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ARTICLE

Epidemiology

Statin use is associated with a reduced incidence of colorectal cancer expressing SMAD4

Sarah Ouahoud[1], Rutger J. Jacobs[1], Ludmilla L. Kodach[1], Philip W. Voorneveld[1], Lukas J. A. C. Hawinkels[1], Nikki L. Weil[1], Britt van Vliet[1], Ron M. Herings[2,3], Lennart R. A. van der Burg[1], Tom van Wezel[4], Hans Morreau[4], Marije Slingerland[5], Esther Bastiaannet[5,6], Hein Putter[7] and James C. H. Hardwick[1]✉

BACKGROUND: Long-term use of statins is associated with a small reduced risk of colorectal cancer but their mechanism of action is not well understood. While they are generally believed to act on KRAS, we have previously proposed that they act via influencing the BMP pathway. The objective of this study was to look for associations between statin use and the risk of developing colorectal cancer of a particular molecular subtype.

METHODS: By linking two registries unique to the Netherlands, 69,272 statin users and 94,753 controls were identified and, if they developed colorectal cancer, their specimens traced. Colorectal cancers were molecularly subtyped according to the expression of SMAD4 and the mutation status of *KRAS* and *BRAF*.

RESULTS: Statin use was associated with a reduction in the risk of developing colorectal cancer regardless of molecular subtype (HR 0.77; 95% CI 0.66–0.89) and a larger reduction in the risk of developing SMAD4-positive colorectal cancer (OR 0.64; 95% CI 0.42–0.82). There was no relationship between statin use and the risk of developing colorectal cancer with a mutation in *KRAS* and/or *BRAF*.

CONCLUSIONS: Statin use is associated with a reduced risk of developing colorectal cancer with intact SMAD4 expression.

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INTRODUCTION

Statin use is associated with a reduced risk of developing colorectal cancer (CRC) but this risk reduction varies widely between studies and in meta-analyses is small, ~10% [1–6]. Statins are attractive drugs for use in chemoprevention because they are well tolerated and have beneficial effects on cardiovascular mortality and morbidity. However, CRC is a molecularly heterogeneous disease and it is likely that statin sensitivity varies between molecular subtypes. Understanding their mechanism of action in preventing CRC could potentially allow statins to be targeted specifically to sensitive subtypes.

The mechanism by which statins influence the risk of CRC is not well understood. Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase the rate-limiting step in mevalonate synthesis [7]. Inhibition of the mevalonate pathway not only disrupts cholesterol synthesis but also farnesyl pyrophosphate important for the prenylation of KRAS. Approximately 40% of CRCs have activating *KRAS* mutations [8]. Therefore, the current dominant hypothesis for the mechanism of action of statins in CRC is that they act by inhibiting KRAS thus inhibiting the RAS/RAF pathway [9].

We have proposed an alternative mechanism of action, that statins act on CRC through activating the Bone Morphogenetic Protein (BMP) pathway [10]. This is based on evidence that statins activate the BMP pathway in bone [11] and on evidence for a crucial role for the BMP pathway in CRC [12, 13]. In studies in vitro and in rodents we have shown that the effects of statins depend on the expression of the central BMP pathway element, SMAD4. Statins suppress the growth of SMAD4-expressing cancer cells but promote the growth of cancer cells lacking SMAD4 [10].

If these findings hold true in humans, then statin use should reduce the risk of SMAD4-expressing tumours, and increase the risk of SMAD4 non-expressing tumours. Similarly, if statins act by inhibition of KRAS then any reduction in cancer risk due to statin use would be expected to be influenced by the presence of *KRAS* and *BRAF* activating mutations. We therefore set out to investigate whether the influence of the use of statins on the risk of CRC varied with the expression of SMAD4 in the tumour or with the presence of *KRAS* and *BRAF* mutations in a large patient cohort. We first performed a large population-based cohort study to investigate the influence of statin use on CRC incidence and

