Energy Balance-Related Factors and Risk of Colorectal Cancer Expressing Different Levels of Proteins Involved in the Warburg Effect

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ABSTRACT

Background: Energy balance–related factors [body mass index (BMI), waist circumference, physical activity] have been associated with colorectal cancer risk. Warburg effect activation via PI3K/Akt signaling is one of the proposed mechanisms. We investigated whether energy balance–related factors were associated with risk of Warburg subtypes in colorectal cancer.

Methods: We investigated this using immunohistochemistry for six proteins involved in the Warburg effect (LDHA, GLUT1, MCT4, PKM2, P53, PTEN) on tissue microarrays of 2,399 incident colorectal cancer cases from the prospective Netherlands Cohort Study (n_{total} = 120,852; $n_{subcohort}$ = 5,000; aged 55–69 in 1986; 20.3 years follow-up). Data analyses included 3,911 subcohort members and 1,972 colorectal cancer cases with complete covariate data. Expression levels of all proteins were combined into a pathway-based sum score and categorized into three "Warburg subtypes" (Warburg-low/moderate/high). Multivariable Cox regression analyses were used to estimate associations of BMI, clothing size (waist circumference proxy), and physical activity with Warburg subtypes in colorectal cancer.

Results: BMI and clothing size were positively associated with Warburg-moderate and Warburg-high colon cancer risk in men ($P_{heterogeneity}$ = 0.192). In women, clothing size was positively associated with Warburg-low and Warburg-high colon cancer ($P_{heterogeneity}$ = 0.005). Nonoccupational physical activity was inversely associated with Warburg-low and Warburg-moderate colon cancer in women ($P_{heterogeneity}$ = 0.045), but positively associated with Warburg-high rectal cancer in men ($P_{heterogeneity}$ = 0.089).

Conclusions: The Warburg effect might be involved in associations between adiposity and colon cancer risk, though additional mechanisms could be at play in women as well. The inverse association between physical activity and colon cancer might be explained by mechanisms other than the Warburg effect.

Impact: Further research is needed to reproduce these results and investigate possible additional mechanisms.

Introduction

Energy balance–related factors are known to influence risk of colorectal cancer. Measures of adiposity, such as body mass index (BMI) and waist circumference, have been associated with increased risk of colorectal cancer (1, 2), whereas physical activity is inversely associated with colorectal cancer risk (2–4). Up till now, the underlying biological mechanisms are not fully understood. Several reviews published on this subject (5–7) implied three main factors: adipocyte-derived cytokines (adipokines), insulin and insulin-like growth factor 1 (IGF-1) signaling, and sex hormones.

Circulating levels of adipokines are influenced by the quantity of adipose tissue, with a larger number of adipocytes leading to higher circulating leptin and lower adiponectin levels (8). Conversely, physical activity has been linked to lower circulating leptin and higher adiponectin levels, even independent of weight loss (9, 10). Similarly, increased serum levels of insulin and free IGF-1 have been reported for overweight and obese individuals (11), whereas reduced levels were observed in more physically active individuals (9). High levels of leptin, insulin, and IGF-1 have all been associated with increased colorectal cancer risk (12–15), whereas high adiponectin levels have been associated with a decreased risk (14, 15).

Adipokine, insulin, and IGF-1 signaling share a common downstream effect, namely activation of the PI3K/Akt signaling pathway (16). Apart from its well-known properties like cell survival and growth, the PI3K/Akt signaling pathway has been associated with the so-called metabolic switch (17, 18). Upon activation, the expression of glucose transporters and enzymes involved in glycolysis increases (19, 20). Upregulation of aerobic glycolysis in cancer cells was first observed in the 1920s by Warburg and colleagues, hence the term "Warburg effect" (21). While it was initially thought that the Warburg effect was an effect rather than a cause of cancer, it is increasingly being considered a carcinogenic step (22). This is further supported by the addition of "Reprogramming Energy Metabolism" as an Emerging Hallmark of Cancer in 2011 (23).

