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# Density of CD3<sup>+</sup> and CD8<sup>+</sup> Cells in the Microenvironment of Colorectal Cancer according to Prediagnostic Physical Activity



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## ABSTRACT

**Background:** Physical activity is associated not only with a decreased risk of developing colorectal cancer but also with improved survival. One putative mechanism is the infiltration of immune cells in the tumor microenvironment. Experimental findings suggest that physical activity may mobilize immune cells to the tumor. We hypothesized that higher levels of physical activity prior to colorectal cancer diagnosis are associated with higher densities of tumor-infiltrating T-lymphocytes in colorectal cancer patients.

**Methods:** The study setting was a northern Swedish population-based cohort, including 109,792 participants with prospectively collected health- and lifestyle-related data. For 592 participants who later developed colorectal cancer, archival tumor tissue samples were used to assess the density of CD3<sup>+</sup> and CD8<sup>+</sup> cytotoxic T cells by IHC. Odds ratios for associations between self-reported, prediagnostic

recreational physical activity and immune cell infiltration were estimated by ordinal logistic regression.

**Results:** Recreational physical activity >3 times per week was associated with a higher density of CD8<sup>+</sup> T cells in the tumor front and center compared with participants reporting no recreational physical activity. Odds ratios were 2.77 (95% CI, 1.21–6.35) and 2.85 (95% CI, 1.28–6.33) for the tumor front and center, respectively, after adjustment for sex, age at diagnosis, and tumor stage. The risk estimates were consistent after additional adjustment for several potential confounders. For CD3, no clear associations were found.

**Conclusions:** Physical activity may promote the infiltration of CD8<sup>+</sup> immune cells in the tumor microenvironment of colorectal cancer.

**Impact:** The study provides some evidence on how physical activity may alter the prognosis in colorectal cancer.

## Introduction

Physical activity reduces the risk of colorectal cancer (1–3) and has been associated with improved survival (4–7). Evidence suggests that these beneficial effects on survival apply to both pre- and postdiagnostic physical activity (4, 5).

The prognosis of colorectal cancer is modulated by the infiltration of immune cells in the tumor microenvironment. A high density of leukocytes expressing the general T-cell marker, CD3, and/or the cytotoxic T-cell marker, CD8, in the tumor microenvironment is associated with longer patient survival (8–14). T-cell densities are also lower in the tumor microenvironment of metastatic compared with nonmetastatic colorectal cancer at diagnosis (11), possibly suggesting that tumor-infiltrating T cells have a role in preventing the dissemination of the disease.

The biological mechanisms behind the inverse relationship between physical activity and colorectal cancer have not been fully elucidated (15), but evidence suggests that physical activity may affect the immune system and immune cell infiltration of the tumor microenvironment in a prognostically favorable manner (16, 17). An acute bout of exercise mobilizes lymphocytes to the bloodstream (18) and to nonlymphoid organs (19). Furthermore, aerobic fitness is associated with lower proportions of senescent CD3<sup>+</sup> and CD8<sup>+</sup> cells in the blood and a higher proportion of naïve CD3<sup>+</sup> and CD8<sup>+</sup> (20). Associations between physical activity and tumor-infiltrating T cells have recently gained scientific attention. In animal models, physical activity has been associated with slower tumor growth (21), alteration of the CD8<sup>+</sup> cells in a cytotoxic and antitumoral efficacy, and increased numbers of CD8<sup>+</sup> cells in the tumor microenvironment (17, 22, 23).

The development of a neoplastic polyp into colorectal cancer can take up to 20 years (24), allowing for a long period of exposure to, and potential modulation by, exogenous and endogenous factors. We hypothesized that physical activity during this asymptomatic, prediagnostic phase of tumor progression may lead to higher densities of tumor-infiltrating T cells in colorectal cancer, which in turn improves cancer-specific survival.

In this study of colorectal cancer patients from a population-based cohort, we investigated self-reported, prediagnostic physical activity in relation to the densities of CD3<sup>+</sup> and CD8<sup>+</sup> immune cells in the tumor microenvironment.

## Materials and Methods

### Study cohort and study population

We conducted a cohort study using retrospectively collected colorectal cancer patients included in a prospectively sampled, population-based cohort from northern Sweden, the Västerbotten Intervention

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**Note:** Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (<http://cebp.aacrjournals.org/>).

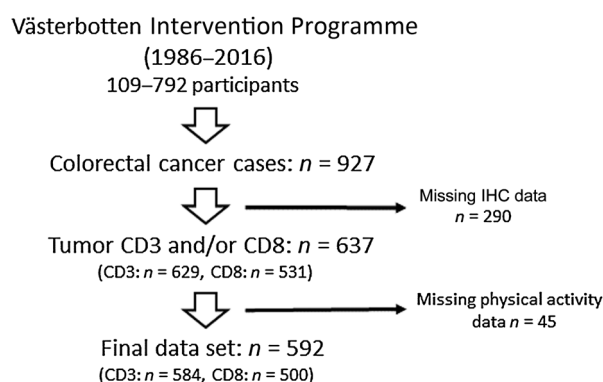
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**Figure 1.**

Selection of participants who developed colorectal cancer after participating in the Västerbotten Intervention Programme cohort and for whom both tumor-infiltrating lymphocyte data and prediagnostic physical activity data were available.

Programme (VIP; ref. 25). In brief, VIP is an ongoing health screening program in Region Västerbotten, initiated in 1986. All inhabitants are invited to participate at ages 40, 50, and 60. The program has a participation rate of approximately 65% and includes a health examination performed by a health professional, a questionnaire on diet, health, and lifestyle, and a blood sample (25). VIP is the largest cohort in the umbrella cohort referred to as the Northern Sweden Health and Disease Study.

The selection of participants for the present study is presented in Fig. 1. At the final recruitment date for the present study, January 19, 2016, the VIP included 109,792 participants (Fig. 1). The Swedish Cancer Registry was used to identify colorectal cancer cases (International Classification of Disease, ICD-10, codes C18.0 and C18.2–18.9 for colon cancer and C19.9 and C20.9 for rectal cancer). Diagnoses were verified, and data on tumor stage and anatomic tumor site were collected using the Swedish Colorectal Cancer Registry and, when necessary, individual patient records. A total of 927 colorectal cancer cases with prediagnostic participation in VIP were available for inclusion in the present study. Of these, formalin-fixed, paraffin-embedded tumor specimens from 637 patients were available and successfully analyzed for CD3 and/or CD8, including 592 with physical activity data.

### Baseline study variables

The exposure variable in this study was self-reported recreational physical activity estimated on a five-level scale (“never,” “now, and then,” “1–2 times per week,” “2–3 times per week,” “>3 times per week”) at baseline. The variable is based on a single questionnaire item, phrased “How often have you exercised in exercise clothing over the past three months, with the intent to improve your fitness and/or well-being?” The questionnaire for physical activity in VIP has not been specifically validated, but it was included in a multicenter validation of self-reported physical activity including the composite Cambridge index (26).