[1]Department of Gastroenterology and Hepatology, Leiden University Medical Centre, Utrecht, The Netherlands. [2]PHARMO Institute for Drug Outcomes Research, Utrecht, The Netherlands. [3]Department of Epidemiology & Data Science, Utrecht, The Netherlands. [4]Department of Pathology, Leiden University Medical Centre, Leiden, The Netherlands. [5]Department of Medical Oncology, Leiden University Medical Centre, Leiden, The Netherlands. [6]Department of Surgery, Leiden University Medical Centre, Leiden, The Netherlands. [7]Department of Medical Statistics, Leiden University Medical Centre, Leiden, The Netherlands. ✉email: j.c.h.hardwick@lumc.nl

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subsequently analysed SMAD4 expression and *KRAS* and *BRAF* mutation status in a subset of the tumour specimens.

METHODS

National registries

The PHARMO record linkage system includes a pharmacy database and anonymously records all dispensed drug prescriptions from more than 450 pharmacies in more than 50 regions scattered over the Netherlands, representative for the Netherlands as a whole. Currently it covers more than 4 million residents regardless of type of insurance, or 25% of the Dutch population. During the study period (2001–2007) patients in the Netherlands still registered at a single pharmacy to which they brought all prescriptions from general practitioners or medical specialists. Dispensing histories for this period are virtually complete [14]. The computerised drug dispensing histories record the type and quantity of the dispensed drug, prescriber details, dispensing date and the prescribed daily dose. These data are linked to the Dutch National Pathology Registry (PALGA), which contains data from all pathological examinations performed in the Netherlands allowing it to serve as the main database source for the Dutch Cancer Registry [15]. PALGA contains abstracts of the pathology reports consisting of encrypted patient identification and a summary of the report and the diagnosis (SNOMED). By means of this unique linkage, drug use in relation to pathology and hospitalisation can be studied.

Study population

Cardiovascular risk is associated with a higher risk of CRC [16]. To control for this major confounder in statin users, the study base included all patients receiving beta-blockers continuously for more than 6 months within the study period defined as between January 1, 2000 and December 31, 2007. Beta-blocker use is not associated with CRC incidence [17]. Within this study base of patients with a similar risk of cardiovascular disease, we performed a nested cohort study. Statin use was coded as a time-dependent covariate and patients receiving more than 6 months of continuous statin treatment during the study period were included into the statin user cohort from the date of the first statin prescription or the begin of the study period whichever came last. Where there were gaps between successive prescriptions, continuous use of either beta-blockers or statins was defined as use on more than 80% of days. Patients receiving no statins during the study period were entered into the control cohort. All patients with a history of CRC, chemotherapy, or radiotherapy before the prospective date of cohort inclusion were excluded. All cases of CRC occurring within the study period were identified Using the Dutch National Pathology Registry (PALGA).

Tumour analysis

We retrieved a sample of 592 CRC pathological specimens, 312 from statin users and 280 from statin non-users. We aimed to retrieve ~280 specimens from each group guided by a prior power analysis. We collected all available specimens from the six largest pathological laboratories geographically spread around the country. Our analysis was limited by the availability of paraffin blocks with amounts of tumour tissue sufficient for immunohistochemical analysis. All samples were handled according to the medical ethical guidelines established by the Dutch Federation of Medical Sciences. In rectal cancer we analysed biopsies from the time of diagnosis rather than irradiated resection specimens.

SMAD4 analysis

SMAD4 immunostaining on whole tissue sections was performed using a monoclonal antibody against SMAD4 (Clone B8, Santa Cruz Biotechnology, Inc.; 1:400). Four micrometres sections were deparaffinised and blocked for endogenous peroxidase activity by immersion in 0.3% H_2O_2 in methanol for 20 min. Antigen retrieval was performed in Tris-EDTA buffer (10 mmol/L/1 mmol/L, pH 9.0) for 30 min at 97 °C. Nonspecific binding was blocked with 10% goat serum in PBS for 10 min. This was followed by 1 h SMAD4 antibody incubation at room temperature. Antibody binding was visualised using the Powervision+poly-HRP detection system (ImmunoVision Technologies, Co.) and 3,3-diamino-benzidine (DAB, Sigma). Sections were counterstained with haematoxylin.