Previous studies investigated the suggested link between energy balance–related factors and colorectal cancer mainly using circulating

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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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Cancer Epidemiol Biomarkers Prev 2022;31:633–46

doi: 10.1158/1055-9965.EPI-21-0678

biomarkers (e.g., leptin or insulin; refs. 12–15). However, differentiating between cause and effect is difficult with circulating biomarkers, especially when they are measured at the time of cancer diagnosis, as the biomarker status may be influenced by the tumor. In the current study, we aimed to investigate whether this suggested link could be captured in the primary tumor itself by upregulation of the Warburg effect.

We aimed to capture the Warburg effect by ensuring that the different steps of the pathway were represented by at least one protein (Supplementary Table S1). These steps include: upstream regulation of the Warburg effect (PTEN, P53), glucose import (GLUT1), glycolysis (PKM2), conversion of pyruvate into lactate (LDHA), and lactate secretion (MCT4). The expression levels of these six proteins (PTEN, P53, GLUT1, PKM2, LDHA, MCT4) were combined into a sum score, which was divided into three subgroups, representing tumors with a low, moderate, or high likelihood of presence of the Warburg effect, hereafter referred to as the Warburg subtypes (Warburg-low, Warburg-moderate, Warburg-high, respectively).

We hypothesized that associations between energy balance–related factors (BMI; lower body clothing size, as a proxy for waist circumference; physical activity) and risk of colorectal cancer differ across Warburg subtypes.

Materials and Methods

Study design and study population

The Netherlands Cohort Study (NLCS) was initiated in 1986 and included 120,852 subjects ages 55–69 years at baseline. All participants completed a mailed, self-administered questionnaire on diet, smoking habits, anthropometry, history of selected diseases, physical activity, and other cancer risk factors (24). The NLCS was approved by Institutional Review Boards from Maastricht University (Maastricht, the Netherlands) and the Netherlands Organization for Applied Scientific Research. Ethical approval was obtained from the Medical Ethical Committee of Maastricht University Medical Center+ (Maastricht, the Netherlands). The NLCS was conducted in accordance with the Declaration of Helsinki. All cohort members consented to participate in the NLCS by completing the questionnaire. For data processing and analysis, the case–cohort method was used (25). Accumulated person-years in the cohort were estimated from a subcohort (n = 5,000), randomly sampled from the whole cohort immediately after baseline. These subcohort members were actively followed up biennially for vital status information and by linkage to municipal population registries. Only one male subcohort member was lost to follow-up.

Follow-up for cancer incidence was established by annual record linkage with the Netherlands Cancer Registry and PALGA, the nationwide Dutch Pathology Registry (26), covering 20.3 years of follow-up (September 17, 1986 until January 1, 2007). Completeness of cancer incidence follow-up by the Netherlands Cancer Registry and PALGA was estimated to be over 96% (27). After excluding cases and subcohort members who reported a history of cancer (except skin cancer) at baseline, a total of 4,597 incident colorectal cancer cases and 4,774 subcohort members were available (**Fig. 1**).

Formalin-fixed paraffin-embedded (FFPE) tissue blocks from primary tumor and matched normal colon tissue from 3,872 colorectal cancer cases were requested from participating laboratories as part of the Rainbow-TMA (tissue microarray) project during 2012–2017 (28). Colorectal cancer cases were selected on the basis of available linkage to a PALGA-record (which provides access to pathology labs) and surgical specimen with pathology report, or coloscopic resection. Cases treated with neoadjuvant therapy were excluded. Tissue blocks from 3,021 colorectal cancer cases were successfully collected from 43 pathology laboratories throughout the Netherlands (78% retrieval rate).

For TMA construction, pathologists reviewed scanned hematoxylin & eosin (H&E)-stained sections and identified areas with the highest tumor density, from which three 0.6-mm-diameter cores were sampled per case along with three normal tissue cores (TMA-Grandmaster, 3D-Histech). In total, tumor tissue of 2,694 colorectal cancer cases was successfully assembled in 78 TMA blocks (**Fig. 1**).

Immunohistochemistry

Five-μm-thick sections were cut from all 78 TMA blocks, H&E stained according to standard protocol, and subjected to immunohistochemistry (IHC). IHC was performed using an automated immunostainer (DAKO Autostainer Link 48) for GLUT1, P53, and PTEN, and manually for LDHA, MCT4, and PKM2. Details of the primary antibodies and staining protocols are shown in Supplementary Table S2. All TMA sections were scanned using an Aperio scanner (Leica Microsystems) at 40× magnification at the University of Leeds (Leeds, United Kingdom) Scanning Facility or at the Department of Pathology, Aachen University Hospital (Aachen, Germany).