Other baseline variables used included age at baseline, body mass index (BMI, kg/m<sup>2</sup>), diabetes (dichotomous), self-reported smoking status (never smoker, previous smoker, or current smoker), self-reported alcohol intake (none, above the sex-specific median and under the sex-specific median), self-reported education level (no secondary, secondary, or post-secondary education), blood pressure, and plasma C-reactive protein concentrations (CRP, mg/l). Alcohol

intake data were obtained from a validated food frequency questionnaire (27) and divided into zero intake or above/below sex-specific medians in grams/day (4.88 g/day for men and 0.95 g/day for women, based on the initial colorectal cancer case population,  $n = 927$ ). Diabetes was defined as self-reported diabetes (yes/no) and/or fasting blood glucose level of >7.0 mmol/L and/or >12.2 mmol/L at two hours after the oral glucose tolerance test. CRP was analyzed quantitatively by immunoassay (Meso-Scale Discovery). When participants had two observations in VIP prior to colorectal cancer diagnosis, the observation closest to the date of diagnosis was selected as the baseline for the exposure variables in this study.

### Tumor tissue analysis

Tumor tissue samples collected during routine clinical diagnostics were acquired from the regional health care biobank in Västerbotten (Biobanken Norr). According to routine procedures, diagnostic colorectal cancer specimens obtained after primary tissue resection were fixed in 4% formaldehyde and embedded in paraffin, and long-termed stored at room temperature. From each patient, one 4- $\mu$ m section was cut, dried, dewaxed, and rehydrated. For IHC procedures, a staining machine (Ventana BenchMark Ultra, Ventana Medical Systems, Inc.) was used with the CC1 standard pretreatment and the iVIEW DAB detection kit (Ventana Medical Systems, Inc.) for visualization. Anti-CD8 polyclonal antibody (clone C8/144B; Dako) and primary polyclonal CD3 antibody (Dako) were used at a dilution of 1:50. The slides were counterstained with hematoxylin. The cases were diagnosed between 1992 and 2016, and the median storage time was approximately 11 years.

The immune cell density was scored as (1) no or sporadic; (2) moderate numbers; (3) abundant occurrence; or (4) highly abundant cells. This scoring was assigned for both cell types in three locations within each tumor: the tumor front (cells localized in stroma adjacent to the invasive margin of the tumor), the tumor center (cells localized in the stroma within the tumor mass), and the intraepithelial compartment (cells localized within tumor cell nests). A total score for each cell type and tumor was calculated as the sum of the scores from each location, ranging from 3 to 12. The total score was classified as low (3–4), intermediate (5–6), or high (7–12), in accordance with previous studies (8, 9, 28). Immune infiltration was scored by one observer (BG) under the supervision of a senior consultant in gastrointestinal pathology (RP). A subsample of 35 consecutively selected tumors was reexamined by a second observer to assess interobserver agreement (weighted Cohen Kappa of 0.61–0.87, with 0.71 for total score).

### Statistical analyses

Baseline characteristics were compared using the  $\chi^2$  test for ordinal and categorical variables. Continuous variables were defined as normally distributed or not using a Shapiro–Wilks test. All continuous variables were nonnormally distributed, and the Kruskal–Wallis test was therefore used for continuous variables.

Odds ratios (OR) were calculated using ordinal logistic regression and generalized ordinal logistic regression. For each multivariable model, we tested if the proportional odds assumption was met using the Brant test. When violated, generalized ordinal logistic regression was used (29) with relaxing of the proportional odds assumption for variables that violated the assumption. Calculations were made for both cell types (CD3<sup>+</sup> and CD8<sup>+</sup>) in each position (tumor front, center, intraepithelial, and total score). In order to test for a dose-response relationship,  $P$  trends were calculated by including the physical activity categories (numbered 1–5 for lowest to highest) as a continuous variable in the regression models.

Three regression models were constructed: crude (univariable), minimally adjusted (sex, age at diagnosis, and tumor stage), and fully adjusted. Physical activity was the exposure variable and treated as an ordinal variable. Several potential confounders of associations between physical activity and tumor immune cell infiltration were available in the data set and considered for inclusion in the fully adjusted model. As summarized in a directed acyclic graph in Supplementary Fig. S1, these included the variables in the minimally adjusted model as well as baseline age, tumor site, BMI, smoking status, diabetes status, alcohol intake, education level, systolic and diastolic blood pressure, CRP, and year of diagnosis. Although additional tumor data were available for some or all of the patients in this study (microsatellite instability status, *BRAF*, and *KRAS* mutations, and CpG island methylator phenotype), we did not consider them to be potential confounders, given the current lack of theory or evidence for an independent association with physical activity (30–33). Variables were categorized as described in the baseline study variables section or left as continuous variables if not otherwise specified. Covariates were selected for the fully adjusted model using bivariable ordinal logistic regressions. Variables that altered the beta-coefficient for the association between recreational physical activity (as a continuous variable) and CD3<sup>+</sup> or CD8<sup>+</sup> total score by more than 10% were included in the respective fully adjusted model (34). Collinearity was tested using variance inflation factor. Variance inflation factor above 5 was considered high collinearity. No collinearity was observed in the minimally or fully adjusted models. The additional covariates included in the final fully adjusted models were, for CD8<sup>+</sup>: tumor site, baseline age, CRP, and year of diagnosis; for CD3<sup>+</sup>: tumor site, BMI, smoking status, systolic blood pressure, and alcohol intake.

Missing data for covariates in the risk analyses were imputed, using sex-specific medians for nonnormally distributed continuous variables, and sex-specific modes for categorical variables based on the final study population ( $n = 592$ ). Due to a higher number of missing observations for alcohol ( $n = 29$ ), missing data were included as a separate category in the regression models.

Secondary analyses included three prespecified subgroup analyses in ordinal logistic regression models. First, stratification by sex was conducted as a sensitivity analysis. Secondly, given our hypothesis of a role for physical activity in tumor progression, we stratified by follow-up time from baseline to diagnosis (at a mean of 9.4 years). Finally, in consideration of the close interrelationship between physical activity and body size, with respect to both colorectal cancer and the immune response and inflammation (35, 36), we ran subgroup analyses stratifying at the BMI cutoff-point between normal weight and overweight ( $\text{BMI} \geq 25 \text{ kg/m}^2$ ). All subgroup analyses were conducted using the minimally adjusted model.

All statistical analyses were conducted using STATA version 15.1 (StataCorp LP).

This study was approved by the Regional Ethics Review Board in Umeå, Sweden (2015-243-31 and amendment 2020-00081).

## Results

In the final study population of 592 colorectal cancer cases (Table 1), 584 (98.6%) had CD3 IHC data, and 500 (84.5%) had CD8 IHC data available.

The proportion of women was 49.3%, and the mean age at diagnosis was 65.6 years. The median time from baseline to diagnosis was 9.4 years (25th–75th percentile 5.2–13.3 years).

Higher CD3<sup>+</sup> and CD8<sup>+</sup> total scores were associated with female gender, right-sided colon cancer, and less-advanced tumor stage (Table 2).

In the ordinal logistic regression models, physical activity >3 times per week was associated with a higher density of CD8<sup>+</sup> immune cells in the tumor front and center (Table 3). ORs were 2.77 (95% CI, 1.21–6.35) and 2.85 (95% CI, 1.28–6.33) for the tumor front and center, respectively, after adjustment for sex, age at diagnosis, and tumor stage. These results were consistent and remained statistically significant after adjustment for additional potential confounders in the fully adjusted model. For the CD8<sup>+</sup> total score, the ORs were attenuated to approximately 2 and no longer statistically significant, due to a null association for CD8<sup>+</sup> intraepithelial immune cell density. *P* trends were not significant.