Two pathologists both unaware of any data concerning the participants, independently interpreted SMAD4 expression, using a standardised grading system (absent, weak, normal). The pathologists classified staining of tumour cells as "normal" if SMAD4 expression was at the same level of intensity as that in stromal cells; "weak" and "absent" staining indicated progressively decreasing degrees of loss of expression. If the intensity of immunostaining was normal, tumours were classified as cancers with intact SMAD4 expression (SMAD4 positive). If immunostaining intensity was weak or absent, tumours were classified as having low SMAD4 expression (SMAD4 negative) (Fig. 1).

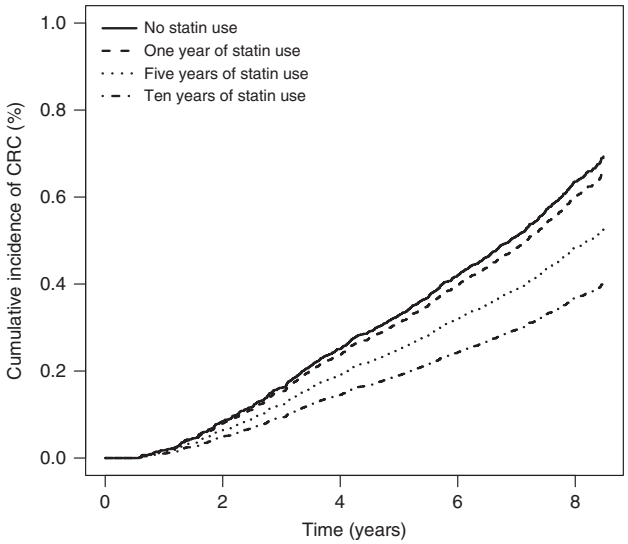

Fig. 1 Colorectal cancer incidence and duration of statin use. Model-based incidence of colorectal cancer depending on the duration of statin use, for subjects with no statin use and 1, 5 and 10 years of statin use. Longer duration of statin use is associated with a lower incidence of colorectal cancer ($p = 0{\cdot}003$, time-dependent Cox model).

KRAS and *BRAF* mutation analysis

DNA was extracted from micro-dissected 4 µm sections of formaldehyde-fixed, paraffin-embedded tumour biopsy or resection specimens using the NucleoSpin FFPE DNA XS kit (Macherey-Nagel, Düren, Germany) according to the manufacturers protocol. The DNA was screened for 7 *KRAS* CRC hotspot mutations in codon 12 and 13 and the BRAF v600e mutation by TaqMan real-time PCR mutation assays (Thermo Fisher Scientific Inc., MA) using TaqMan minor groove binder (MGB) probes as described previously [18]. An overview of the sequences used is given in Supplementary table 1.

Statistical analysis

For the initial analysis of the effect of statin use on unselected CRC incidence, time-dependent Cox regression, adjusted for age, gender and Non-Steroidal Anti-Inflammatory Drug (NSAID) use, with time since beta-blocker use as time scale, was used employing the Andersen-Gill method. Statin use was coded as time-dependent exposure taking values 0 (no statin use), 1 (less than 6 months of statin use), 2 (between 6 and 12 months of statin use) and 3 (12 months or more of statin use). This methodology was chosen in order to avoid immortal time bias. Statin use was considered to be cumulative; interrupting use of statin did not reset the value of the time-dependent covariate. The primary analysis compared the value 0 as defined above to the values 1, 2 and 3 together, but sensitivity analyses were conducted comparing different combinations. NSAID use and age were also added as time-dependent covariates. NSAID use was coded as 0 (no current use) or 1 (current use). Age was discretised and updated at the start of every year. Using age as a continuous time-dependent covariate would not have been feasible, given the large sample size. Multivariate hazard ratios were adjusted for age, sex, length of follow-up, diabetes, inflammatory bowel disease, adenoma removal and NSAID use. For the analyses restricted to women, the multivariate models were additionally adjusted for postmenopausal hormone-replacement therapy. All p values are two-sided. R, version 4·0·0, was used for this analysis.

