that included these four studies found aspirin use after a diagnosis of *PIK3CA*m CRC reduced total mortality without a significant improvement in cancer specific survival[25]. The evidence suggests aspirin may be useful in the secondary prevention of *PIK3CA*m CRC but this benefit is yet to be confirmed.

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In cancer research, analyses of real world data provide insights into medication usage and the impact of interventions on cancer outcomes. This is particularly valuable where randomized controlled trials have not addressed specific questions or where data from randomized studies are not yet available. While randomized studies are the 'gold standard' for establishing efficacy, they operate in an idealized environment and can only measure efficacy in selected populations, typically younger and fitter than routine care patients. Consequently, caution must be exercised when applying results achieved in the context of clinical trials to the general population.

Aspirin is a cheap, relatively safe and possibly effective adjuvant treatment for stage 2 colon cancer. Our study examines the impact of regular aspirin use on survival in a real world cohort of stage 2 CC patients; we explore the effect of aspirin use on RFS and OS, in both *PIK3CA*m and *PIK3CA*wt subgroups, in order to confirm the role of *PIK3CA* mutations as a biomarker for benefit from aspirin use.

Materials and Methods

Patient cohort

Patients were identified using the Australian Comprehensive Cancer Outcomes and Research Database (ACCORD) for CRC. This multidisciplinary prospective database collects demographic, histopathological and clinical data on patients at participating sites. All patients who underwent surgical resection for stage 2 CC between 1 January 2000 and 31 December 2011 were identified. Patients were excluded if they had synchronous tumours or underwent preoperative chemo-radiotherapy.

Data collection

Clinico-pathological data was extracted from ACCORD and included age, American Society of Anaesthetists (ASA) risk classification score (surrogate measure of fitness), tumour site (right versus left), T-stage, number of lymph nodes sampled and presence of lymphovascular invasion (LVI). ASA classification assesses the fitness of patients before surgery, assigning the following scores: 1) Healthy person; 2) Mild systemic disease; 3) Severe systemic disease; 4)

Severe systemic disease that is a constant threat to life; 5) A moribund person not expected to survive without the operation; 6) A declared brain-dead person whose organs are being removed for donation.

Chart review was conducted to identify regular aspirin use and Neutrophil-Lymphocyte Ratio (NLR) at diagnosis. Regular aspirin use was defined as ≥75mg aspirin daily at time of diagnosis. Data regarding duration of aspirin use following diagnosis was not collected. Neutrophil and lymphocyte counts were recorded if performed within 30 days prior to surgery. NLR was calculated as absolute neutrophil count divided by the absolute lymphocyte count, with "high NLR", representative of systemic inflammation, defined as a ratio of greater than 5 (as selected by numerous other studies[26-28]). High NLR is a validated poor prognostic factor in CRC[27, 29, 30].

Tumour analysis

Where available, tumour blocks were retrieved and *PIK3CA* mutation testing was conducted using next generation sequencing. A stained section was examined by a pathologist who marked the region of tumour, for macro-dissection from unstained slides. DNA was extracted and successful DNA extraction was confirmed. Targeted regions of exons 9 and 20 of the *PIK3CA* gene were amplified using multiplex PCR. Sequencing of the final library was performed on an Illumina MiSeq Next Generation Sequencer. Mutations were detected using MiSeq Reporter Software. The mutation allele fraction was determined by comparing the mutation allele read count with the total allele read count. In our laboratory, this assay has a limit of detection of approximately 3% mutation allele fraction. It is 100% specific for a mutation allele fraction over 5%, and 100% reproducible for a mutation allele fraction between 3% and 5%.

Mismatch repair status (MMR) was assessed as per standard of care and recorded where available.

Statistical analyses

Descriptive statistics were used to evaluate features observed in patients taking regular aspirin compared with those who were not. Where appropriate, Fisher's exact test or Pearson's Chi Square test were used to determine the significance of any differences observed. Similar analyses were undertaken when stratifying patients by both *PIK3CA* mutation and aspirin use.

Survival data was available through ACCORD. Data cut-off date for follow up was 1-Jan-2015. Patients who had not developed recurrence or were still alive were censored for recurrence and death respectively. RFS was defined as time from date of diagnosis to date of recurrence. OS was defined as time from date of diagnosis to date of death. Univariate and multivariate survival analyses were conducted using the Cox proportional hazards method. The multivariate model for RFS and OS analyses included factors with p-values <0.5 identified in the univariate analyses. Median RFS and OS for regular Aspirin use versus non-use were determined by the Kaplan Meier method.

Statistical software used included GraphPad® Prism 6 (GraphPad Software Inc., La Jolla, CA) and MedCalc 16.2.1 (MedCalc Software, Belgium). This study was approved by the relevant Human Research Ethics Committees.

Results

Clinico-pathological characteristics

We identified 488 patients with stage 2 CC diagnosed between 2000 and 2011. Patient and tumour characteristics are described in **Table 1**. Median age was 72 years (range 24.6 – 101.3 years). Regular aspirin use or non-use was recorded in 393 patients (81%). *PIK3CA*m were identified in 70 patients (14%). Data was generally complete, except for ASA score (27% unknown) and MMR status (54% unknown).

Table 1 also outlines differences between aspirin users and non-users. Aspirin users were significantly older (median age 76.4 versus 68.3 years, p<0.001), had a higher proportion of right sided cancers (62% versus 45%, p=0.005), a higher proportion of patients with ASA score 3-4 (58% versus 31%, p≤0.001) and a higher proportion of patients with

NLR>5 (39% versus 27%, p=0.027). Given these significant differences, we assessed for interaction and determined that patients with right sided cancers, ASA score 3-4 and NLR>5 were significantly more likely to be older (right sided cancers: median age 73 versus 69, p=0.0006, ASA score 3-4: median age 75 versus 67, p<0.001, NLR>5: median age 75.9 versus 70.0, p<0.0001).