For CD3<sup>+</sup>, there were no clear associations between recreational physical activity and immune cell infiltration, with ORs generally around 1 (Table 3).

In analyses stratified by sex, time from baseline to colorectal cancer diagnosis, and BMI (Table 4), subgroup results were similar to each other, and the overall findings in Table 3.

## Discussion

In this population-based study of 592 colorectal cancer cases from a prospectively sampled cohort, the highest category of prediagnostic self-reported recreational physical activity (>3 times/week) was associated with a higher density of CD8<sup>+</sup> immune cells in the tumor front and center. Overall, these findings provide some suggestive support for our primary hypothesis.

In our study, higher immune cell infiltration at higher recreational physical activity was observed for CD8<sup>+</sup> but not for CD3<sup>+</sup> cells. Animal studies have shown that physical activity mobilizes CD8<sup>+</sup> cells to the bloodstream (18), as well as to the tumor microenvironment (17, 22, 23). In one recent murine study, physical activity increased the infiltration of CD8<sup>+</sup> cells in the tumor microenvironment, and the presence of CD8<sup>+</sup> cells was associated with enhanced survival and suppressed tumor growth (23). Similar results have not been demonstrated for CD3<sup>+</sup> cells. Because CD3<sup>+</sup> is a pan T-cell marker, whereas CD8<sup>+</sup> is a marker of the subpopulation of cytotoxic T cells, physical activity appears to influence different T-cell populations in different patterns. Our results indicate that the effect of prediagnostic physical activity on tumor-infiltrating lymphocytes may be more pronounced for CD8<sup>+</sup> compared with CD3<sup>+</sup> immune cells. A recent study by Koh and colleagues (37) hypothesized that the survival benefits seen with increased postdiagnosis physical activity might be stronger in tumors with lower T-cell densities. Koh and colleagues concluded this association between postdiagnosis physical activity and improved survival was seen in tumors with lower CD3<sup>+</sup> T-cell densities, but no association was seen for CD8<sup>+</sup>. Their results in perspective to the present study suggest that physical activity alters the immune infiltration in the microenvironment of the tumor by increasing the densities of CD8<sup>+</sup> cells. However, in patients with low CD3<sup>+</sup> density tumors, postdiagnosis physical activity has the most beneficial survival effects.

The lack of association between recreational physical activity and intraepithelial infiltration of CD8<sup>+</sup> cells was not consistent with our hypothesis or with the results for CD8<sup>+</sup> in the tumor front and center. There are, broadly, four subsets of CD8<sup>+</sup> T cells, ranging from naïve to effector memory subtypes which reflect different maturation steps. These cell types express different surface proteins (38, 39). The naïve CD8<sup>+</sup> T cells have not been introduced to an antigen, whereas the

**Table 1.** Baseline and clinical variables, according to levels of self-reported prediagnostic physical activity, in the final data set of 592 colorectal cancer cases and for an additional 45 cases excluded due to missing physical activity data.

	Recreational physical activity							<i>p</i> <sup>b</sup>
	Total <i>n</i> = 637 <sup>a</sup>	Never <i>n</i> = 294	Now and then <i>n</i> = 147	1–2 times/wk <i>n</i> = 71	2–3 times/wk <i>n</i> = 51	>3 times/wk <i>n</i> = 29	Missing <sup>b</sup> <i>n</i> = 45	
Sex, <i>n</i> (%)								
Man	323	153 (52.0)	82 (55.8)	33 (46.5)	24 (47.1)	13 (44.8)	18 (40.0)	0.601 <sup>c</sup>
Woman	314	141 (48.0)	65 (44.2)	38 (53.5)	27 (52.9)	16 (55.2)	27 (60.0)	
Age at baseline <sup>d</sup>	637	59.9 (50.2–60.1)	59.9 (50.1–60.1)	59.8 (50.0–60.0)	59.9 (50.0–60.1)	59.9 (50.4–60.0)	59.5 (50.0–60.0)	0.335 <sup>e</sup>
Age at diagnosis, <i>n</i> (%)								
<55	86	31 (10.5)	23 (15.7)	15 (21.1)	8 (15.7)	3 (10.3)	6 (13.3)	0.326 <sup>c</sup>
55–65	182	85 (28.9)	39 (26.5)	23 (32.4)	14 (27.5)	13 (44.8)	8 (17.8)	
65–75	284	135 (45.9)	65 (44.2)	25 (35.2)	25 (49.0)	11 (37.9)	23 (51.1)	
>75	85	43 (14.6)	20 (13.6)	8 (11.3)	4 (7.8)	2 (6.9)	8 (17.8)	
Time from baseline to diagnosis, years <sup>d</sup>	637	9.44 (5.38–13.7)	8.92 (5.75–13.2)	8.30 (4.63–13.3)	6.49 (4.61–12.0)	6.15 (3.50–8.65)	12.4 (8.44–18.8)	0.009 <sup>e</sup>
BMI <sup>f</sup> , kg/m <sup>2</sup>	634	27.0 (3.9)	26.5 (3.7)	26.3 (4.4)	26.7 (4.9)	26.5 (5.3)	26.0 (4.3)	0.215 <sup>e</sup>
Missing, ( <i>n</i> )	3	2	0	0	0	0	0	
Diabetes, <i>n</i> (%)								
No	561	263 (89.8)	126 (86.3)	63 (88.7)	46 (92.0)	26 (89.7)	37 (82.2)	0.785 <sup>c</sup>
Yes	73	30 (10.2)	20 (13.7)	8 (11.3)	4 (8.0)	3 (10.3)	8 (17.8)	
Missing <sup>b</sup> , ( <i>n</i> )	3	1	1	0	1	0	0	
Smoker, <i>n</i> (%)								
Never smoker	258	106 (36.8)	72 (50.4)	28 (40.0)	21 (42.0)	15 (57.7)	16 (38.1)	0.152 <sup>c</sup>
Ex-smoker	246	128 (44.4)	47 (32.9)	33 (47.1)	20 (40.0)	8 (30.8)	10 (23.8)	
Current smoker	115	54 (18.8)	24 (16.8)	9 (12.9)	9 (18.0)	3 (11.5)	16 (38.1)	
Missing <sup>b</sup> , ( <i>n</i> )	18	6	4	1	1	3	3	
Alcohol intake, <i>n</i> (%)								
None	48	25 (8.9)	9 (6.5)	2 (2.9)	6 (12.2)	4 (14.8)	2 (33.3)	0.080 <sup>c</sup>
Under median <sup>f</sup>	255	131 (46.8)	70 (50.4)	26 (38.2)	18 (36.7)	7 (25.9)	3 (50.0)	
Above median <sup>f</sup>	266	124 (44.3)	60 (43.2)	40 (58.8)	25 (51.0)	16 (59.3)	1 (16.7)	
Missing <sup>b</sup> , ( <i>n</i> )	68	14	8	3	2	2	39	
Education level, <i>n</i> (%)								
No secondary	433	223 (76.6)	99 (67.8)	38 (54.3)	32 (64.0)	14 (50.0)	27 (73.0)	0.002 <sup>c</sup>
Secondary	90	31 (10.7)	26 (17.8)	16 (22.9)	7 (14.0)	5 (17.9)	5 (13.5)	
Post-secondary	99	37 (12.7)	21 (14.4)	16 (22.9)	11 (22.0)	9 (32.1)	5 (13.5)	
Missing <sup>b</sup> , ( <i>n</i> )	15	3	1	1	1	1	8	
Systolic BP <sup>d</sup> , mm Hg	627	135 (122–148)	130 (120–142)	131 (118–140)	125 (116–140)	130 (120–145)	135 (120–145)	0.043 <sup>e</sup>
Missing <sup>b</sup> , ( <i>n</i> )	10	7	1	0	1	0	1	
Diastolic BP <sup>d</sup> , mm Hg	625	85 (76–90)	84 (76–90)	80 (75–90)	80 (70–88)	81 (74–90)	84 (74–90)	0.287 <sup>e</sup>
Missing <sup>b</sup> , ( <i>n</i> )	12	8	1	0	1	1	1	
CRP <sup>d</sup> , mg/L	637	1.87 (0.77–3.67)	1.59 (0.81–2.88)	1.13 (0.64–2.73)	1.86 (0.70–4.59)	1.27 (0.71–2.97)	1.39 (0.62–2.71)	0.231 <sup>e</sup>
Tumor site, <i>n</i> (%)								
Right colon	225	103 (35.2)	56 (38.1)	22 (31.0)	13 (25.5)	8 (27.6)	23 (51.1)	0.234 <sup>c</sup>
Left colon	194	97 (33.1)	47 (32.0)	23 (32.4)	12 (23.5)	8 (27.6)	7 (15.6)	
Rectum	217	93 (31.7)	44 (29.9)	26 (36.6)	26 (51.0)	13 (44.8)	15 (33.3)	
Missing <sup>b</sup> , ( <i>n</i> )	1	1	0	0	0	0	0	
Stage, <i>n</i> (%)								
I and II	345	160 (55.9)	80 (55.2)	40 (56.3)	29 (58.0)	14 (48.3)	22 (50.0)	0.940 <sup>c</sup>
III and IV	280	126 (44.1)	65 (44.8)	31 (43.7)	21 (42.0)	15 (51.7)	22 (50.0)	
Missing <sup>b</sup> , ( <i>n</i> )	12	8	2	0	1	0	1	

