Prognostic and predictive biomarkers in colorectal cancer. Towards precision medicine

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CHAPTER 7

Expression of HLA Class I, Aspirin Use and Survival after a diagnosis of Colon Cancer

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ABSTRACT

Background

Use of aspirin (which inhibits platelet function) after a colon cancer diagnosis is associated with improved overall survival. Identifying predictive biomarkers of this effect could individualize therapy and decrease toxicity. Platelets are thought to protect circulating tumor cells from natural killer cells which preferentially eliminate targets with low or absent human leukocyte antigen (HLA) class I expression. We hypothesized that the survival benefit associated with low dose aspirin use after a diagnosis of colorectal cancer might depend upon HLA class I expression.

Methods

A cohort study with tumor blocks from 999 colon cancer patients (surgically resected between 2002 and 2008), analyzed for HLA class I and PTGS2 expression using a Tissue Micro Array (TMA). *PIK3CA* mutation analysis was also performed. Aspirin use post-diagnosis was obtained from a prescription database. Parametric survival models with exponential (Poisson) distribution were used to model overall survival.

Results

The overall survival benefit associated with aspirin use after a diagnosis of colon cancer had an adjusted Rate Ratio (RR) of 0.53 (95% CI 0.38-0.74, $p<0.001$) when tumors expressed HLA class I compared to a RR of 1.03 (95% CI 0.66-1.61 $p=0.9$) when HLA expression was lost. The benefit of aspirin was similar for tumors with strong PTGS2 expression (RR 0.68 95% CI 0.48-0.97, $p=0.03$), weak expression (RR 0.59 95% CI 0.38-0.97, $p=0.02$), and wild-type *PIK3CA* tumors (RR 0.55, 95% CI 0.40-0.75, $p<0.001$). With mutated *PIK3CA* tumors a non-significant trend was observed (RR 0.73 (95% CI 0.33-1.63, $p=0.4$).

Conclusions

Aspirin use after colon cancer diagnosis was associated with improved survival if tumors expressed HLA class I contrary to the original hypothesis. Increased PTGS2 expression or the presence of mutated *PIK3CA* did not predict benefit from aspirin. HLA class I might serve as a predictive biomarker for adjuvant aspirin therapy in colon cancer.

INTRODUCTION

There is a significant body of preclinical, epidemiological and randomized data demonstrating that aspirin has anti-cancer effects [1-7]. Several studies have shown that aspirin use after a diagnosis of colorectal cancer improves colorectal cancer specific and overall survival [2;4;8-10]. Randomized trials designed to assess the cardiovascular benefits of aspirin demonstrate that allocation to aspirin reduces the risk of distant metastasis when cancer is diagnosed (hazard ratio (HR) 0.69 (95% CI 0.5–0.95) p=0.02) and on subsequent follow-up in patients without metastasis at diagnosis (HR 0.45 (95% CI 0.28–0.72) p=0.0009), with the largest effects seen for colorectal cancer (HR at diagnosis 0.36 (95% CI 0.18–0.74) p=0.005 and at follow-up HR 0.26 (95% CI 0.11–0.57) p=0.0008) [7]. Although questions remain about the optimal dose and duration of aspirin use, its efficacy in prediagnostic users and the localization of tumors most likely to benefit, the data suggest that aspirin is a potential adjuvant therapy to prevent distant metastasis in colorectal cancer, and possibly other tumors.

The precise biological mechanisms underlying the anti-cancer effects are unknown. PTGS2 (prostaglandin-endoperoxide synthase 2, also known as cyclo-oxygenase -2) overexpression has been associated with a poor prognosis in colorectal cancer [11;12]. Aspirin inhibits PTGS, at low doses (75-300 mg once daily), given the short half-life of around 30 minutes, this effect is manifest as a permanent inhibition of PTGS1 in the anucleate platelet, which is unable to resynthesize the enzyme. Higher and more frequent dosing for example 600 mg qds would be required to constantly inhibit PTGS2 in systemic tissues [13-16]. Despite this, data from two observational cohorts (the Nurses' Health Study (NHS) and Health Professionals Follow-Up Study (HPFS)), have indicated that the survival benefits of regular low-dose aspirin use after a diagnosis of colorectal cancer are associated with the molecular characteristics of the tumor particularly mutations in the gene *PIK3CA* (a component of the PTGS2 pathway) with a multivariable HR for aspirin users compared to non-users in tumors with mutated *PIK3CA* of 0.18 (95% CI 0.06–0.61; P <0.001) for cancer death and 0.54 (95% CI 0.31–0.94; p = 0.01) for death from any cause [17].