Three non-pathologists (G.E. Fazzi: histology technician; K. Offermans: PhD student; J.C.A. Jenniskens: PhD student) were trained by a senior histopathologist (H.I. Grabsch) in recognizing adenocarcinoma and IHC scoring (29). Presence of adenocarcinoma was confirmed for every individual core by reviewing H&E-stained TMA sections, in combination with pan-cytokeratin stained sections if necessary. Requiring at least one core per case, 2,497 cases passed quality control (**Fig. 1**).

After quality control, all cores were scored by at least two assessors (Supplementary Table S3 shows contribution of each assessor), independently and blinded for case characteristics. IHC scoring protocols for all markers are described in the Supplementary Materials and Methods and shown in Supplementary Fig. S1. Kappa values on interobserver and intraobserver scoring agreement are shown in Supplementary Table S4.

Figure 2 illustrates the stepwise process of combining multiple core-level scores into case-level Warburg subtypes. If at least two assessors assigned the same score to a core, this score became the "combination score." Remaining discrepancies were resolved by consensus agreement of two non-pathologist assessors or by an experienced pathologist, resulting in a final score for each core. Case-level protein expression was determined by taking the average of the final scores of available cores (range: 1–3 cores per case) and rounding it to the nearest scoring category. The average score per case was subdivided into three subgroups, representing low, moderate, or high expression. Cutoffs for PTEN and P53 were based on previous literature (30, 31), cutoffs for other proteins were determined on the basis of distribution of cases (Supplementary Table S4 shows cutoffs per protein).

Creating Warburg subtypes

To create Warburg subtypes, we used a pathway-based sum score of case-level protein expression levels of LDHA, GLUT1, MCT4, PKM2, P53, and PTEN (**Fig. 2**). Cases with incomplete protein expression data were excluded (**Fig. 1**). Expression of LDHA, GLUT1, MCT4, PKM2, and P53 are positively associated with the Warburg effect (18, 32), whereas PTEN expression is inversely associated with the Warburg effect (32). Therefore, for all proteins, except PTEN, high protein expression was given a score of 2, moderate expression a score of 1, and low expression a score of 0. For PTEN, this score was reversed; high PTEN expression was given a score of 0, moderate expression a score of