Abbreviations: BMI, body mass index; BP, blood pressure; CRP, C-reactive protein; wk, week.

<sup>a</sup>Final data set (*n* = 592) and cases excluded due to missing recreational physical activity data (*n* = 45).<sup>b</sup>Missing categories not included in statistical comparisons.<sup>c</sup>Chi-square test.<sup>d</sup>Results displayed as median (25–75 percentile).<sup>e</sup>Kruskal–Wallis test.<sup>f</sup>Sex-specific medians, 4.88 g/day for men and 0.95 g/day for women, calculated from the initial colorectal cancer population diagnosed after participation in the cohort (*n* = 927).

effector memory subtypes are those that, to the highest extent, exert the cytotoxic function. A previous study on 13 healthy and active humans showed that effector memory subtypes were mobilized to a greater extent than the naïve cells following exercise (18). Different subtypes also

express different adhesion molecules and thus infiltrate different tissues to a different extent. Perhaps, in a similar manner, different CD8<sup>+</sup> subtypes respond differently to physical activity and infiltrate different localizations in the tumor microenvironment in colorectal cancer.

**Table 2.** Clinical variables and characteristics of colorectal cancer patients according to CD3<sup>+</sup> and CD8<sup>+</sup> total score.

	CD8 <sup>+</sup> total score <sup>a</sup>				P value	CD3 <sup>+</sup> total score <sup>a</sup>				P value
	N = 519	1 (low) n = 207	2 (med) n = 139	3 (high) n = 173		N = 617	1 (low) n = 191	2 (med) n = 174	3 (high) n = 252	
Age at diagnosis, n (%)										
<55	58	25 (12.1)	20 (14.4)	13 (7.5)	0.100 <sup>b</sup>	83	26 (13.6)	29 (16.7)	28 (11.1)	0.057 <sup>b</sup>
55–65	132	57 (27.5)	34 (24.5)	41 (23.7)		176	61 (31.9)	51 (29.3)	64 (25.4)	
65–75	244	101 (48.8)	62 (44.6)	81 (46.8)		274	86 (45.0)	75 (43.1)	113 (44.8)	
>75	85	24 (11.6)	23 (16.6)	38 (22.0)		84	18 (9.4)	19 (10.9)	47 (18.7)	
Sex, n (%)										
Man	254	119 (57.5)	66 (47.5)	69 (39.9)	0.003 <sup>b</sup>	313	98 (51.3)	107 (61.5)	108 (42.9)	0.001 <sup>b</sup>
Woman	265	88 (42.5)	73 (52.5)	104 (60.1)		304	93 (48.7)	67 (38.5)	144 (57.1)	
Tumor site, n (%)										
Right colon	195	68 (32.9)	42 (30.4)	85 (49.1)	0.001 <sup>b</sup>	224	66 (34.6)	56 (32.2)	102 (40.6)	0.051 <sup>b</sup>
Left colon	161	64 (30.9)	46 (33.3)	51 (29.5)		190	55 (28.8)	51 (29.3)	84 (33.5)	
Rectum	162	75 (36.2)	50 (36.2)	37 (21.4)		202	70 (36.7)	67 (38.5)	65 (25.9)	
Missing <sup>c</sup>	1	0	1	0		1	0	0	1	
Stage, n (%)										
I and II	284	95 (46.6)	75 (54.7)	114 (68.3)	<0.001 <sup>b</sup>	334	74 (39.0)	94 (54.3)	166 (68.3)	<0.001 <sup>b</sup>
III and IV	224	109 (53.4)	62 (45.3)	53 (31.7)		272	116 (61.1)	79 (45.7)	77 (31.7)	
Missing <sup>c</sup>	11	3	2	6		11	1	1	9	

Abbreviations: CD, cluster of differentiation; med, medium.

<sup>a</sup>Calculated as the sum of the scores from each location, ranging from 3 to 12. The total score was classified as low (3–4), intermediate (5–6), or high (7–12).<sup>b</sup>Chi-square test.<sup>c</sup>Missing categories not included in statistical comparisons.

In our study, the association between higher recreational physical activity and higher tumor immune cell infiltration was primarily seen in participants reporting recreational physical activity >3 times per week (Table 3), and *P* trends were not statistically significant. Physical activity may, therefore, have a threshold rather than a dose–response relationship with tumor immune cell infiltration, occurring at higher physical activity levels. The amount of physical activity required to lower the risk and mortality of colorectal cancer is not known, and the evidence to date does not permit distinction between a threshold or dose–response effect (1, 5, 40–42).

Physical activity is associated with a lower risk of, and improved survival in, colorectal cancer (3, 4). Colorectal cancer is also associated with several lifestyle factors such as obesity, diabetes, and intake of processed meat (43). The development of a neoplastic polyp into a colorectal adenocarcinoma can take up to 20 years (24). It is plausible that differences in the tumor microenvironment begin long before the colorectal cancer is diagnosed. Our baseline data consist of self-reported physical activity data prior to the date of diagnosis. We do not know if the participants maintain their physical activity level until tumor tissue sample collection, but in cases with shorter time from baseline to diagnosis, the reported physical activity is more likely to have occurred in the presence of a neoplastic lesion. Our subgroup analysis of time from baseline to diagnosis showed no material differences compared with the total data set, except the loss of significant results in some analyses (Table 4), possibly due to loss of statistical power.