To model the association of statin use (versus no statin use) with the molecular subtype of CRC we used a generalised linear model (GLM) with a binomial distribution stratified within the subgroups of KRAS, BRAF, combination of KRAS and BRAF and SMAD4. Effect size was expressed as Odds Ratio (OR) with corresponding 95% Confidence Interval (95% CI).

Table 1. Baseline characteristics of the study cohort.

Characteristics	Statin users (*N* = 68,948)	Statin non-users (*N* = 94,272)
Gender (%) (no.)		
Female	43 (29,666)	63 (59,357)
Male	57 (39,282)	37 (34,915)
Median age (yr)	64	63
Follow-up yrs mean (no.)	4.3 (293,129)	4.4 (413,870)
NSAIDs % (no.)[a]	37 (25,126)	37 (35,164)
Hormone-replacement therapy % (no.)[b]	7.6 (4907)	9.8 (9249)
Inflammatory Bowel Disease % (no.)[c]	1.7 (1202)	1.8 (1732)
Diabetes Mellitus % (no.)[d]	27.8 (19,289)	10.4 (9897)
Previous polypectomy % (no.)[e]	1.3 (885)	1.0 (928)

[a]NSAID use was defined as at least two prescriptions of any NSAID and more than one prescription per year of follow-up.
[b]Hormone-replacement therapy was defined as at least two prescriptions of HRT (estrogens with or without progestogens).
[c]IBD was inferred by receipt of at least two prescriptions of Mesalazine.
[d]DM was inferred by receipt of at least two prescriptions of insulin or non-insulin medication.
[e]Previous polypectomy was inferred from a pathology diagnosis of benign neoplasia of the colon, rectum or anus (ICD-9 codes 211.3 and 211.4).

Models were adjusted for age, sex, postmenopausal hormone-replacement therapy, diabetes, inflammatory bowel disease, adenoma removal and NSAID use. We used STATA software (STATA/SE version 12·0) for these analyses; *p*-values are two-sided and considered statistically significant if $p < 0{\cdot}05$.

To estimate the age-standardised incidence rate we used the age-standardised mortality rate calculation template, which uses the 2013 European Standard Population (ESP) from the UK Office of National Statistics. https://www.ons.gov.uk.

Power analysis

Forty percent of CRCs have normal expression of SMAD4. If the ~10% reduction seen in unselected cancers in meta-analyses is exclusively due to reductions in SMAD4-expressing cancers, we would expect this proportion to drop to ~30%. With alpha = 0.05 and a beta = 0.8, a sample size of 281 tumours in each group is required.

Patient and public involvement

Patients were not involved with this study.

RESULTS

Colorectal cancer incidence in statin users

We identified 164,025 beta-blocker users to provide the study base. Sixty nine thousands two hundred seventy two had received at least one prescription for statins and 94,753 had never used statins. One thousand one hundred eighty eight developed colorectal cancer (CRC) during the study period. The group of statin users included more men and more diabetics than the control group but otherwise the groups showed no clinically significant differences in age, follow-up, inflammatory bowel disease, adenoma removal, use of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) and postmenopausal hormones (among women) (Table 1). We observed a significantly lower risk of developing CRC among statin users than among controls, after adjusting for age, sex, follow-up, diabetes, inflammatory bowel disease, adenoma removal, NSAID use and postmenopausal hormone-replacement therapy (Table 2). Statin use was associated with a multivariate hazard ratio of CRC of 0.78 (95% confidence interval [CI], 0.66–0.92). The reduction in CRC incidence increases with the duration of statin therapy (Fig. 1).