Supplemental Table 1 outlines the differences between aspirin users and non-users in the *PIK3CAm* and *PIK3CAwt* cohorts. Aspirin users with *PIK3CA*m were significantly older than aspirin users in the *PIK3CAwt* subgroup.

Survival analyses

Univariate survival analyses determined that aspirin use was not significantly associated with a benefit in RFS or OS in the *PIK3CAm* **(Table 2)** or the *PIK3CAwt* cohort **(Table 3)**.

Figure 1 demonstrates a possible trend towards a RFS benefit with aspirin use in the *PIK3CAm* cohort (HR 0.45, 0.06-3.70, p=0.42); there were 16 aspirin users with only 1 RFS event, 40 non-users with 7 RFS events and 3 events in 14 patients whose aspirin use was unknown.

Figure 2 demonstrates no significant RFS benefit with aspirin use in the *PIK3CAwt* cohort (HR 0.77, 0.34-1.73, p=0.53); there were 66 aspirin users with 7 events and 235 non-users with 36 events. There were 13 events in 69 patients whose aspirin use was unknown.

In the *PIK3CA*m cohort **(Table 2)**, T4 stage and presence of LVI were significantly associated with poorer RFS, while increasing age, ASA score 3-4 and NLR>5 were significantly associated with poorer OS. In the larger *PIK3CAwt* cohort **(Table 3)**, again T4 stage and presence of LVI were significantly associated with poorer RFS, while in the OS analysis, increasing age, right sided primary cancer, aspirin use, T4 stage, ASA score 3-4 and baseline NLR>5 were all significantly associated with poorer OS. Given the large proportion of missing data MMR was not assessed in these patient subgroups.

OS analyses were conducted noting the interaction between aspirin, age, ASA score 3-4 and NLR>5. Aspirin use was associated with a trend towards poorer OS in the *PIK3CAm* group (HR 1.76, 0.51-6.04, p=0.37) and significantly poorer OS in the *PIK3CAwt* group (HR 2.50, 1.46-4.28, p=0.0008).

Table 4 outlines the univariate survival analysis of the entire cohort and **Table 5** outlines the multivariate survival analysis. Given the large proportion of missing data, MMR status was excluded from multivariate analyses. There were relatively few events for the *PIK3CA*m subgroup (11 RFS events, 17 OS events) therefore multivariate analyses were not conducted for either the *PIK3CA*m or *PIK3CA*wt subgroups. T4 stage and presence of LVI were significantly associated with poorer RFS whereas deficient MMR was significantly associated with improved RFS. In the multivariate model, MMR was excluded due to a large proportion of missing data, therefore only LVI remained as a significant poor prognostic factor for RFS. Aspirin use was not a significant prognostic factor in the multivariate model for RFS. In the OS analyses, increasing age, right sided primary, aspirin use, T4 stage, <12 LN sampled, ASA score 3-4, baseline NLR>5 were all significantly associated with poorer OS. In the multivariate model for OS, increasing age, right sided primary, T4 stage, <12 LN sampled and baseline NLR>5 remained significant poor prognostic factors. Aspirin use was not a significant prognostic factor in the multivariate model for OS. *PIK3CA* mutation status had no impact on RFS or OS.

Discussion

Data supporting the role of aspirin as an anti-cancer agent in CRC has been building. Its potential use as secondary chemoprevention has come under increased scrutiny with meta-analyses and a recent retrospective cohort study of 23 000 Norwegian patients suggesting post-diagnosis aspirin use improves overall and cancer-specific survival[31-33]. Prospective clinical trials are now underway to examine its role in early stage CC[13, 34, 35]. Retrospective analyses from clinical trial and selected health professional cohorts suggest the benefit of aspirin in CRC is limited to patients with *PIK3CA*m cancers[21, 22]. While these data are compelling, they have been contradicted by two other studies[23, 24]. The entirety of this data was recently examined by Palaeri and colleagues in a meta-analysis which suggested, but could not confirm, a definitive benefit to aspirin use in *PIK3CA*m disease[25]. Our study examined the impact of aspirin use on survival in stage 2 CC patients only. In particular, we investigated the role of *PIK3CA* mutations as a predictive biomarker for benefit from aspirin as secondary prevention. In our cohort of unselected stage 2 CC patients, aspirin use did not result in a statistically significant survival advantage in the *PIK3CA*m subgroup. A possible trend towards improved RFS was observed in these patients. Aspirin use was significantly associated with poorer OS and further analyses demonstrated this was a reflection of aspirin users being both older and frailer, as assessed by ASA score.

As the weight of recent evidence favoured a role for aspirin in *PIK3CA*m cancers, we sought an explanation for our discrepant results. Our finding that regular aspirin use was not associated with improved RFS and OS in *PIK3CA*m stage 2 CC patients is still consistent with previously published data if the low event rate and large confidence intervals are taken into account. Comparing our cohort to that studied by Domingo and colleagues demonstrates some of the challenges faced when interpreting real world data, even with what appears initially to be a large sample size. Domingo *et al* studied 896 patients with stage 2 and 3 CC (443 were stage 2 CC) and identified 104 patients with *PIK3CA*m cancers; of these, 14 were aspirin users with 0 RFS events, while 90 were non-users with 23 RFS events. We studied 488 stage 2 CC patients and identified 70 patients with *PIK3CA*m cancers; of these, 16 were aspirin users with 1 RFS event, while 40 were non-users with 7 RFS events. Both cohorts are quite similar, with comparable median follow up, 61.5 months by Domingo and 63.6 months for our cohort. There were fewer events

observed in our non-users (7 of 40 in our cohort versus 23 of 90 in Domingo's) which likely accounts for the discrepancy in survival outcomes. Our study was limited to the better prognosis stage 2 patients where events are less frequent, while Domingo and colleagues included both stage 2 and stage 3 patients. Additionally, the small numbers of aspirin users in both studies accentuates the statistical impact of the solitary event in our *PIK3CA*m aspirin user group.