Obesity affects the immune system and is associated with chronic, low-grade, systemic inflammation (35). There is also an established association between overweight and increased risk of colorectal cancer (44). A previous study investigated the association between BMI and immune infiltration of CD3<sup>+</sup>, CD8<sup>+</sup>, CD45RO<sup>+</sup>, and FOXP3<sup>+</sup> cells in the tumor microenvironment in colorectal cancer and showed no association (45). In contrast, another study reported an association between obesity and higher immune infiltration of CD8<sup>+</sup> cells in the colorectal cancer tumor microenvironment (46). Therefore, in addi-

tion to considering BMI as a potential confounder, we conducted a subgroup analysis stratifying at a cutoff of BMI 25 kg/m<sup>2</sup>. The results were consistent with those for the total data set (Table 4), suggesting that the immune infiltration of CD8<sup>+</sup> is associated with physical activity regardless of BMI.

There are limitations in the present study. Probably the most important weakness was the use of a single self-reported recreational physical activity questionnaire item as the primary exposure variable. Objective measurements of usual physical activity, such as with an accelerometer, or a fitness test (e.g., 6-minute walking test or VO<sub>2</sub>-max cycling test) as a proxy of usual physical activity levels, are more reliable than self-reported estimations (47). Self-reported physical activity may overestimate the actual physical activity when compared with accelerometer data (48, 49). The VIP questionnaire includes several questions on physical activity, including occupational physical activity, as well as several specific types of activities. However, they are difficult to combine (into MET-hours/week, for example) and have varied over the years. Also, occupational physical activity may be more reflective of other factors than overall physical activity, e.g., socioeconomic status (with accompanying differences in other lifestyle-related risk factors), as well as gender differences in type of occupational physical activity, and results have been conflicting regarding whether occupation affects how physically active one is (50, 51). In order to address our hypothesis while taking into consideration the risk of chance findings due to multiple testing if several exposure variables were to be used, we selected recreational activity as the variable most likely to best represent overall physical activity level and to best capture moderate to vigorous activity. The questionnaire for physical activity in the VIP has not been specifically validated, but it was included in a multicenter validation of self-reported physical activity including the composite Cambridge index (26). Another weakness, common in molecular epidemiology studies such as this one, is the risk of selection bias due to missing tumor data. Although essentially no patients were unaccounted for in our study, IHC data were lacking for approximately one third of the potentially eligible cases, due largely to lack of tumor

**Table 3.** Odds ratios (95% confidence intervals) for self-reported, prediagnostic, recreational physical activity in relation to tumor immune cell infiltration in colorectal cancer<sup>a</sup>.

		Recreational physical activity					<i>P</i> trend <sup>b</sup>	
		Never	Now and then	1-2 times/wk	2-3 times/wk	>3 times/wk		
<b>CD8<sup>a</sup></b>								
Front <sup>c</sup>	Cases ( <i>n</i> )	237	125	55	49	22		
	Crude	1 (Ref)	1.31 (0.88–1.94)	1.00 (0.58–1.70)	0.74 (0.42–1.30)	2.33 (1.05–5.18)	0.593	
	Minimally adj. <sup>d</sup>	1 (Ref)	1.36 (0.92–2.02)	1.02 (0.59–1.78)	0.75 (0.42–1.32)	2.77 (1.21–6.35)	0.449	
	Fully adj. <sup>e</sup>	1 (Ref)	1.32 (0.89–1.98)	1.13 (0.64–1.97)	0.81 (0.45–1.45)	2.91 (1.25–6.75)	0.264	
Center <sup>f</sup>	Cases ( <i>n</i> )	247	127	55	49	22		
	Crude	1 (Ref)	1.25 (0.84–1.86)	0.98 (0.56–1.72)	0.98 (0.55–1.73)	2.25 (1.03–4.92)	0.251	
	Minimally adj. <sup>d</sup>	1 (Ref)	1.38 (0.92–2.06)	1.03 (0.58–1.81)	1.05 (0.59–1.87)	2.85 (1.28–6.33)	0.107	
	Fully adj. <sup>e</sup>	1 (Ref)	1.39 (0.93–2.08)	1.05 (0.59–1.86)	1.04 (0.58–1.86)	2.92 (1.31–6.50)	0.095	
Intra-ep. <sup>g</sup>	Cases ( <i>n</i> )	247	127	55	49	22		
	Crude	1 (Ref)	1.03 (0.67–1.58)	1.17 (0.67–2.03)	1.22 (0.67–2.21)	0.95 (0.41–2.20)	0.624	
	Minimally adj. <sup>d,h</sup>							
	1 vs. 2, 3, 4	1 (Ref)	0.97 (0.62–1.54)	1.20 (0.65–2.13)	1.17 (0.61–2.23)	1.18 (0.47–2.97)	0.497	
	1, 2 vs. 3, 4	1 (Ref)	1.66 (1.00–2.78)	1.13 (0.55–2.35)	2.09 (1.04–4.19)	1.42 (0.48–4.16)	0.102	
	1, 2, 3 vs. 4	1 (Ref)	1.38 (0.75–2.56)	1.25 (0.53–2.92)	1.14 (0.46–2.81)	0.37 (0.05–2.88)	0.844	
	Fully adj. <sup>e,i</sup>							
	1, 2, 3, and 4	1 (Ref)	0.95 (0.60–1.50)	1.25 (0.67–2.31)	1.16 (0.60–2.25)	1.18 (0.46–3.00)	0.485	
	1, 2 vs. 3, 4	1 (Ref)	1.55 (0.92–2.59)	1.21 (0.58–2.51)	2.02 (1.00–4.10)	1.49 (0.51–4.36)	0.094	
	1, 2, 3 vs. 4	1 (Ref)	1.28 (0.69–2.39)	1.25 (0.52–2.99)	1.14 (0.45–2.87)	0.44 (0.06–3.44)	0.946	
	TS <sup>j</sup>	Cases ( <i>n</i> )	237	125	55	49	22	
	Crude	1 (Ref)	1.09 (0.73–1.63)	1.06 (0.62–1.81)	0.97 (0.54–1.75)	1.82 (0.81–4.09)	0.386	
	Minimally adj. <sup>d</sup>	1 (Ref)	1.12 (0.75–1.69)	1.10 (0.63–1.91)	1.00 (0.55–1.82)	2.19 (0.55–5.10)	0.247	
	Fully adj. <sup>e</sup>	1 (Ref)	1.11 (0.74–1.68)	1.16 (0.66–2.02)	1.03 (0.55–1.90)	2.34 (0.99–5.52)	0.185	
<b>CD3<sup>a</sup></b>								
Front <sup>c</sup>	Cases ( <i>n</i> )	282	144	68	51	29		
	Crude	1 (Ref)	1.16 (0.80–1.67)	0.95 (0.59–1.53)	1.08 (0.64–1.82)	0.76 (0.38–1.52)	0.722	
	Minimally adj. <sup>d,k</sup>							
	1, 2, 3, and 4	1 (Ref)	1.02 (0.64–1.63)	0.95 (0.51–1.75)	1.31 (0.63–2.71)	0.77 (0.33–1.80)	0.992	
	1, 2 vs. 3, 4	1 (Ref)	1.09 (0.71–1.66)	0.85 (0.48–1.50)	1.11 (0.59–2.08)	0.69 (0.29–1.67)	0.635	
	1, 2, 3 vs. 4	1 (Ref)	2.27 (1.29–4.01)	1.18 (0.51–2.74)	0.73 (0.24–2.20)	1.73 (0.55–5.46)	0.643	
	Fully adj. <sup>k,l</sup>							
	1 vs. 2, 3, 4	1 (Ref)	0.91 (0.57–1.47)	0.97 (0.52–1.80)	1.54 (0.74–3.24)	0.87 (0.36–2.10)	0.686	
	1, 2 vs. 3, 4	1 (Ref)	0.97 (0.63–1.50)	0.89 (0.50–1.58)	1.30 (0.68–2.47)	0.75 (0.30–1.85)	0.949	
	1, 2, 3 vs. 4	1 (Ref)	2.12 (1.19–3.77)	1.25 (0.54–2.92)	0.81 (0.27–2.47)	1.86 (0.57–6.03)	0.488	
	Center <sup>f</sup>	Cases ( <i>n</i> )	290	145	69	51	29	
	Crude	1 (Ref)	1.08 (0.75–1.54)	1.02 (0.63–1.65)	1.44 (0.85–2.42)	1.43 (0.73–2.83)	0.153	
	Minimally adj. <sup>d,m</sup>							
	1 vs. 2, 3, 4	1 (Ref)	1.37 (0.83–2.28)	0.92 (0.50–1.70)	1.88 (0.80–4.39)	2.20 (0.73–6.64)	0.965	
	1, 2 vs. 3, 4	1 (Ref)	0.88 (0.58–1.34)	1.04 (0.60–1.80)	1.43 (0.78–2.63)	1.60 (0.72–3.54)	0.172	
	1, 2, 3 vs. 4	1 (Ref)	1.53 (0.88–2.66)	1.19 (0.57–2.50)	1.04 (0.43–2.49)	1.74 (0.61–4.98)	0.392	
	Fully adj. <sup>l</sup>	1 (Ref)	1.07 (0.74–1.55)	0.98 (0.60–1.60)	1.41 (0.83–2.42)	1.66 (0.81–3.41)	0.132	
	Intra-ep. <sup>g</sup>	Cases ( <i>n</i> )	290	145	69	51	28	
	Crude	1 (Ref)	0.99 (0.67–1.45)	0.97 (0.59–1.59)	1.35 (0.77–2.36)	0.73 (0.33–1.61)	0.931	
	Minimally adj. <sup>d</sup>	1 (Ref)	1.04 (0.70–1.54)	0.94 (0.56–1.57)	1.41 (0.81–2.48)	0.77 (0.34–1.74)	0.805	
	Fully adj. <sup>l</sup>	1 (Ref)	0.96 (0.64–1.44)	0.96 (0.57–1.60)	1.52 (0.86–2.70)	0.73 (0.32–1.67)	0.757	
	TS <sup>j</sup>	Cases ( <i>n</i> )	282	144	68	51	28	
	Crude	1 (Ref)	0.97 (0.67–1.41)	0.94 (0.58–1.55)	1.29 (0.75–2.21)	1.07 (0.53–2.16)	0.569	
	Minimally adj. <sup>d,n</sup>	1 (Ref)	0.99 (0.68–1.45)	0.94 (0.57–1.55)	1.29 (0.74–2.23)	1.18 (0.57–2.45)	0.481	
	Fully adj. <sup>l</sup>	1 (Ref)	0.91 (0.62–1.34)	0.94 (0.57–1.56)	1.44 (0.82–2.52)	1.27 (0.61–2.66)	0.319	