The metastatic potential of cancer cells that are shed into the bloodstream can be modified by environmental conditions, including platelets and bone marrow-derived cells in the vasculature [18]. Platelets are thought to protect disseminating tumor cells from natural killer (NK) cells which preferentially recognize and eliminate cells with low or absent expression of HLA class I [19]. We hypothesized that the survival benefit associated with low dose aspirin use after a cancer diagnosis would be associated with tumors that have low or absent HLA class I expression. We analyzed tumors from a cohort where we had previously shown an association between overall survival (OS) and low-dose aspirin use after diagnosis (adjusted HR 0.65 (95% CI 0.50-0.84; p=0.001) with an even

larger effect in older colon cancer patients ((>70 years) adjusted HR 0.59 (95% CI = 0.44-0.81, $p = 0.001$))[8;10] for HLA class I and PTGS2 expression, and *PIK3CA* mutations.

METHODS

Study cohort

The Eindhoven Cancer Registry was initially used to identify patients diagnosed with colorectal cancer and linked to data on aspirin use from the PHARMO database network (PHARMO, Netherlands). As previously reported, compared with non-users, aspirin initiated or continued after diagnosis was associated with improved survival for colon cancer patients but not rectal cancer patients [8]. Paraffin-embedded tissue blocks were retrieved from 1026 colon cancer patients who had a surgical resection between 2002 and 2008. For this study, 27 patients with more than one colon tumor at the time of diagnosis were excluded thus n=999. There were no significant demographic differences between the patients included in this study and the whole colon cancer cohort in the registry (n= 3586) (Supplementary Table I).

TMA production and immuno-histochemistry

Three 1.0 mm diameter cores were obtained from formalin-fixed paraffin-embedded (FFPE) tumor blocks using haematoxylin and eosin stained sections for tumor identification (with a qualified pathologist confirming the identification of the tumor) and transferred into a receiver paraffin block using the TMA Master (3D Histech, Budapest, Hungary). Immuno-histochemical staining was performed on 4 µm sections, cut from each receiver block and mounted on glass. For each primary antibody, all slides were stained simultaneously to avoid inter-assay variation.

Immuno-histochemical analyses to detect HLA class I expression with mouse monoclonal antibodies HCA2 and HC10 using diaminobenzidine solution (DAB+) (DAKO, Glostrup, Denmark) for visualization of the antibodies, were performed by two independent observers, M.R and R.V, as previously described [20], with good inter-observer agreement (kappa value of 0.5-0.7). The mouse monoclonal antibodies HCA2 and HC10 used recognize the heavy chains of HLA Class I. Their reactivity spectrum has been described in detail before [18]. HLA class I expression status was determined according to the International HLA and Immunogenetics Workshop [21], with tumor cell HLA class I status defined as follows: loss <5% expressing both HCA2 or HC10 or <5% expressing either of the markers and expression as $\geq$ 5% expressing either marker. Normal epithelial, stromal or lymphoid cells served as positive internal controls. PTGS2 expression was analyzed automatically with a double staining to separately visualize stromal cells (using DAB+ for visualization of anti-collagen I, anti-collagen VI and elastin (all polyclonal rabbit antibod-

ies obtained from AbCam)) and positive tumor cells (with the monoclonal mouse antibody anti-PTGS2 (Cayman Chemical Co., Ann Arbor, MI, USA)), using Vector Blue ((Vector Laboratories, Burlingame, USA) for visualization of the PTGS2 antibody. Slides were scanned with the Panoramic Midi scanner (3D-Histech, Hungary) and PTGS2 expression was assessed using the criteria proposed by Buskens *et al.* [22] using AxioVision 4.6 (Zeiss, Jena, Germany), and comparable to the scoring method used by Chan *et al.* [2].

Microsatellite stability status was determined by immuno-histochemical analyses as previously described [23]. In short, four antibodies directed against MutL homolog21 (*MLH1,* clone ES05, DAKO Cytomation, Glostrup, Denmark), MutS homolog 2 (*MSH2,* clone g219-1129, BD Biosciences, Franklin Lakes, USA), MutS homolog 6 (*MSH6,* clone EPR3945, Epitomics, Burlingame, USA) and postmeiotic segregation of *Saccharomyces cerevisia* 2 (*PMS2,* clone A16-4, BD Biosciences, Franklin Lakes, USA) were used. The