There are clear differences between the populations studied by both Domingo and colleagues (randomised clinical trial participants), Liao and colleagues (health professionals) and the patients included in our study which may also account for our conflicting results. These differences include age, tumour location and fitness. Domingo and colleagues studied a clinical trial cohort, where patients are generally of better performance status. Liao and colleagues studied a cohort of health professionals, who may be more health conscious, more vigilant with preventative medicine strategies and come from higher socioeconomic groups, all of which can impact outcomes. Our cohort were considerably older: median age 72 years compared to 64 years and 68 years in the Domingo and Liao cohorts respectively. In our study aspirin users were also significantly older than non-users (76 vs 68 years, p <0.001) whereas there was no difference in the Liao cohort and, in the Domingo cohort, the difference was smaller (69 vs 63 years, p<0.001). Aspirin use has been shown to reduce the incidence of right sided colon cancers[19], which carry a poorer prognosis than left sided tumours[36]. In both the Domingo and Liao studies, there was no difference in primary tumour site between aspirin users and non-users, but in our study, aspirin users were *more* likely to have right sided cancers. This is best explained by the increased age of aspirin users in our cohort, as right sided cancers are more common in older patients[37]. A unique aspect of our study is the inclusion of ASA score as a measure of patient fitness. ASA score was available for 73% of our cohort and this data demonstrated aspirin users were significantly less fit than non-users (ASA 3-4 in 58% Aspirin users vs 31% non-users, p <0.001).

Our cohort included all consenting patients with Stage 2 CC treated at our centres and as such provided a true representation of the diversity of the general population and their clinical outcomes. The differences in age, primary

tumour site and fitness of aspirin users in our cohort are likely to have influenced our ability to demonstrate a survival benefit. This assertion is supported by our statistical analyses demonstrating interaction between these factors. We believe these differences provide a reasonable explanation why, in contrast to earlier reports and acknowledging the limitations of cross trial comparisons, we were unable to demonstrate a survival advantage for aspirin use in patients with Stage 2 *PIK3CA*m CRC.

A further limitation of our study was its retrospective design. While the clinico-pathological data was collected prospectively, data regarding aspirin use was collected retrospectively by chart review, and was missing in 19% of patients. Data was also incomplete for ASA scores, MSI status and *PIK3CA* mutation status (**Table 1**). We collected data on aspirin use at the time of diagnosis whereas previous studies have suggested the benefit of aspirin in CRC may be restricted to patients who commence treatment after their cancer diagnosis[18, 33, 38, 39]. Although we did not demonstrate a survival advantage with regular aspirin use, a real and significant benefit in stage 2 CC cannot be excluded based on our findings. Clinical trials, such as ASPREE, ASCOLT and ADD-ASPIRIN should include analyses of tumour tissue, blood markers and other translational endpoints[13, 14, 34]. This research is critical to further our understanding of the mechanism of action of aspirin and its effects in CRC and hopefully provide a definitive answer to the question of aspirin's utility in early stage *PIK3CA*m disease.

Conclusion

Currently we have no reliable biomarkers to predict which patients will benefit from adjuvant therapies in Stage 2 CC. Aspirin is a relatively safe, cost-effective intervention that may reduce the risk of recurrence in patients with *PIK3CA*m disease. Data demonstrating survival benefits associated with aspirin use in *PIK3CA*m CRC have been impressive but our study was unable to replicate these findings. The 'real world' nature of our cohort and the subsequent uncontrolled differences in age and comorbidities are likely to have contributed to our negative results. Clinical trials examining the role of adjuvant aspirin use in early stage CC are already underway and their results are eagerly anticipated.

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References

1 Benson AB, 3rd, Schrag D, Somerfield MR, Cohen AM, Figueredo AT, Flynn PJ*, et al.* American Society of Clinical Oncology recommendations on adjuvant chemotherapy for stage II colon cancer. *J Clin Oncol*. 2004; **22**: 3408-19.

2 Clinical Practice Guidelines in Oncology (NCCN Guidelines) Colon Cancer v2.2016. Vol. 2015.

3 Betge J, Pollheimer MJ, Lindtner RA, Kornprat P, Schlemmer A, Rehak P*, et al.* Intramural and extramural vascular invasion in colorectal cancer: prognostic significance and quality of pathology reporting. *Cancer*. 2012; **118**: 628-38.

4 McKenzie S, Nelson R, Mailey B, Lee W, Chung V, Shibata S*, et al.* Adjuvant chemotherapy improves survival in patients with American Joint Committee on Cancer stage II colon cancer. *Cancer*. 2011; **117**: 5493-9.

5 Betge J, Rehak P, Langner C. Adjuvant chemotherapy improves survival in patients with American Joint Committee on Cancer stage II colon cancer. *Cancer*. 2012; **118**: 2184; author reply 84-5.

6 Gray RG, Quirke P, Handley K, Lopatin M, Magill L, Baehner FL*, et al.* Validation study of a quantitative multigene reverse transcriptase-polymerase chain reaction assay for assessment of recurrence risk in patients with stage II colon cancer. *J Clin Oncol*. 2011; **29**: 4611-9.

7 Tie J. Circulating tumor DNA (ctDNA) as a marker of recurrence risk in stage II colon cancer (CC). *American Society of Clinical Oncology Annual Meeting*. Vol. 32. Journal of Clinical Oncology 2014.

8 Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M*, et al.* Circulating mutant DNA to assess tumor dynamics. *Nat Med*. 2008; **14**: 985-90.

9 Galon J, Pages F, Marincola FM, Thurin M, Trinchieri G, Fox BA*, et al.* The immune score as a new possible approach for the classification of cancer. *J Transl Med*. 2012; **10**: 1.

10 Pages F, Kirilovsky A, Mlecnik B, Asslaber M, Tosolini M, Bindea G*, et al.* In situ cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer. *J Clin Oncol*. 2009; **27**: 5944-51.

11 Dalerba P, Sahoo D, Paik S, Guo X, Yothers G, Song N*, et al.* CDX2 as a Prognostic Biomarker in Stage II and Stage III Colon Cancer. *N Engl J Med*. 2016; **374**: 211-22.