Abbreviations: Adj., adjusted; CD, cluster of differentiation; Intra-ep, intraepithelial; TS, total score; wk, week.

<sup>a</sup>Odds ratios were calculated per one-level increase in the category of immune cell infiltration (on a 4-level scale) using ordinal logistic regression or when the proportional odds assumption was not met as tested by the Brant test, generalized ordinal logistic regression.<sup>b</sup>*P* trends were calculated by including physical activity categories (numbered 1-5, lowest to highest) as a continuous variable in the regression models.<sup>c</sup>Invasive front of the tumor.<sup>d</sup>Variables included sex, age at diagnosis, and tumor stage.<sup>e</sup>Variables included sex, age at diagnosis, tumor stage, tumor site, age at baseline, CRP, and year when diagnosed.<sup>f</sup>Center/core of the tumor.<sup>g</sup>Within tumor cell nests.<sup>h</sup>Generalized ordinal logistic regression. Relaxed proportional odds assumption for recreational physical activity.<sup>i</sup>Generalized ordinal logistic regression. Relaxed proportional odds assumption for physical activity and tumor site.<sup>j</sup>Calculated as the sum of the scores from each location, ranging from 3 to 12. The total score was classified as low (3-4), intermediate (5-6), or high (7-12).<sup>k</sup>Generalized ordinal logistic regression. Relaxed proportional odds assumption for recreational physical activity, sex, and age at diagnosis.<sup>l</sup>Variables included sex, age at diagnosis, tumor stage, tumor site, BMI, smoking, systolic blood pressure, and alcohol intake.<sup>m</sup>Generalized ordinal logistic regression. Relaxed proportional odds assumption for recreational physical activity and sex.<sup>n</sup>Generalized ordinal logistic regression. Relaxed proportional odds assumption for sex.

**Table 4.** Odds ratios (95% confidence intervals) for self-reported, prediagnostic recreational physical activity in relation to tumor immune cell infiltration in colorectal cancer, according to subgroups based on sex, time from baseline to diagnosis, and body size.