Table 2. Colorectal cancer incidence in statin users vs non-users.

Patient factor	Adjusted HR[a]	*p*-value	Adjusted HR[a] 95% CI
Statin use	0.78	0.0025	0.66–0.92
Age	1.06	<0.0001	1.06–1.07
Female	0.55	<0.0001	0.47–0.65
NSAID use	5.43	<0.0001	4.65–6.34
Hormone-replacement therapy	0.86	0.315	0.63–1.16
Adenoma removal	4.65	<0.0001	3.55–6.09
Diabetes Mellitus	0.69	0.361	0.31–1.54
Inflammatory Bowel Disease	0.82	0.784	0.21–3.30

[a]Multivariate hazard ratios are adjusted for age, sex, NSAID use, Hormone-replacement therapy use, Diabetes Mellitus, previous adenoma removal and Inflammatory Bowel Disease.

Statin use and the incidence of colorectal cancer according to molecular subtype

We next collected a sample of 592 tumour specimens for molecular subtype analysis guided by a prior power analysis. The patient characteristics of the tumours we analysed did not differ significantly from those we did not analyse (Supplementary Table 2). We analysed SMAD4 expression and mutations in *BRAF* and *KRAS*. Two hundred fifteen (37%) had normal SMAD4 expression (SMAD4 positive), whereas 375 (63%) had weak or absent SMAD4 expression (SMAD4 negative). The concordance between the two pathologists was 0.86 ($\kappa = 0.70$, 95% CI 0.61–0.79). Representative examples of SMAD4 expression in tumours are shown in Fig. 2. We evaluated the influence of statin use on the risk of developing CRC subtyped according to the expression of SMAD4 in the tumour (Table 3). Statin use was associated with a reduced risk of developing CRC with normal SMAD4 expression (multivariate odds ratio, 0.64; 95% CI, 0.45–0.92). At the same time statin use was associated with an increased risk of developing CRC with weak or absent SMAD4 expression (multivariate odds ratio, 1.54; 95% CI, 1.08–2.19). The same sample of tumours was analysed for *KRAS* and *BRAF* mutations. Of the 592 tumours, 88 showed a *BRAF* mutation and 144 a *KRAS* mutation. DNA extraction was of insufficient quality to perform the *KRAS* or *BRAF* mutation analysis in 59 and 62 samples, respectively. We evaluated the influence of statin use on the risk of CRC subtyped according to the *KRAS* and *BRAF* mutation status (Table 2). There was no association between statin use and the development of cancers with mutations in *KRAS* or *BRAF* or the combination of both.

To show the overall effect of statin use on CRC incidence we estimated the age-standardised incidence rate for unselected CRC, CRC with normal SMAD4 expression (SMAD4 positive) and CRC with weak or absent SMAD4 expression (SMAD4 negative) in relation to statin use in the entire cohort. The age-standardised incidence rate of unselected CRC was 42 per 100,000 person-years in statin users and 54 per 100,000 person-years in non-users. The age-standardised incidence rate of SMAD4-positive tumours was 13 per 100,000 person-years in statin users compared to 23 per 100,000 person-years in non-users. In contrast, the age-standardised incidence rate of SMAD4-negative tumours was 29 per 100,000 person- years in statin users compared to 31 per 100,000 person-years in non-users.

DISCUSSION

In this large population-based cohort study we find that statin users have a significant reduction in the risk of developing colorectal cancer (CRC) compared with non-users. However, the primary aim of the study was to molecularly analyse the tumour specimens to look for