12 Chia WK, Ali R, Toh HC. Aspirin as adjuvant therapy for colorectal cancer--reinterpreting paradigms. *Nat Rev Clin Oncol*. 2012; **9**: 561-70.

13 Ali R, Toh HC, Chia WK, Investigators AT. The utility of Aspirin in Dukes C and High Risk Dukes B Colorectal cancer--the ASCOLT study: study protocol for a randomized controlled trial. *Trials*. 2011; **12**: 261.

14 Langley RE. Add-Aspirin trial: A phase III, double-blind, placebo-controlled, randomized trial assessing the effects of aspirin on disease recurrence and survival after primary therapy in common nonmetastatic solid tumors. *American Society of Clinical Oncology Annual Meeting*. Vol. 32. Journal of Clinical Oncology 2014.

15 Burn J, Gerdes AM, Macrae F, Mecklin JP, Moeslein G, Olschwang S*, et al.* Long-term effect of aspirin on cancer risk in carriers of hereditary colorectal cancer: an analysis from the CAPP2 randomised controlled trial. *Lancet*. 2011; **378**: 2081-7.

16 Movahedi M, Bishop DT, Macrae F, Mecklin JP, Moeslein G, Olschwang S*, et al.* Obesity, Aspirin, and Risk of Colorectal Cancer in Carriers of Hereditary Colorectal Cancer: A Prospective Investigation in the CAPP2 Study. *J Clin Oncol*. 2015; **33**: 3591-7.

17 Ait Ouakrim D, Dashti SG, Chau R, Buchanan DD, Clendenning M, Rosty C*, et al.* Aspirin, Ibuprofen, and the Risk of Colorectal Cancer in Lynch Syndrome. *J Natl Cancer Inst*. 2015; **107**.

18 Chan AT, Ogino S, Fuchs CS. Aspirin use and survival after diagnosis of colorectal cancer. *JAMA*. 2009; **302**: 649-58.

19 Rothwell PM, Wilson M, Elwin CE, Norrving B, Algra A, Warlow CP*, et al.* Long-term effect of aspirin on colorectal cancer incidence and mortality: 20-year follow-up of five randomised trials. *Lancet*. 2010; **376**: 1741-50.

20 Rothwell PM, Fowkes FG, Belch JF, Ogawa H, Warlow CP, Meade TW. Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patient data from randomised trials. *Lancet*. 2011; **377**: 31-41.

21 Liao X, Lochhead P, Nishihara R, Morikawa T, Kuchiba A, Yamauchi M*, et al.* Aspirin use, tumor PIK3CA mutation, and colorectal-cancer survival. *N Engl J Med*. 2012; **367**: 1596-606.

22 Domingo E, Church DN, Sieber O, Ramamoorthy R, Yanagisawa Y, Johnstone E*, et al.* Evaluation of PIK3CA mutation as a predictor of benefit from nonsteroidal anti-inflammatory drug therapy in colorectal cancer. *J Clin Oncol*. 2013; **31**: 4297-305.
23 Reimers MS, Bastiaannet E, Langley RE, van Eijk R, van Vlierberghe RL, Lemmens VE*, et al.* Expression of HLA class I antigen, aspirin use, and survival after a diagnosis of colon cancer. *JAMA Intern Med*. 2014; **174**: 732-9.
24 Kothari N, Kim R, Jorissen RN, Desai J, Tie J, Wong HL*, et al.* Impact of regular aspirin use on overall and cancer-specific survival in patients with colorectal cancer harboring a PIK3CA mutation. *Acta Oncol*. 2015; **54**: 487-92.
25 Paleari L, Puntoni M, Clavarezza M, DeCensi M, Cuzick J, DeCensi A. PIK3CA Mutation, Aspirin Use after Diagnosis and Survival of Colorectal Cancer. A Systematic Review and Meta-analysis of Epidemiological Studies. *Clin Oncol (R Coll Radiol)*. 2016; **28**: 317-26.
26 Turner N, Wong HL, Templeton A, Tripathy S, Whiti Rogers T, Croxford M*, et al.* Analysis of local chronic inflammatory cell infiltrate combined with systemic inflammation improves prognostication in stage II colon cancer independent of standard clinicopathologic criteria. *Int J Cancer*. 2016; **138**: 671-8.
27 Guthrie GJ, Roxburgh CS, Farhan-Alanie OM, Horgan PG, McMillan DC. Comparison of the prognostic value of longitudinal measurements of systemic inflammation in patients undergoing curative resection of colorectal cancer. *Br J Cancer*. 2013; **109**: 24-8.
28 Kishi Y, Kopetz S, Chun YS, Palavecino M, Abdalla EK, Vauthey JN. Blood neutrophil-to-lymphocyte ratio predicts survival in patients with colorectal liver metastases treated with systemic chemotherapy. *Ann Surg Oncol*. 2009; **16**: 614-22.
29 McMillan DC, Canna K, McArdle CS. Systemic inflammatory response predicts survival following curative resection of colorectal cancer. *Br J Surg*. 2003; **90**: 215-9.
30 Canna K, McMillan DC, McKee RF, McNicol AM, Horgan PG, McArdle CS. Evaluation of a cumulative prognostic score based on the systemic inflammatory response in patients undergoing potentially curative surgery for colorectal cancer. *Br J Cancer*. 2004; **90**: 1707-9.
31 Bains SJ, Mahic M, Myklebust TA, Smastuen MC, Yaqub S, Dorum LM*, et al.* Aspirin As Secondary Prevention in Patients With Colorectal Cancer: An Unselected Population-Based Study. *J Clin Oncol*. 2016; **34**: 2501-8.
32 Ye XF, Wang J, Shi WT, He J. Relationship between aspirin use after diagnosis of colorectal cancer and patient survival: a meta-analysis of observational studies. *Br J Cancer*. 2014; **111**: 2172-9.
33 Li P, Wu H, Zhang H, Shi Y, Xu J, Ye Y*, et al.* Aspirin use after diagnosis but not prediagnosis improves established colorectal cancer survival: a meta-analysis. *Gut*. 2015; **64**: 1419-25.
34 Group AI. Study design of ASPirin in Reducing Events in the Elderly (ASPREE): a randomized, controlled trial. *Contemp Clin Trials*. 2013; **36**: 555-64.
35 http://www.addaspirintrial.org/.
36 Venook AP, Niedzwiecki D, Innocenti F, Fruth B, Greene C, O'Neill B*, et al.* Impact of primary (1º) tumor location on overall survival (OS) and progression-free survival (PFS) in patients (pts) with metastatic colorectal cancer (mCRC): Analysis of CALGB/SWOG 80405 (Alliance). *J Clin Oncol*. 2016; **34**.
37 Pappas AV, Lagoudianakis EE, Dallianoudis IG, Kotzadimitriou KT, Koronakis NE, Chrysikos ID*, et al.* Differences in colorectal cancer patterns between right and left sided colorectal cancer lesions. *J BUON*. 2010; **15**: 509-13.
38 Bastiaannet E, Sampieri K, Dekkers OM, de Craen AJ, van Herk-Sukel MP, Lemmens V*, et al.* Use of aspirin postdiagnosis improves survival for colon cancer patients. *Br J Cancer*. 2012; **106**: 1564-70.
39 McCowan C, Munro AJ, Donnan PT, Steele RJ. Use of aspirin post-diagnosis in a cohort of patients with colorectal cancer and its association with all-cause and colorectal cancer specific mortality. *Eur J Cancer*. 2013; **49**: 1049-57.