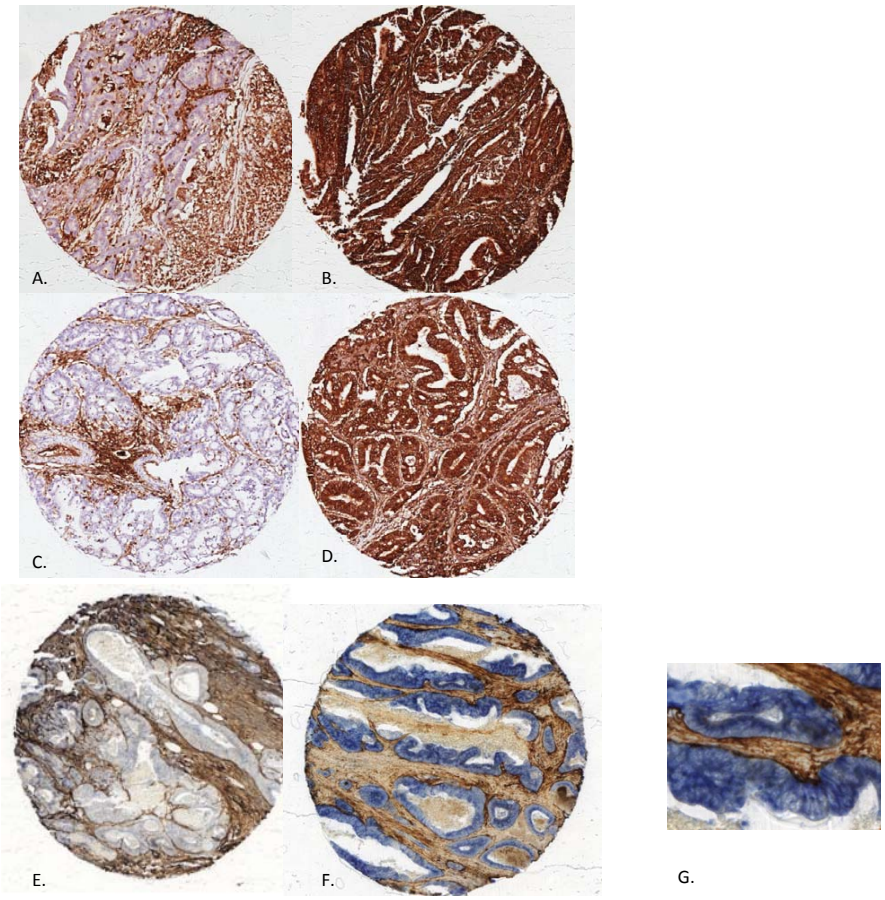

Figure 1: Representative images of HCA2, HC10 and PTGS2 staining in colon cancer.
Representative images of immunohistochemical stainings for HLA Class I expression (HCA2 and HC10) and PTGS2 performed according to standard protocols (details in Material and Methods).(A) HC10-negative tumor (B) HC10-positive tumor (C) HCA2- negative tumor (D) HCA2- positive tumor (E) Tumor with weak PTGS2 expression (F) Tumor with strong PTGS2 expression, with a magnification in (G).

criteria used to confirm microsatellite instability (MSI) in the tissues are described elsewhere [23;24].

All slides were stained simultaneously to avoid interassay variation. Slides that did undergo the whole immunohistochemical staining procedure but without primary antibodies served as negative controls. The quality of the staining, the scoring method and discrepancies between the two observers were checked by a pathologist (H.M).

Representative images of the immuno-histochemical stainings are shown in Figure 1.

PIK3CA mutation analysis

DNA was extracted from 1 to 2, 2.0 mm diameter and variable length cores taken from 663 of the 999 blocks randomly chosen, with a ratio 1:2 for aspirin user: non-user, using a fully automated system (Tissue Preparation System with VERSANT Tissue Preparation Reagents, Siemens Healthcare Diagnostics, Tarrytown, NY, USA) as described previously [25].

Hydrolysis probes assays were performed for the major known mutations (hotspots) in exon 9, c.1624G>A; *p*.E542K, c.1633G>A; p.E545K and in exon 20 the c.3140A>G; p.H1047R as described before [26]. Hydolysis probe assays were analyzed using qPCR analysis software (CFX manager version 3/0, Bio-Rad). To identify additional non-hotspot mutations, Sanger sequencing was performed on exon 9 and exon 20 of all samples. Mutation detection was performed by two observers independently (M.R and R.E) using DNA variant analysis software (Mutation Surveyor version 4.0.9, Softgenetics, State College, PA, USA). All primers and probes used for the assays can be found in Supplementary Table II.