	Recreational physical activity					P trend <sup>a</sup>
	Never	Now and then	1-2 times/wk	2-3 times/wk	>3 times/wk	
<b>Women<sup>b</sup></b>						
<b>CD8<sup>c,d</sup></b>	114	61	32	27	11	
Front <sup>e</sup>	Ref (1.0)	1.31 (0.74-2.32)	0.71 (0.33-1.51)	1.00 (0.47-2.13)	2.15 (0.71-6.55)	0.636
Center <sup>f</sup>	Ref (1.0)	1.59 (0.89-2.84)	0.98 (0.46-2.10)	1.11 (0.52-2.39)	3.53 (1.15-10.2)	0.176
Intra-ep. <sup>g</sup>	Ref (1.0)	1.40 (0.76-2.59)	0.89 (0.41-1.95)	1.27 (0.55-2.93)	0.88 (0.24-3.28)	0.866
TS <sup>h</sup>	Ref (1.0)	1.26 (0.70-2.27)	0.87 (0.42-1.81)	1.20 (0.54-2.66)	2.62 (0.72-9.56)	0.366
<b>CD3<sup>c,d</sup></b>	135	64	36	27	16	
Front <sup>e</sup>	Ref (1.0)	1.29 (0.74-2.26)	0.73 (0.37-1.42)	1.24 (0.59-2.57)	0.81 (0.32-2.08)	0.808
Center <sup>f</sup>	Ref (1.0)	1.25 (0.73-2.16)	1.15 (0.60-2.21)	1.81 (0.86-3.82)	1.35 (0.50-3.62)	0.169
Intra-ep. <sup>g</sup>	Ref (1.0)	0.92 (0.51-1.64)	0.65 (0.32-1.30)	1.44 (0.67-3.10)	0.50 (0.15-1.59)	0.556
TS <sup>h</sup>	Ref (1.0)	0.92 (0.52-1.64)	0.89 (0.44-1.81)	1.47 (0.66-3.28)	1.28 (0.45-3.59)	0.472
<b>Men<sup>b</sup></b>						
<b>CD8<sup>c,i</sup></b>	123	64	23	22	11	
Front <sup>e</sup>	Ref (1.0)	1.40 (0.80-2.45)	1.62 (0.73-3.63)	0.49 (0.20-1.19)	3.95 (1.11-14.1)	0.533
Center <sup>f</sup>	Ref (1.0)	1.22 (0.70-2.14)	1.15 (0.49-2.69)	1.01 (0.42-2.42)	2.26 (0.71-7.23)	0.339
Intra-ep. <sup>g</sup>	Ref (1.0)	0.90 (0.48-1.70)	1.94 (0.82-4.61)	1.27 (0.53-3.07)	1.26 (0.53-4.09)	0.335
TS <sup>h</sup>	Ref (1.0)	1.00 (0.56-1.76)	1.55 (0.67-3.58)	0.78 (0.31-1.99)	1.91 (0.61-5.97)	0.469
<b>CD3<sup>c,i</sup></b>	147	80	32	24	12	
Front <sup>e</sup>	Ref (1.0)	1.14 (0.68-1.91)	1.24 (0.61-2.51)	0.93 (0.43-2.00)	0.88 (0.29-2.70)	0.986
Center <sup>f</sup>	Ref (1.0)	1.04 (0.63-1.71)	0.90 (0.44-1.87)	1.12 (0.52-2.41)	2.49 (0.91-6.82)	0.257
Intra-ep. <sup>g</sup>	Ref (1.0)	1.13 (0.65-1.95)	1.46 (0.69-3.10)	1.33 (0.57-3.10)	1.20 (0.37-3.89)	0.363
TS <sup>h</sup>	Ref (1.0)	1.01 (0.61-1.69)	0.99 (0.48-2.06)	1.09 (0.50-2.38)	1.18 (0.41-3.39)	0.765
<b>Time from baseline to diagnosis &lt; mean (9.4 years)<sup>b</sup></b>						
<b>CD8<sup>c,i</sup></b>	100	57	26	32	17	
Front <sup>e</sup>	Ref (1.0)	1.66 (0.91-3.03)	1.56 (0.68-3.58)	0.81 (0.39-1.68)	2.39 (0.89-6.45)	0.456
Center <sup>f</sup>	Ref (1.0)	1.79 (0.97-3.29)	2.51 (1.10-5.72)	1.43 (0.68-3.02)	3.27 (1.26-8.53)	0.018
Intra-ep. <sup>g</sup>	Ref (1.0)	1.07 (0.56-2.05)	2.08 (0.91-4.77)	1.12 (0.50-2.47)	0.87 (0.30-2.52)	0.705
TS <sup>h</sup>	Ref (1.0)	1.16 (0.63-2.17)	2.12 (0.95-4.72)	1.08 (0.50-2.34)	2.12 (0.80-5.63)	0.149
<b>CD3<sup>c,i</sup></b>	139	77	40	34	23	
Front <sup>e</sup>	Ref (1.0)	1.05 (0.62-1.77)	0.83 (0.43-1.61)	1.01 (0.53-1.95)	0.67 (0.30-1.52)	0.464
Center <sup>f</sup>	Ref (1.0)	1.08 (0.65-1.79)	0.90 (0.47-1.73)	1.71 (0.87-3.35)	1.50 (0.68-3.30)	0.153
Intra-ep. <sup>g</sup>	Ref (1.0)	0.96 (0.56-1.63)	1.04 (0.53-2.03)	1.03 (0.50-2.12)	0.53 (0.50-1.37)	0.447
TS <sup>h</sup>	Ref (1.0)	0.85 (0.50-1.43)	0.71 (0.36-1.40)	1.11 (0.56-2.20)	0.90 (0.41-2.00)	0.847
<b>Time from baseline to diagnosis ≥ mean (9.4 years)<sup>b</sup></b>						
<b>CD8<sup>c,i</sup></b>	137	68	29	17	5	
Front <sup>e</sup>	Ref (1.0)	1.17 (0.68-2.00)	0.68 (0.32-1.45)	0.68 (0.26-1.78)	4.14 (0.75-22.7)	0.923
Center <sup>f</sup>	Ref (1.0)	1.14 (0.66-1.94)	0.47 (0.21-1.05)	0.78 (0.30-2.02)	3.92 (0.76-20.2)	0.720
Intra-ep. <sup>g</sup>	Ref (1.0)	1.19 (0.65-2.15)	0.67 (0.29-1.55)	1.55 (0.59-4.07)	1.48 (0.28-7.81)	0.682
TS <sup>h</sup>	Ref (1.0)	1.10 (0.64-1.90)	0.57 (0.26-1.25)	0.95 (0.35-2.58)	4.34 (0.44-42.6)	0.941
<b>CD3<sup>c,i</sup></b>	143	67	28	17	5	
Front <sup>e</sup>	Ref (1.0)	1.36 (0.80-2.32)	0.99 (0.48-2.05)	1.03 (0.42-2.58)	1.08 (0.20-5.78)	0.804
Center <sup>f</sup>	Ref (1.0)	1.18 (0.69-2.02)	1.14 (0.55-2.39)	0.98 (0.41-2.32)	3.15 (0.61-16.2)	0.158
Intra-ep. <sup>g</sup>	Ref (1.0)	1.06 (0.59-1.92)	0.75 (0.33-1.68)	2.17 (0.86-5.47)	1.08 (0.17-6.76)	0.452
TS <sup>h</sup>	Ref (1.0)	1.10 (0.63-1.92)	1.21 (0.57-2.59)	1.56 (0.60-4.02)	2.53 (0.26-24.8)	0.239
<b>BMI ≥ 25 kg/m<sup>2</sup> <sup>b</sup></b>						
<b>CD8<sup>c,i</sup></b>	166	74	32	29	14	
Front <sup>e</sup>	Ref (1.0)	1.33 (0.80-2.22)	1.43 (0.71-2.87)	1.10 (0.54-2.26)	3.07 (1.10-8.52)	0.086
Center <sup>f</sup>	Ref (1.0)	1.60 (0.96-2.66)	1.65 (0.79-3.43)	1.44 (0.69-3.01)	2.67 (0.97-7.35)	0.028
Intra-ep. <sup>g</sup>	Ref (1.0)	1.12 (0.64-1.95)	1.20 (0.57-2.55)	1.63 (0.76-3.51)	1.02 (0.36-2.90)	0.374
TS <sup>h</sup>	Ref (1.0)	1.12 (0.66-1.88)	1.77 (0.88-3.56)	1.63 (0.76-3.47)	1.94 (0.69-5.46)	0.046
<b>CD3<sup>c,i</sup></b>	196	85	40	29	19	
Front <sup>e</sup>	Ref (1.0)	1.26 (0.79-2.02)	1.35 (0.73-2.51)	1.28 (0.65-2.53)	0.82 (0.34-1.95)	0.642
Center <sup>f</sup>	Ref (1.0)	1.07 (0.68-1.70)	1.20 (0.64-2.26)	1.59 (0.79-3.19)	1.49 (0.63-3.52)	0.141
Intra-ep. <sup>g</sup>	Ref (1.0)	1.05 (0.64-1.71)	1.09 (0.56-2.10)	1.30 (0.61-2.76)	0.89 (0.35-2.26)	0.770
TS <sup>h</sup>	Ref (1.0)	1.00 (0.62-1.60)	1.11 (0.59-2.11)	1.41 (0.68-2.91)	1.21 (0.50-2.91)	0.385
<b>BMI &lt; 25 kg/m<sup>2</sup> <sup>b</sup></b>						
<b>CD8<sup>c,i</sup></b>	71	51	23	20	8	
Front <sup>e</sup>	Ref (1.0)	1.31 (0.69-2.50)	0.58 (0.23-1.48)	0.40 (0.16-1.03)	2.61 (0.61-11.1)	0.353
Center <sup>f</sup>	Ref (1.0)	1.03 (0.53-2.01)	0.49 (0.20-1.24)	0.59 (0.23-1.51)	3.14 (0.83-11.9)	0.816
Intra-ep. <sup>g</sup>	Ref (1.0)	1.11 (0.54-2.30)	1.10 (0.44-2.76)	0.91 (0.34-2.44)	1.40 (0.29-6.79)	0.876
TS <sup>h</sup>	Ref (1.0)	1.09 (0.55-2.15)	0.49 (0.19-1.25)	0.45 (0.16-1.22)	2.85 (0.64-12.8)	0.457