Figure Legends

Figure 1. Recurrence free survival for regular Aspirin use versus non-use in *PIK3CA* mutated stage 2 colon cancer

Abbreviations: RFS recurrence free survival; (mo) months

Figure 2. Recurrence free survival for regular Aspirin use versus non-use in *PIK3CA* wildtype stage 2 colon cancer

Abbreviations: RFS recurrence free survival; (mo) months

Table 1. Baseline patient and tumour characteristics for the entire cohort and stratified by Aspirin use

	Entire cohort		Aspirin use		Non use		P-value
	N=488		N=95† (20%)		N=296† (61%)		
Age							
Median age	72		76.4		68.3		<0.001*
Sex							
Male	266	55%	56	59%	151	51%	0.19
Female	222	45%	39	41%	145	49%	
Tumour site							
Right colon	243	50%	59	62%	133	45%	0.005*
Left colon	245	50%	36	38%	163	55%	
T-stage							
T3	409	84%	82	86%	246	83%	0.52
T4	79	16%	13	14%	50	17%	
LN sampled							
≥12	384	79%	68	72%	238	80%	0.09
<12	104	21%	27	28%	58	20%	
LVI							
Yes	126	26%	21	22%	77	26%	0.49
No	328	67%	68	72%	201	68%	
Unknown	34	7%	6	6%	18	6%	
ASA							
1-2	186	38%	22	23%	132	45%	<0.001*

3-4	171	35%	55	58%	92	31%	
Unknown	131	27%	18	19%	72	24%	
PIK3CA							
Mutation	70	14%	16	17%	40	14%	0.30
Wildtype	370	76%	66	70%	235	79%	
Unknown	48	10%	13	13%	21	7%	
Baseline NLR							
NLR>5	147	30%	37	39%	80	27%	0.027*
NLR<=5	333	68%	55	58%	213	72%	
Unknown	8	2%	3	3%	3	1%	
MMR							
Deficient	48	10%	10	11%	33	11%	0.83
Proficient	176	36%	30	32%	110	37%	
Unknown	264	54%	55	57%	153	52%	

†regular Aspirin use at diagnosis unknown in 97 patients (19%)

LN = Lymph Node; LVI = Lymphovascular invasion; ASA = American Society of Anaesthesiologists physical status classification; NLR = Neutrophil-Lymphocyte ratio; MMR = Mismatch repair

Supplemental Table S1. Clinico-pathological features stratified by *PIK3CA* mutation and Aspirin use

	***PIK3CA* mutation (N=70)**				***PIK3CA* wildtype (N=370)**				
	Aspirin use		**Non use**		**Aspirin use**		**Non use**		**P-value**
	N=16†		**N=40†**		**N=66‡**		**N=235‡**		
Age									
Median age	80.8		64.4		75.4		69.7		0.005*
Sex									
Male	9	56%	17	42%	37	56%	119	50%	0.23
Female	7	44%	23	58%	29	44%	116	50%	
Tumour site									
Right colon	13	81%	22	55%	40	60%	102	43%	0.31
Left colon	3	19%	18	45%	26	40%	133	57%	
T-stage									
T3	15	94%	33	83%	56	85%	198	84%	0.19
T4	1	6%	7	17%	10	15%	37	16%	
LN sampled									
≥12	16	100%	34	85%	45	68%	186	79%	0.06
<12	0	0%	6	15%	21	32%	49	21%	
LVI									
Yes	1	6%	10	25%	15	23%	60	26%	0.68
No	14	88%	30	75%	47	71%	158	67%	
Unknown	1	6%	0	-	4	6%	17	7%	
ASA									

1-2	6	38%	24	60%	14	21%	99	42%	0.14
3-4	8	50%	6	15%	41	62%	81	34%	
Unknown	2	12%	10	25%	11	17%	55	24%	
Baseline NLR									
NLR>5	5	31%	9	23%	26	39%	64	27%	0.75
NLR<=5	11	69%	31	77%	38	58%	168	71%	
Unknown	0	-	0	-	2	3%	3	2%	
MMR									
Deficient	2	12%	5	13%	8	12%	25	11%	1.00
Proficient	3	19%	16	40%	22	33%	91	39%	
Unknown	11	71%	19	47%	36	55%	119	50%	

†Aspirin use unknown in 14 (20%) of patients with colon cancer harbouring *PIK3CA* mutation