Statistics

The vital status of patients (alive/dead) was established from medical records or through linkage of cancer registry data with the municipal population registries. Follow-up started 30 days from diagnosis of colorectal cancer (T0), as information on hospital prescriptions was not available, and was continued until last contact date (January 2012) or date of death. Patients who died within 30 days were excluded from the survival analyses (2.4% for colon cancer). Non-users were classified as those who never had a prescription for aspirin or had a prescription for less than 14 days after diagnosis of colon cancer. Users were defined as those who had been given a prescription for aspirin for 14 days or more after a colon cancer diagnosis. The median duration of prescriptions was 30 days and the mean number of prescriptions was 12 (range 1- 220). Non-users were defined from T0 to first use and users from first use to the end of the follow-up in the time-dependent exposure survival analysis.

As the data was split in two episodes for users (multiple ID rows for one patient), we were not able to model a Cox proportional hazard model and used a parametric

survival model with an exponential (Poisson) distribution after the data was declared as survival-time data (stset) and split at the time to first prescription.

Adjustments for potential confounders were made for sex, age (continuous), stage (pathological stage and clinical stage if pathological stage was unknown), adjuvant chemotherapy (yes/no), co-morbidity (yes/no), tumor grade and year of diagnosis. Stratified analyses were performed for HLA class I expression, weak or strong PTGS2 expression and for wild-type *PIK3CA* / *PIK3CA* mutation.

RESULTS

Aspirin use, survival and tumor HLA class I expression

Of the 999,18.2% (182/999) were defined as aspirin users and there had been 465 deaths recorded until January 2012. There were 396 deaths in 817 nonusers (48.5%) and 69 deaths in 182 aspirin users (37.9%) after diagnosis. In this cohort, aspirin use after diagnosis was associated with an improved OS (RR 0.64, 95% CI 0.49-0.83, p=0.001), when compared to nonusers.

36/999 tumors could not be analyzed for HLA class I expression due to staining artefacts or loss of material. Table I summarizes the clinical characteristics of the patients presented by HLA class I expression and according to aspirin use/non-use after diagnosis. Loss of HLA class I expression was found in 33.2% (320/963) and expression in 66.8% (643/963), in accord with results from previous studies [27;28]. Aspirin use was similar in both groups, loss of HLA 18% (57/320) and expression of HLA class I 19% (122/643),

Table I: Baseline Characteristics of the Colon Cancer Patients according to Tumor HLA Class I Expression and Use of Aspirin after Diagnosis

	All patients (N=999)	HLA Loss (N=320)			HLA Expression (N=643)		
		No aspirin	Aspirin	*p*-value	No aspirin	Aspirin	*p*-value
Sex							
Male	505 (50.6)	121 (46.0)	35 (61.4)	0.04	260 (49.9)	78 (63.9)	0.005
Female	494 (49.4)	142 (54.0)	22 (38.6)		261 (50.1)	44 (36.1)	
Age							
<65	342 (34.2)	110 (41.8)	8 (14.0)	<0.001	188 (36.1)	23 (18.8)	0.001
66-74	304 (30.4)	66 (25.1)	26 (45.6)		155 (29.7)	45 (36.9)	
75 and older	353 (35.4)	87 (33.1)	23 (40.4)		178 (34.2)	54 (44.3)	
Year of diagnosis							
2002-2004	451 (45.2)	102 (38.8)	27 (47.4)	0.2	232 (44.5)	65 (53.3)	0.08
2005-2007	548 (54.8)	161 (61.2)	30 (52.6)		289 (55.5)	57 (46.7)	

Table I: Baseline Characteristics of the Colon Cancer Patients according to Tumor HLA Class I Expression and Use of Aspirin after Diagnosis *(Continued)*

	All patients (N=999)	HLA Loss (N=320)			HLA Expression (N=643)		
		No aspirin	Aspirin	*p*-value	No aspirin	Aspirin	*p*-value
Disease stage							
I	138 (13.8)	24 (9.1)	5 (8.8)	0.2	71 (13.6)	33 (27.0)	<0.001
II	402 (40.2)	108 (41.1)	30 (52.6)		210 (40.3)	39 (32.0)	
III	287 (28.7)	77 (29.3)	17 (29.8)		142 (27.3)	40 (32.8)	
IV	169 (16.9)	54 (20.5)	5 (8.8)		95 (18.2)	10 (8.2)	
Unknown	3 (0.3)				3 (0.6)		
Comorbidity							
No	443 (44.3)	138 (52.5)	14 (24.6)	<0.001	253 (48.6)	25 (20.5)	<0.001
Yes	556 (55.7)	125 (47.5)	43 (75.4)		268 (51.4)	97 (79.5)	
Microsatellite status							
MSI	90 (9.0)	28 (10.7)	11 (19.3)	0.2	38 (7.3)	8 (6.6)	0.9
MSS	870 (87.1)	227 (86.3)	45 (78.9)		472 (90.6)	112 (91.8)	
Unknown	39 (3.9)	8 (3.0)	1 (1.8)		11 (2.1)	2 (1.6)	

Overall aspirin use: 182 patients (18.2%).