(Continued on the following page)



**Table 4.** Odds ratios (95% confidence intervals) for self-reported, prediagnostic recreational physical activity in relation to tumor immune cell infiltration in colorectal cancer, according to subgroups based on sex, time from baseline to diagnosis, and body size. (Cont'd)

	Recreational physical activity					P trend <sup>a</sup>
	Never	Now and then	1-2 times/wk	2-3 times/wk	>3 times/wk	
<b>CD3<sup>+</sup></b> <sup>c,i</sup>	86	59	28	22	9	
Front <sup>e</sup>	Ref (1.0)	1.06 (0.57-1.97)	0.53 (0.24-1.17)	0.80 (0.35-1.85)	0.90 (0.24-3.33)	0.346
Center <sup>f</sup>	Ref (1.0)	1.26 (0.68-2.35)	0.80 (0.37-1.72)	1.20 (0.53-2.76)	2.46 (0.71-8.52)	0.415
Intra-ep. <sup>g</sup>	Ref (1.0)	1.07 (0.55-2.11)	0.76 (0.33-1.73)	1.59 (0.67-3.75)	0.52 (0.09-2.95)	0.920
TS <sup>h</sup>	Ref (1.0)	0.97 (0.51-1.85)	0.71 (0.31-1.61)	1.10 (0.46-2.58)	1.11 (0.30-4.09)	0.970

Abbreviations: BMI, body mass index; CD, cluster of differentiation; Intra-ep, intraepithelial; TS, total score; wk – week.

<sup>a</sup>P trends were calculated by including physical activity categories (numbered 1-5, lowest to highest) as a continuous variable in the regression models.

<sup>b</sup>Numbers of observations for total score.

<sup>c</sup>Odds ratios were calculated per one-level increase in the category of immune cell infiltration using ordinal logistic regression.

<sup>d</sup>Covariates included age at diagnosis and tumor stage.

<sup>e</sup>Invasive front of the tumor.

<sup>f</sup>Center/core of the tumor.

<sup>g</sup>Within tumor cell nests.

<sup>h</sup>Calculated as the sum of the scores from each location, ranging from 3 to 12. The total score was classified as low (3-4), intermediate (5-6), or high (7-12).

<sup>i</sup>Covariates included sex, age at diagnosis, and tumor stage.

tissue available. Among these patients, older age, rectal cancer, and higher tumor stage were more common (Supplementary Table S1), consistent with lack of a surgical specimen, which might limit the generalizability of the results. The higher frequency of missing data for CD8<sup>+</sup> compared with CD3<sup>+</sup> was due to the order of analysis and insufficient tumor tissue volumes remaining after CD3<sup>+</sup> IHC was completed. Although this is a potential source of information bias, it seems unlikely to have affected the main results. We have only analyzed immune infiltration of CD3<sup>+</sup> and CD8<sup>+</sup> in the tumor microenvironment.

We included all cases with prediagnostic data available, including those with baseline during the potentially symptomatic final months prior to diagnosis. Any association between overtly symptomatic cancer and tumor immune infiltration should be adequately accounted for by the multivariable adjustment for tumor site and stage. Also, we do not believe that reduced physical activity due to cancer symptoms contributed to our results, as the expected effect would be a dilution of the risk estimates toward the null. Furthermore, only 13 cases (2.2%) were diagnosed within six months prior to diagnosis, and the analyses stratified by median time from baseline to diagnosis (9.4 years) yielded similar results.

A major strength of our study is the prospectively collected baseline data, which reduces the risk of reverse causality and recall bias. We were also able to account for several potential confounders, including lifestyle-related factors. Although distinguishing between potential confounders and mediators of an association between physical activity and tumor immune cell infiltration is difficult, the consistent results in the crude, minimally adjusted and fully adjusted models suggest that covariate selection did not affect our findings. Furthermore, we used a population-based cohort that gives a representative patient material. Although the tumor sample missingness was not completely at random, a higher total score for the infiltration of both CD3<sup>+</sup> and CD8<sup>+</sup> immune cells were associated with right-sided colon cancer, female gender, and lower tumor stage (Table 2). This is in line with previous studies that have reported that tumor-infiltrating CD3<sup>+</sup> and CD8<sup>+</sup> immune cells are associated with proximal tumor localization (8, 28, 52-55), female gender (12, 56, 57), and lower tumor stage (8, 9, 28, 55, 58-60), supporting the generalizability of our findings. The use of full tumor

tissue sections in our study, though more resource-demanding than tumor tissue microarrays, may also imply a lower risk of sampling error and can, therefore, also be considered a strength of the study. Finally, this is, to our knowledge, the first study to investigate prediagnostic recreational physical activity and tumor immune cell infiltration in colorectal cancer.

## Conclusion

In this study based on a population-based cohort, self-reported, prediagnostic recreational physical activity >3 times per week was associated with a higher density of CD8<sup>+</sup> immune cells in the front and center of the tumor in colorectal cancer, independent of factors such as age, tumor site, and lifestyle factors. The present study provides some evidence for a potential association between high degree of recreational physical activity and infiltration of CD8<sup>+</sup> immune cells in the colorectal cancer tumor microenvironment. However, the results should be interpreted with caution, because there were few observations in the highest degree of physical activity and the use of self-reported physical activity. Thus, a future prospective study with a larger data material is warranted to investigate this association thoroughly.

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## Authors' Contributions

**D. Renman:** Conceptualization, data curation, formal analysis, validation, investigation, visualization, methodology, writing—original draft, project administration, writing—review and editing. **B. Gylling:** Formal analysis, supervision, investigation, methodology, writing—original draft, writing—review and editing. **L. Vidman:** Validation. **S. Bodén:** Data curation, writing—review and editing. **K. Strigard:** Conceptualization, supervision, writing—review and editing. **R. Palmqvist:** Formal analysis, supervision, methodology, writing—review and editing. **S. Harlid:** writing—review and editing. **U. Gunnarsson:** Conceptualization, formal analysis, supervision, validation, project administration, writing—review and editing. **B. van Guelpen:** Conceptualization, formal analysis, supervision, validation, investigation, methodology, writing—original draft, project administration, writing—review and editing.

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