‡Aspirin use unknown in 69 (19%) patients with *PIK3CA* wildtype colon cancer

Table 2. Survival analyses for *PIK3CA* mutation cohort

	RFS (11 events)			OS (17 events)		
	HR	**95%CI**	**P-value**	**HR**	**95%CI**	**P-value**
Older age	0.99	0.94, 1.04	0.63	1.05	1.00, 1.10	0.03*
Sex						
Male	-	-	-	-	-	-
Female	1.68	0.49, 5.76	0.40	0.92	0.35, 2.40	0.87
Tumour site						
Right colon	-	-	-	-	-	-
Left colon	1.50	0.46, 4.90	0.51	1.65	0.63, 4.36	0.31
Aspirin use†						
No use	-	-	-	-	-	-
Aspirin	0.45	0.06, 3.70	0.42	1.76	0.51, 6.04	0.37
T-stage						
T3	-	-	-	-	-	-
T4	3.86	1.00, 14.8	0.05	1.57	0.45, 5.56	0.48
LN sampled						
≥12	0.81	0.17, 3.78	0.79	0.85	0.25, 3.01	0.81
<12	-	-	-	-	-	-
LVI†						
Yes	5.15	1.50, 17.6	0.0078	0.93	0.29, 2.91	0.91
No	-	-	-	-	-	-
ASA†						

1-2	-	-	-	-	-	-
3-4	1.00	0.20, 5.02	0.99	3.59	1.13, 11.42	0.03*
Baseline NLR†						
NLR>5	1.62	0.43, 6.13	0.48	3.18	1.22, 8.26	0.018*
NLR<=5	-	-	-	-	-	-

†missing data as per table 1

‡ MMR not included in this analysis due to small numbers and small numbers of events

Table 3. Survival analyses for *PIK3CA* wildtype cohort

	RFS (56 events)			OS (80 events)		
	HR	**95%CI**	**P-value**	**HR**	**95%CI**	**P-value**
Age	1.01	0.99, 1.04	0.22	1.09	1.06, 1.11	<0.0001*
Sex						
Male	-	-	-	-	-	-
Female	0.88	0.52, 1.49	0.64	0.90	0.58, 1.41	0.65
Tumour site						
Right colon	-	-	-	-	-	-
Left colon	1.21	0.71, 2.06	0.48	0.53	0.34, 0.83	0.0059*
Aspirin use†						
Aspirin	0.77	0.34, 1.73	0.53	2.50	1.46, 4.28	0.0008*
No use	-	-	-	-	-	-
T-stage						
T3	-	-	-	-	-	-
T4	2.05	1.12, 3.76	0.020	2.22	1.36, 3.63	0.0015*
LN sampled						
≥12	0.92	0.49, 1.70	0.78	0.59	0.37, 0.94	0.028*
<12	-	-	-	-	-	-
LVI†						
Yes	2.46	1.41, 4.30	0.0016	1.38	0.84, 2.25	0.20
No	-	-	-	-	-	-
ASA†						

1-2						
3-4	1.29	0.71, 2.35	0.41	3.54	1.89, 6.64	0.0001*
Baseline NLR†						
NLR>5	1.29	0.68, 2.10	0.53	2.75	1.76, 4.30	<0.0001*
NLR<=5						

†missing data as per table 1

‡ MMR not included in this analysis due to small numbers and small numbers of events

Table 4. Univariate Survival analyses for entire cohort

	RFS (74 events)			OS (117 events)		
	HR	**95%CI**	**P-value**	**HR**	**95%CI**	**P-value**
Age	1.01	0.99, 1.03	0.28	1.08	1.06, 1.10	<0.001*
Sex						
Male	-	-	-	-	-	-
Female	0.92	0.58, 1.46	0.73	0.81	0.56, 1.18	0.27
Tumour site						
Right colon	-	-	-	-	-	-
Left colon	1.06	0.67, 1.68	0.78	0.64	0.44, 0.92	0.016*
Aspirin use†						
Aspirin	0.75	0.38, 1.50	0.42	2.34	1.51, 3.62	<0.001*
No use	-	-	-	-	-	-
T-stage						
T3	-	-	-	-	-	-
T4	2.23	1.33, 3.72	0.002*	1.98	1.31, 3.01	0.001*
LN sampled						
≥12	0.83	0.49, 1.40	0.48	0.65	0.44, 0.96	0.03*
<12	-	-	-	-	-	-
LVI†						
Yes	2.71	1.69, 4.37	<0.001*	1.20	0.80, 1.79	0.38
No	-	-	-	-	-	-
ASA†						

1-2	-	-	-	-	-	-
3-4	1.22	0.71, 2.09	0.46	3.05	1.88, 4.95	<0.001*
PIK3CA†						
Mutation	1.05	0.55, 2.01	0.88	1.24	0.73, 2.10	0.42
Wildtype	-	-	-	-	-	-
Baseline NLR†						
NLR>5	1.29	0.79, 2.10	0.31	2.73	1.89, 3.94	<0.001*
NLR<=5	-	-	-	-	-	-
MMR†						
Deficient	0.23	0.07, 0.75	0.014*	0.81	0.41, 1.60	0.54
Proficient	-	-	-	-	-	-

†missing data as per table 1

Table 5. Multivariate analyses for entire cohort

	RFS (74 events)		
	HR	**95%CI**	**P-value**
Age	1.02	0.99, 1.05	0.09
Aspirin use†			
Aspirin	0.63	0.30, 1.32	0.22
No use	-	-	-
T-stage			
T3	-	-	-
T4	1.67	0.88, 3.16	0.11
LN sampled			
≥12	0.67	0.36, 1.25	0.21
<12	-	-	-
LVI†			
Yes	2.79	1.57, 4.92	0.0004*
No	-	-	-
ASA†			
1-2	-	-	-
3-4	1.25	0.65, 2.41	0.51
Baseline NLR†			
NLR>5	0.90	0.47, 1.72	0.75
NLR<=5	-	-	-
	OS (117 events)		