Table II: Rate Ratio for Death (Time-Dependent Analysis Overall Survival), According to Tumor HLA Class I Expression, PTGS2 Expression and *PIK3CA* Mutation Status and Use or Nonuse of Aspirin after Diagnosis

	Patients	Deaths	Univariate RR (95%CI)	*p*-value	Adjusted RR* (95%CI)	*p*-value	*p*-value (interaction)**
HLA Class I							0.007
Loss							
No aspirin use	263	123	1.00 (reference)	0.7	1.00 (reference)	0.9	
Aspirin use	57	26	1.08 (0.70-1.64)		1.03 (0.66-1.61)		
Expression							
No aspirin use	521	257	1.00 (reference)	0.003	1.00 (reference)	<0.001	
Aspirin use	122	42	0.61 (0.44-0.85)		0.53 (0.38-0.74)		
PTGS2							0.12
Low							
No aspirin use	360	190	1.00 (reference)	0.05	1.00 (reference)	0.02	
Aspirin use	66	25	0.66 (0.44-1.01)		0.59 (0.38-0.91)		
High							
No aspirin use	434	192	1.00 (reference)	0.2	1.00 (reference)	0.03	
Aspirin use	114	42	0.80 (0.57-1.12)		0.68 (0.48-0.97)		
PIK3CA							0.004
Wild-type							
No aspirin use	384	200	1.00 (reference)	0.007	1.00 (reference)	<0.001	
Aspirin use	147	55	0.66 (0.49-0.89)		0.55 (0.40-0.75)		
Mutation							
No aspirin use	73	34	1.00 (reference)	0.3	1.00 (reference)	0.4	
Aspirin use	27	9	0.70 (0.34-1.46)		0.73 (0.33-1.63)		

*Aspirin use = use of aspirin after diagnosis. *Adjusted for sex, age, comorbidity, year of incidence, histological grade, stage and chemotherapy. ** Interaction between the markers and aspirin use status (user/nonuser).*

though aspirin users were older and more likely to have co-morbidity. In the HLA class I expression group, there were more lower stage tumors in the aspirin users compared to non-users (p<0.001).

The effect of HLA class I expression status on the survival benefit associated with post-diagnosis aspirin use was examined (Table II and Figure 2). For patients whose tumors expressed HLA class I, aspirin use after diagnosis was associated with a significantly

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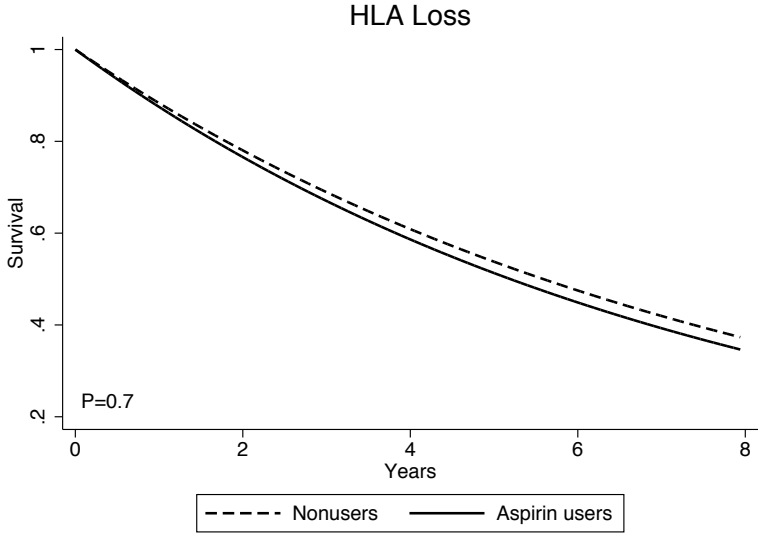