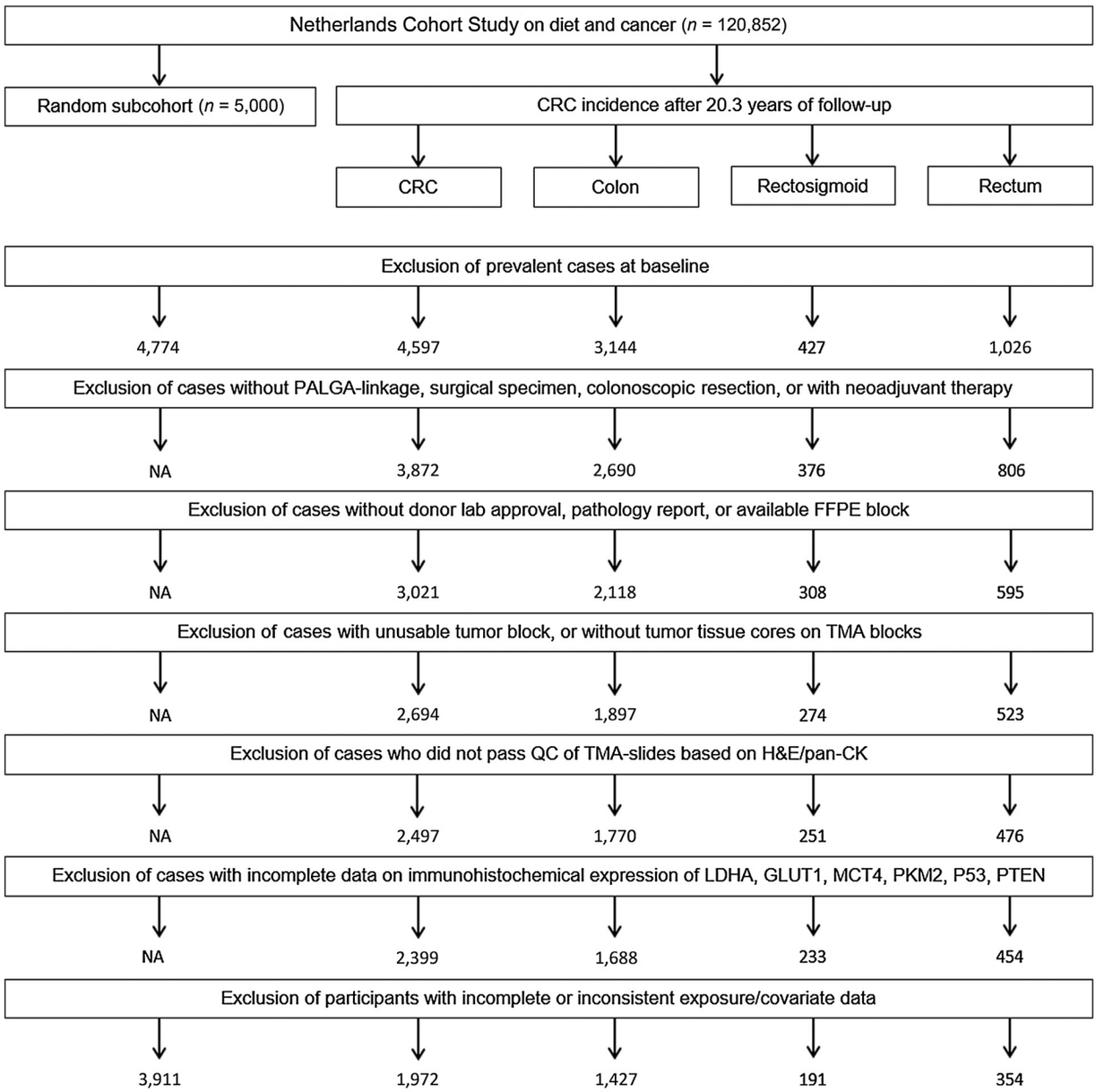

Figure 1.

Flow diagram of the number of colorectal cancer cases and subcohort members; NLCS, 1986–2006. CRC, colorectal cancer; NA, not applicable; PALGA, Dutch Pathology Registry; FFPE, formalin-fixed paraffin-embedded; TMA, tissue microarray; QC, quality control; H&E, hematoxylin & eosin; pan-CK, pan-cytokerin.

1, and low expression a score of 2. The sum score is the sum of scores of all proteins (range: 0–12), whereby a higher score indicates a higher likeliness of presence of the Warburg effect. For statistical efficiency, cases were then divided into tertiles based on the sum score to establish Warburg subtypes. Distribution of the sum score did not differ according to sex or tumor location, leading to the following cutoffs for all cases: cases with sum scores 0–3 were classed as "Warburg-low" ($n = 698$, 29.1%), sum scores 4–5 as "Warburg-moderate" ($n = 859$, 35.8%), and sum scores 6–12 as "Warburg-high" ($n = 842$, 35.1%; **Fig. 2**). Clinical characteristics of the cases stratified on Warburg subtypes are shown in Supplementary Table S5.

Energy balance–related factors

All NLCS participants returned a mailed, self-administered questionnaire on anthropometry, physical activity, diet, and other risk factors at baseline in 1986 (24). BMI at baseline (kg/m^2) was calculated using baseline weight (kg) divided by height squared (m^2). Participants were asked to report their lower body clothing size (trouser/skirt) from their clothing label (Dutch sizes). This has previously been shown to be an adequate proxy for waist circumference when predicting cancer risk in the NLCS (33). To estimate levels of nonoccupational physical activity, participants were asked to report the average daily time spent on activities like walking,