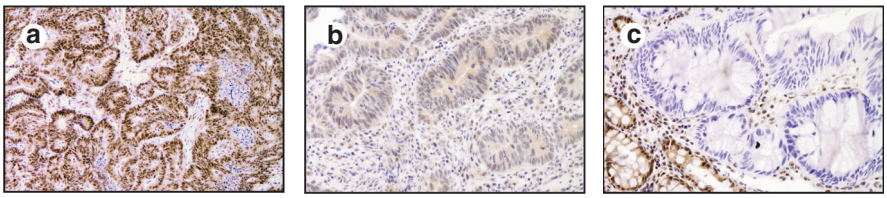

Fig. 2 SMAD4 expression in immunohistochemical assays of colorectal cancer tissue. Panel **a** shows normal expression of SMAD4 with strong (brown) nuclear staining. Tumours with this staining pattern are classified as SMAD4 positive. Panel **b** shows weak expression of SMAD4 in the nuclei of cancer cells. Tumours with this staining pattern are classified as SMAD4 negative. Panel **c** shows absent expression of SMAD4 with normal expression in adjacent non-malignant cells (bottom left). Tumours with this staining pattern are classified as SMAD4 negative.

Table 3. Statin use in relation to the molecular subtype of incident colorectal cancer.

Molecular subtype	Statin non-users	Statin users	*p*-value	OR (95% CI); *p*-value	Adjusted OR[a] (95% CI)
KRAS mutation % (no./total no.[b])					
KRAS wild-type	73 (178/245)	71 (203/287)		1.07 (0.76–1.49); $p = 0.70$	1.17 (0.82–1.66); $p = 0.38$
KRAS mutant	27 (67/245)	29 (84/287)	0.694	1.17 (0.81–1.69); $p = 0.40$	1.10 (0.74–1.61); $p = 0.64$
BRAF mutation % (no./total no.[b])					
BRAF wild-type	89 (218/246)	91 (257/283)		1.33 (0.88–1.99); $p = 0.17$	1.21 (0.79–1.87); $p = 0.38$
BRAF mutant	11 (28/246)	9 (26/283)	0.492	0.82 (0.47–1.43); $p = 0.48$	1.09 (0.59–1.99); $p = 0.79$
KRAS/BRAF % (no./total no.[b])					
KRAS/BRAF wild-type	61 (149/243)	61 (174/284)		1.11 (0.80–1.53); $p = 0.53$	1.09 (0.78–1.53); $p = 0.61$
KRAS/BRAF-pathway defect	39 (94/243)	39 (110/284)	0.991	1.08 (0.77–1.51); $p = 0.67$	1.13 (0.79–1.61); $p = 0.50$
SMAD4 IHC % (no./total no.[b])					
SMAD4 negative[c]	57 (159/279)	69 (216/311)		1.69 (1.20–2.36); $p = 0.002$	1.54 (1.08–2.19); $p = 0.017$
SMAD4 positive[d]	43 (120/279)	31 (95/311)	0.00225	0.58 (0.42–0.82); $p = 0.002$	0.64 (0.45–0.92); $p = 0.015$

[a]Multivariate odds ratios are adjusted for age, sex, NSAID use, Diabetes Mellitus, Hormone-replacement therapy use, Inflammatory Bowel Disease and previous polypectomy.
[b]Totals represent the total number of cancer specimens where the specific molecular analysis was possible.
[c]Cancers with absent or low-intensity Immunohistochemical SMAD4 staining are classified as SMAD4-negative cancers.
[d]Cancers with Immunohistochemical SMAD4 staining of normal intensity are classified as SMAD4-positive cancers.

associations, which might point to a molecular mechanism of action of statins in CRC. This analysis revealed that the overall risk reduction is entirely due to a reduction in the incidence of SMAD4-positive CRCs among statin users. In contrast, there is no change in the incidence of SMAD4-negative CRCs in statin users. This correlates well with our studies in vitro and in rodents [10] where we find that statins decrease the growth of SMAD4-positive CRCs but increase the growth of SMAD4-negative CRCs.

We found no association between the risk of developing CRC harbouring *KRAS* or *BRAF* mutations and statin use. The efficacy of other therapies targeting the RAS/RAF pathway such as epidermal growth factor receptor (EGFR) inhibitors, is dependent on the mutational status of *KRAS* and *BRAF* [19]. While there are other elements of this pathway that could also be assessed we have limited our analysis to those established as predicting response to the biological therapies known to target this pathway.