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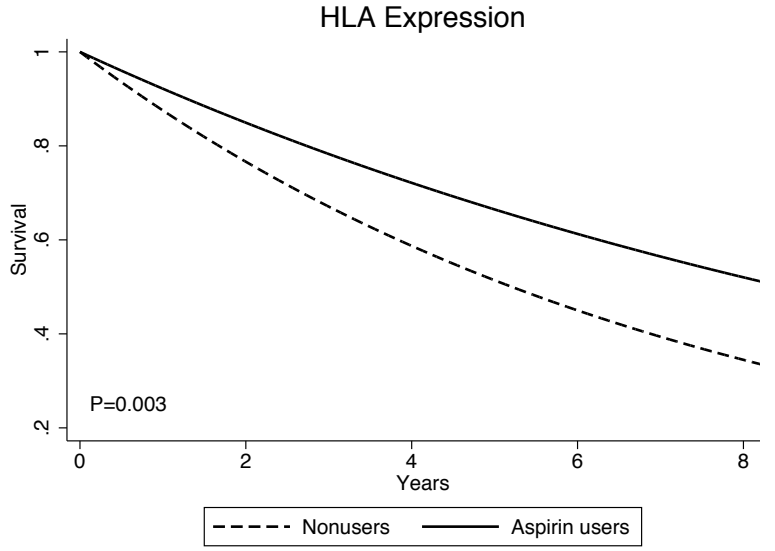

Figure 2: Overall Survival Curves Aspirin Use versus No Aspirin Use Stratified by HLA Class I.
Survival curves for overall survival in colon cancer patients, according to aspirin use after diagnosis or nonuse of aspirin after diagnosis and HLA Class I expression. Above: Overall survival among colon cancer patients with loss of HLA class I in their tumor sections. Below: Survival among colon cancer patients with expression of HLA Class I in their tumor sections.

longer OS, RR 0.61 (95% CI 0.44-0.85, p=0.003), and when adjusted for potential confounders, this effect remained with an adjusted RR of 0.53 (95% CI 0.38-0.74, p<0.001). In contrast, for patients whose tumors had loss of HLA class I expression, aspirin use after diagnosis was not associated with a survival benefit (Adjusted RR 1.03 (95% CI 0.66-1.61, p=0.9).

Aspirin use, survival and tumor PTGS2 expression and *PIK3CA* mutations

25/999 samples could not be analyzed for PTGS2 expression due to staining artefacts or loss of material. Weak expression of PTGS2 was seen in 43.7% (426/974) of samples, and strong PTGS2 expression in 56.3% (548/974) in accord with the literature [2;29]. Use of aspirin after diagnosis was significantly associated with a survival benefit, both when tumors showed weak PTGS2 expression (Adjusted RR 0.59, 95% CI 0.38-0.91, p=0.02) and with strong PTGS2 expression (Adjusted RR 0.68, 95% CI 0.48-0.97, p=0.03) (Table II).

DNA was extracted from 663 tumor blocks and *PIK3CA* mutation status (wild-type/mutation) was established in 95% (631/663) of the samples. Baseline characteristics among participants with colon cancer whom we analyzed for *PIK3CA* were largely similar as the baseline characteristics of the PTGS2/HLA class I cohort (mean age at inclusion, 70.38 vs. 69.01 years; male, 54% vs. 51%; stage I, 15% vs. 14%; stage II, 40% vs. 40%; stage III, 29% vs. 29%; stage IV 15% vs. 17%; presence of comorbidity, 60% vs. 56%; adjuvant chemotherapy, 28% vs. 31%; histological grade I, 11% vs. 11%; grade II, 70% vs. 68%; grade III 19% vs. 21%; p>0.091 for all comparisons). A *PIK3CA* mutation was found in 15.8% (100/663), also in accord with what has been found previously [30]. Aspirin use was 27% (27/100) among patients with a mutated *PIK3CA* tumor and 28% (147/531) in patients with a *PIK3CA* wild-type tumor. Aspirin use after a colon cancer diagnosis was significantly associated with a better OS among patients with a wild-type *PIK3CA* tumor (Adjusted RR 0.55, 95% CI 0.40-0.75, p<0.001). In patients with a *PIK3CA* mutation, post-diagnosis aspirin use showed the same trend (though non-significant) with an adjusted RR of 0.73 (95% CI 0.33-1.63, p=0.4). The small number of deaths (9) among patients with mutated *PIK3CA* tumors precludes robust statistical assessment (Table II).

DISCUSSION

We found that the survival benefit associated with low-dose aspirin use after a diagnosis of colon cancer was significantly associated with HLA class I positive tumors. In contrast, in patients whose tumors had lost their HLA expression, aspirin use did not change outcome. PTGS2 expression and *PIK3CA* mutation analysis could not identify patients with a high likelihood of benefit from aspirin in contrast to previous studies [2;17;31].