	HR	95%CI	P-value
Age	1.07	1.04, 1.10	<0.0001*
Sex			
Male	-	-	-
Female	1.36	0.81, 2.27	0.25
Tumour site			
Right colon	-	-	-
Left colon	0.56	0.33, 0.97	0.038*
Aspirin use†			
Aspirin	1.26	0.72, 2.21	0.41
No use	-	-	-
T-stage			
T3	-	-	-
T4	3.59	1.99, 6.46	<0.0001*
LN sampled			
≥12	0.47	0.27, 0.81	0.0061*
<12	-	-	-
LVI†			
Yes	1.09	0.60, 1.97	0.79
No	-	-	-
ASA†			
1-2	-	-	-
3-4	1.79	0.99, 3.91	0.053
PIK3CA†			

Mutation	1.74	0.86, 3.55	0.126
Wildtype	-	-	-
Baseline NLR†			
NLR>5	2.27	1.34, 3.88	0.0024*
NLR<=5	-	-	-

†missing data as per table 1

‡ MMR excluded from multivariate analysis due to large proportion of missing data

§only factors with p-values <0.5 included in multivariate analysis

Abstract

Background

Data suggest aspirin improves survival in CRC harbouring *PIK3CA* mutations. The impact of aspirin is thought predominantly to be via an anti-inflammatory effect.

Aims

To explore the effect of aspirin use on survival in a real world cohort of Stage 2 colon cancer (CC) patients.

Methods

A prospective CRC database identified patients diagnosed with stage 2 CC between 2000 and 2011. *PIK3CA* mutation status was determined by next generation sequencing. Neutrophil-Lymphocyte ratio (NLR) greater than 5 at diagnosis represented systemic inflammation. Chart review was used to record regular aspirin use at diagnosis. Clinico-pathological features and survival data were available. Survival analyses utilised the Cox proportional hazards method.

Results

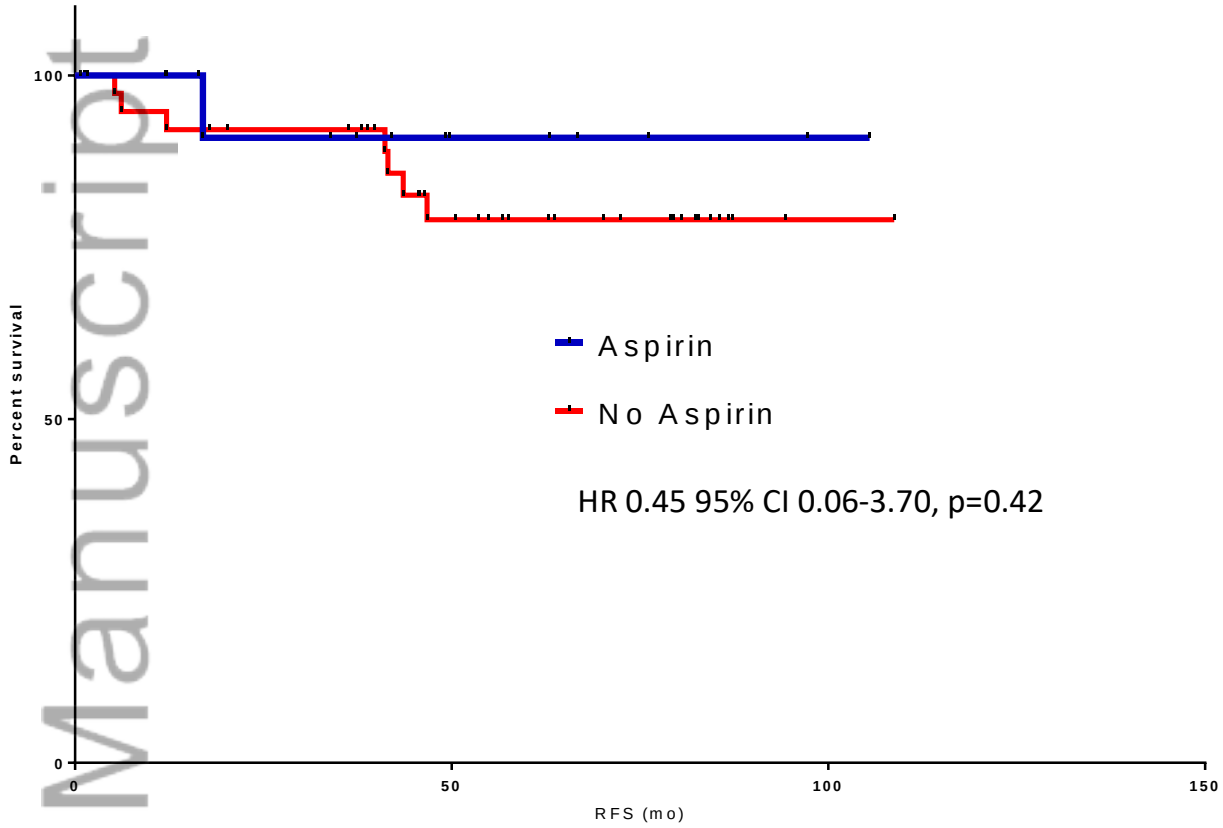
Of 488 patients with stage 2 CC, 95 patients were aspirin users and 70 patients had PIK3CA mutations. Aspirin users were more likely to be older (median: 76.4 years versus 68.3 years, $p<0.001$), to be less fit (American Society of Anaesthetists Score 3-4: 58% versus 31%, $p<0.001$), and to have systemic inflammation (NLR>5: 39% versus 27%, $p=0.027$). Regular aspirin use did not significantly improve recurrence free survival: In the PIK3CA mutated group, there was a trend towards improved recurrence free survival (HR 0.45, $p=0.42$).

Conclusions

Our study did not demonstrate a significant survival advantage from aspirin use in Stage 2 *PIK3CA* mutated CC. The 'real world' nature of our cohort and the subsequent uncontrolled differences in age and fitness in aspirin users are likely to have contributed to this result. Defining the true impact of aspirin in CRC requires prospective randomised clinical trials.