The methodology used in this study is analogous to that used to further elucidate the mechanism of action of aspirin for chemoprevention of CRC [20]. Here comparison of cyclooxygenase (COX)−2 expression in tumour specimens in aspirin users and controls showed a reduction in the incidence of COX-2 expressing tumours in NSAID users.

Previous studies have assessed whether statins can prevent colorectal polyp formation. A planned secondary analysis of large randomised controlled study of Celecoxib for colorectal adenoma chemoprevention showed no reduction in the incidence of polyps with statin use after 5 years follow-up [21]. This suggests that statins have no effect on the initiation and early progression of colorectal adenomas. A recent observational study showed that statin use is associated with a reduction in the development of post colonoscopy CRC [22] and we have previously shown that the use of statins after the diagnosis of CRC is associated with improved cancer-specific survival [23]. This suggests an effect of statins in the later stages of the adenoma to carcinoma sequence. SMAD4 is lost at a late stage during the stepwise sequence from adenoma to carcinoma suggesting that SMAD4-dependent signalling is most critical in the later stages of adenoma development [24]. Our data suggest that the anticancer benefit of statins is mediated, at least in part, through effects on SMAD4-dependent signalling. SMAD4 is the central signalling element in the transforming growth factor (TGF)β, Activin and BMP pathways. In vitro studies where the BMP-specific inhibitor Noggin fully antagonises the effects of statins on SMAD4-positive CRC cells, point towards the BMP pathway being the essential SMAD4-dependent pathway through which statins exert their effects [10]. Mutations in SMAD4 leading to low or absent protein expression are associated with a poor prognosis [25]. High SMAD4 expression is found in tumours with Microsatellite instability (MSI) and hypermethylated tumours (CIMP) [26].

Our study has several strengths. First, this is the first population-based cohort study to specifically look at molecular pathological epidemiological associations between statin use and the risk of developing CRC. Second, because the prescription data is collected directly from the pharmacy, errors in recall inherent to the assessment of drug use by questionnaire are avoided. Third, by performing the analysis in beta-blocker users we have tried to control for the higher rates of CRC seen in those with cardiovascular disease for which most patients take statins. Failure to do this will tend to underestimate any effect of statins. Fourth, despite the lack of a widely accepted, standardised classification scheme for SMAD4

expression in CRC the proportion of cancers with low SMAD4 expression is similar to those found by other investigators [27].

There are several limitations to our study. The study is observational, and makes use of anonymous patient information precluding adjustment for factors not registered in the databases such as dietary influences, smoking and body mass index. Likewise, we cannot adjust for the use of over-the-counter medication such as Non-Steroidal Anti-Inflammatory Drugs. We obtained tumour tissue from a convenience sample of confirmed cases of CRC detected in the two cohorts, but the baseline characteristics of these cases did not differ appreciably from those which we did not examine suggesting that we succeeded in analysing a representative pseudo-random sample of the tumours.

In summary, our results provide evidence in humans that statins act on CRC via a SMAD4-dependent mechanism. Chemoprevention with statins could potentially be targeted to those at risk of developing SMAD4 high tumours such as patients with Lynch Syndrome.

DATA AVAILABILITY

The data included in this study are available directly from the PHARMO database network (https://pharmo.nl).

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AUTHOR CONTRIBUTIONS

SO, RJ, LRAB and PV performed experiments and wrote the manuscript. NW, BW and TW performed experiments. EB and HP performed the statistical analyses. RH, LJACH, LK and HM designed the study. MS critically assessed the manuscript. JH conceived the study, acquired funding, wrote the manuscript.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Formal Medical Ethical permission for this study was deemed unnecessary after consultation with the Medical Ethical Committee of the Leiden University Medical Centre as it was retrospective and anonymous and complied with the ethical requirements of the 2 databases used, PALGA and PHARMO.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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Correspondence and requests for materials should be addressed to James C. H. Hardwick.