Currently, the molecular mechanism(s) underlying the anti-cancer effects of aspirin are incompletely understood. Given that the majority of our cohort (> 80%) were diagnosed as stage III or less at the time of diagnosis the predominant effect of aspirin on cancer outcomes is likely to result from an effect on circulating tumor cells and their ability to develop into metastatic deposits. Natural killer (NK) cells play an important role in tumor immune-surveillance, preferentially eliminating targets with low or absent expression of HLA class I [19]. Adhesion of HLA expressing platelets to tumor cells with absent or low HLA class I expression is thought to result in a "pseudonormal phenotype" and reduced NK mediated lysis [19]. We originally hypothesized that aspirin might inhibit platelet adhesion to tumor cells leaving those with absent or low HLA Class I expression susceptible to immune clearance, however we unexpectedly found that the effect of aspirin is dependent on intact HLA class I expression within the original primary tumor, and assuming that circulating tumor cells retain the same HLA phenotype as the original tumor does not support the hypothesis that the attenuation of metastases by aspirin and possibly other anticoagulants is a result of enhanced NK activity [19].

A possible explanation for this intriguing observation is that HLA expression might be necessary for platelet mediated NF-κB signaling in circulating tumor cells resulting in an epithelial-mesenchymal-like phenotype with enhanced metastatic potential [18]. In this model direct contact of platelets and tumor cells results in secretion of TGF-β and activation of the NF-κB pathway, which, in synergistic action, prime circulating tumor cells for subsequent metastases. In a breast cancer model acquisition of an epithelial-mesenchymal phenotype markedly reduced susceptibility of cancer cells to T-cell mediated immune surveillance in-vitro [32]. Our data would be compatible with the hypothesis that aspirin inhibits platelet-tumor cell signaling (which is dependent upon intact HLA expression) and prevents epithelial-mesenchymal transition in circulating tumor cells, thereby reducing the metastatic potential.

Our data has not confirmed previous reports that the benefits of aspirin after a colorectal cancer diagnosis are associated with strong PTGS2 expression in the original tumor and the presence of mutations in *PIK3CA*, with no benefit for patients whose tumors had wild-type *PIK3CA* [2;17]. Liao *et al.* postulated that by blocking the *PIK3CA* pathway PTGS2 activity decreases, which leads to apoptosis of colon cancer cells and which was in accord with their previous work demonstrating a clinical benefit of aspirin in patients with PTGS2 positive tumors [2]. In a separate study, benefits of aspirin in *PIK3CA* mutated tumors were seen but the correlation with strong expression of PTGS2 expression was not confirmed [31].

Pharmacological data on aspirin indicate that systemic concentrations of aspirin, reached with low-doses, (75-325 mg once daily) are inadequate to permanently acetylate PTGS2, but are optimal for platelet inhibition [13]. It is possible that there may be more than one mechanism of action that accounts for the anti-cancer effects of aspirin. A

direct anti-platelet effect due to PTGS1 inhibition that is responsible for the reduction in metastases and only requires a dose of aspirin that inhibits platelets, and a second mechanism, possibly mediated through platelets again, or perhaps activated with higher or more frequent dosing that inhibits the PTGS2 pathway in systemic tissues and may partly explain the differences between the results of our study and that of Liao *et al*. In breast cancer it has also been reported that PTGS2 expression could not identify a subgroup of patients where aspirin decreased recurrence [33]. Furthermore, in breast cancer low dose aspirin did not influence local recurrence, but was significantly associated with a decrease in metastatic disease [34].

Aspirin use has also been associated with a decreased risk of developing a colorectal tumor with an intact *BRAF* gene but no association between post-diagnosis aspirin use, *BRAF* mutation status and clinical outcome has been found [35]. *BRAF* is a member of RAF-MAPK signaling pathway and involved in the up-regulation of PTGS2 again suggesting aspirin may have differential effects on carcinogenesis and prevention of metastatic spread [35].

Strengths of our study include a more precise definition of regular aspirin use and dose as this information was derived from prescriptions (rather than patient recall), noting also that low-dose aspirin is not available as an over-the-counter medication in the Netherlands, thereby minimizing non-differential misclassification of exposure. Higher dose over-the-counter aspirin use is unknown, which could have biased our results towards the null hypothesis. However, it has been shown that, pharmacy data can give valid associations even though a high proportion (25%) of the drugs are available over-the-counter [36]. Other limitations of our study include the inherent issue that this is non-randomized data, compliance is unknown, and some subgroups contained small numbers of events, although our series is the largest study thus far that has reported on aspirin use in colon cancer patients.