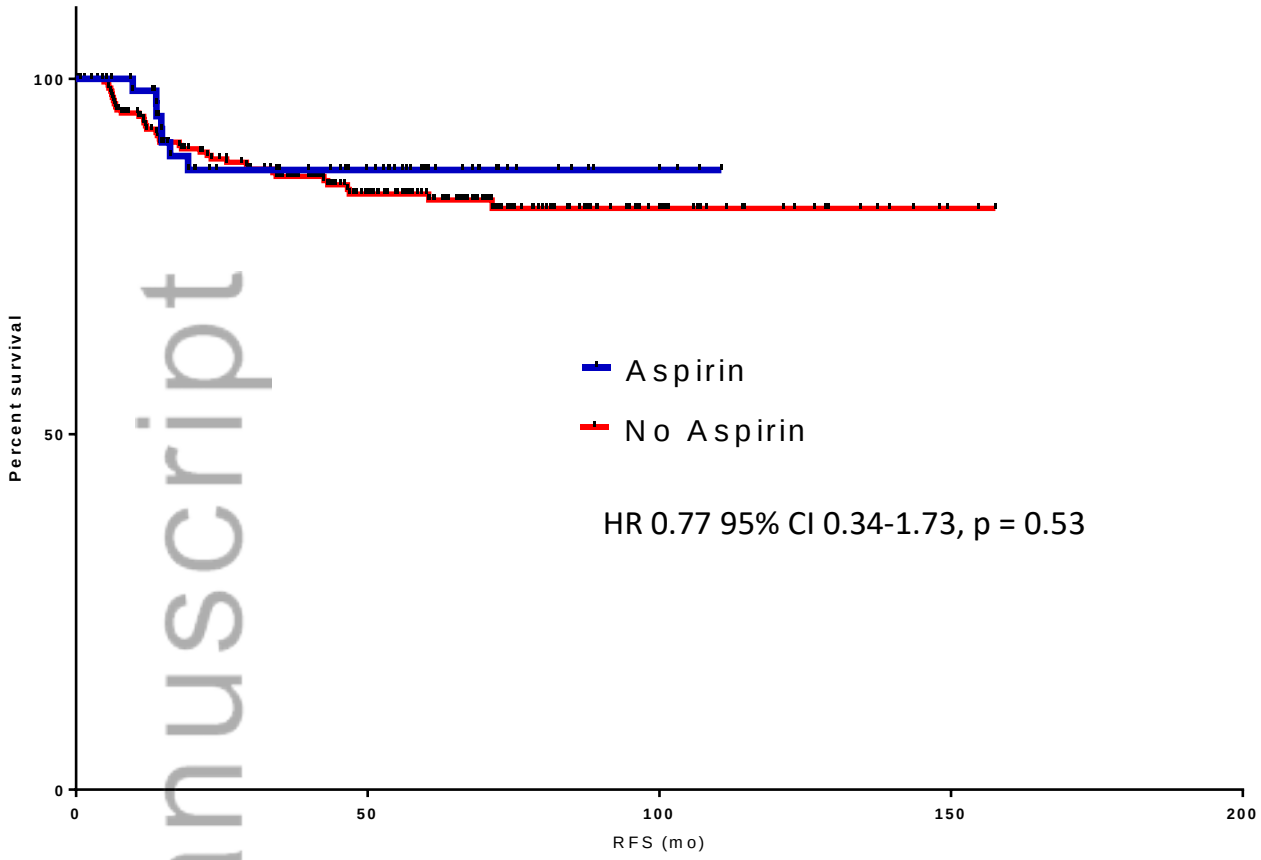
Key words: Aspirin, Colon Cancer, Colorectal Cancer, PIK3CA, Real World data

Figure 1. Recurrence free survival for regular Aspirin use versus non-use in PIK3CA mutated stage 2 colon cancer



No. at risk				
Aspirin	16	15	15	15
No Aspirin	40	33	33	33

Figure 2. Recurrence free survival for regular Aspirin use versus non-use in PIK3CA wildtype stage 2 colon cancer



No. at risk					
Aspirin	66	59	59	59	59
No Aspirin	235	201	199	199	199

Examining the Impact of Regular Aspirin Use and PIK3CA Mutations on Survival in Stage 2 Colon Cancer

Authors: Caitlin Murphy (1), Natalie Turner (2), Hui-Li Wong (2), Mathu Sinnathamby (3), Jeanne Tie (1,2,3), Belinda Lee (2), Jayesh Desai (1,2,3), Iain Skinner (4), Michael Christie (2,5,6), Ryan Hutchinson (6), Sebastian Lunke (6), Paul Waring (6), Peter Gibbs (1,2,3), Ben Tran (1,2,3)

Positions:

Medical Oncologist: Caitlin Murphy, Natalie Turner, Hui-Li Wong, Jeanne Tie, Belinda Lee, Jayesh Desai, Peter Gibbs, Ben Tran

Pathologist: Michael Christie, Ryan Hutchinson, Sebastian Lunke, Paul Waring

Colorectal Surgeon: Iain Skinner

Medical Student: Mathu Sinnathamby

Affiliations:

1) Department of Medical Oncology, Western Health, Furlong Rd, St Albans VIC 3021
2) Colorectal Translational Oncology Group, Walter and Eliza Hall Institute, Parkville VIC 3052
3) Department of Medical Oncology, Royal Melbourne Hospital, Grattan St, Parkville VIC 3050
4) Colorectal Unit, Department of Surgery, Western Hospital, Gordon St, Footscray VIC 3068
5) Department of Anatomical Pathology, Royal Melbourne Hospital, Grattan St, Parkville VIC 3050
6) Centre for Translational Pathology, University of Melbourne, Grattan St, Parkville VIC 3052

Contributions:

All authors meet the International Committee of Medical Journal Editors criteria for authorship.

Corresponding Author:

Ben Tran

Consultant Medical Oncologist

Head, Uro-Oncology Trials Program

Victorian Comprehensive Cancer Centre

305 Grattan St, Melbourne VIC 3000

Phone: +61-3-8559-7810

Fax: +61-3-8559-7739

Ben.Tran@petermac.org

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