The molecular profiling of tumors for example *KRAS* testing in colorectal cancer and HER-2 testing in breast cancer has become standard clinical practice and the basis of therapeutic decisions. If the association of HLA expression and benefit from aspirin is confirmed in other datasets it could be used in clinical practice, where, our data may have important clinical implications for both the dose and timing of aspirin as an anti-cancer agent. First, low dose daily aspirin may suffice as an anti-metastatic therapy in early stage cancer patients. Second, as circulating tumor cells are found in the peri-operative period, it could be argued that aspirin should be commenced as soon as considered clinically appropriate after diagnosis.

In conclusion, we report the novel finding that the survival benefit associated with low-dose aspirin use after diagnosis of colon cancer is dependent on intact HLA class I expression in the original tumor. Randomized trials of the use of aspirin in the adjuvant

setting may provide key information about platelet-tumor interactions and the signaling pathways they elicit.

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SUPPLEMENTARY MATERIAL

Supplementary Table I: Baseline characteristics of the patients included in the population-based registry and the patients included in this study

		Population-based registry (n=3586)	Patients (n=999)	*p*-value
Sex				
	Male	1849 (51.6)	505 (50.6)	0.57
	Female	1737 (48.4)	494 (49.4)	
Age				
	1	1180 (32.9)	342 (34.2)	0.73
	2	1107 (30.9)	304 (30.4)	
	3	1299 (36.2)	353 (35.4)	
Comorbidity				
	No	1627 (45.4)	443 (44.3)	0.56
	Yes	1959 (54.6)	556 (55.7)	
Stage				
	I / II	1878 (52.4)	540 (54.1)	0.69
	III / IV	1541 (43.0)	456 (45.6)	
	Missing	167 (4.6)	3 (0.3)	
Grade				
	1	452 (12.6)	100 (10.0)	0.12
	2	2053 (57.3)	629 (62.9)	
	3	635 (17.7)	193 (19.3)	
	Missing	446 (12.4)	77 (7.7)	
Localization				
	Proximal	1482 (41.3)	422 (42.2)	0.25
	Distal	588 (16.4)	169 (16.9)	
	Sigmoid	1433 (40.0)	395 (39.5)	
	Other	83 (2.3)	13 (1.3)	

Supplementary Table II: primer overview of the primers used for the *PIK3CA* mutation analysis

Assay Name	Primer Name	Primer sequence
p.E542K*	PIK3CA_p.E542K _F	AGCTCAAAGCAATTTCTACACGAGAT
	PIK3CA_p.E542K _R	GCACTTACCTGTGACTCCATAGAAA
p.E545K*	PIK3CA_p.E545K_F	TCAAAGCAATTTCTACACGAGATCCT
	PIK3CA_p.E545K_R	GCACTTACCTGTGACTCCATAGAAA
p.H1047R*	PIK3CA_p.H1047R_F	GCAAGAGGCTTTGGAGTATTTCATG
	PIK3CA_p.H1047R_R	GCTGTTTAATTGTGTGGAAGATCCAA
Exon 9**	PIK3CA_x9_M13F	TGTAAAACGACGGCCAGTGGGAAAATGACAAAGAACAGC
	PIK3CA_x9_M13R	CAGGAAACAGCTATGACCTCCATTTTAGCACTTACCTGTGAC
Exon 20**	PIK3CA_x20_M13F	TGTAAAACGACGGCCAGTCTGAGCAAGAGGCTTTGGAG
	PIK3CA_x20_M13R	CAGGAAACAGCTATGACCCCTATGCAATCGGTCTTTGC
***	PR_M13F	TGTAAAACGACGGCCAGT
***	PR_M13R	CAGGAAACAGCTATGACC

** Hydrolysis probes assays, ** Genomic PCR, *** Sanger sequencing, F=Forward primer, R=Reverse primer.*

Reporter 1 name	Dye	Reporter 1 Sequence	Reporter 2 name	Dye	Reporter 2 Sequence
PIK3CA_p.E542K _V	VIC	CCTCTCTCTGAAATCA	PIK3CA_p.E542K _M	FAM	CCTCTCTCTAAAATCA
PIK3CA_p.E545K_V	VIC	CTCTCTGAAATCACTGAGCAG	PIK3CA_p.E545K_M	FAM	CTCTGAAATCACTAAGCAG
PIK3CA_p.H1047R_V	VIC	CCACCATGATGTGCATC	PIK3CA_p.H1047R_M	FAM	CACCATGACGTGCATC