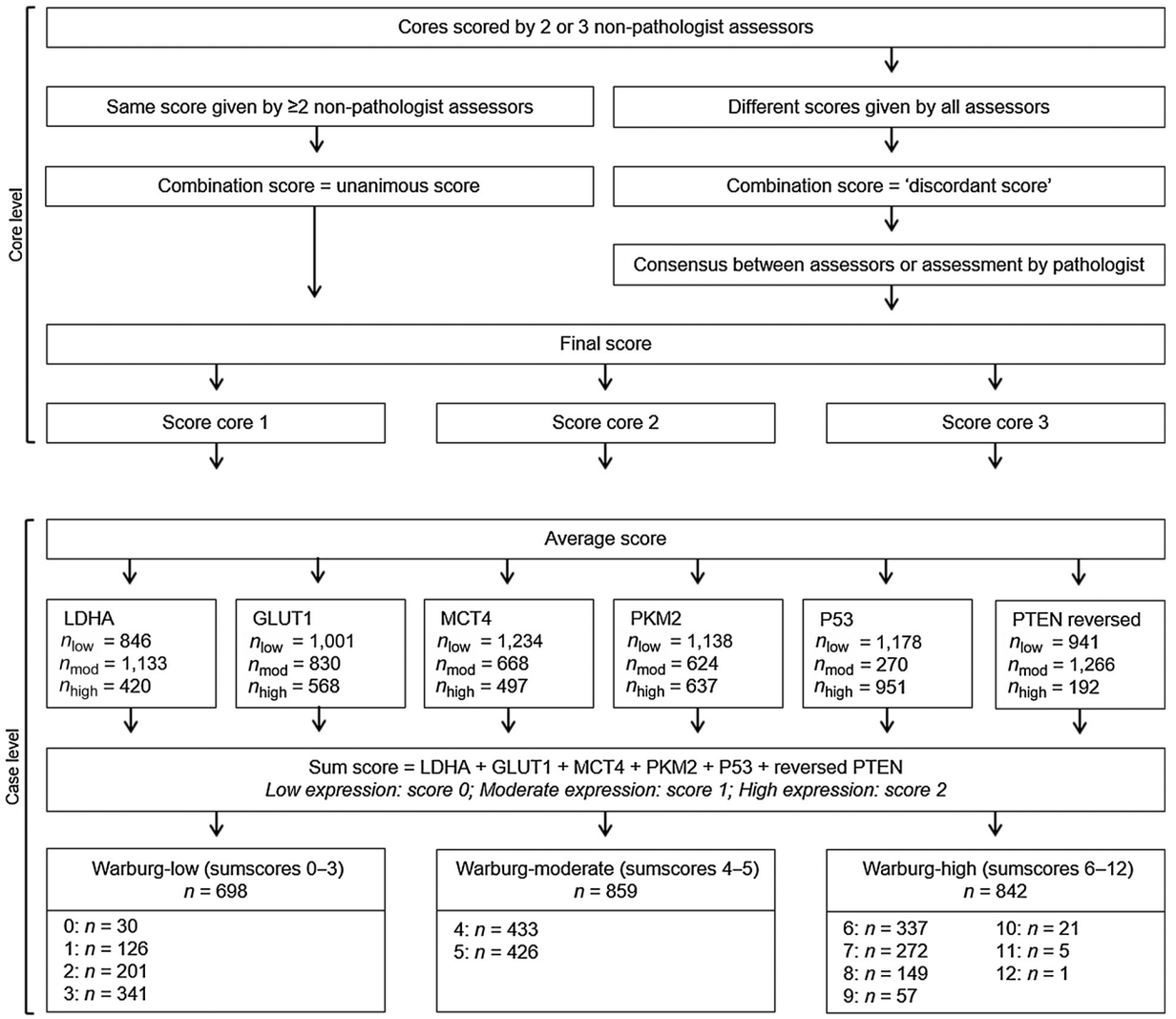

Figure 2.

Flow diagram of getting from multiple core-level scores to case-level Warburg subtypes. n_{low} = number of people with low expression; n_{mod} = number of people with moderate protein expression; n_{high} = number of people with high protein expression.

cycling, or doing sports, as described in more detail previously (34). For occupational physical activity, energy expenditure and sitting time were estimated for the longest held job, which was self-reported at baseline. Jobs were classified as low, moderate, or high activity, as described previously (34). Energy expenditure was classified as <8, 8–12, and >12 kJ/minute, and sitting time as sitting for >6, 2–6, and <2 working hours/day. Data on occupational physical activity were only available for the subcohort and for cases until 17.3 years of follow-up, because funding for later data entry and classification of occupations was unavailable. Furthermore, we did not analyze occupational physical activity measures in women because many did not have paid jobs (34).

Cox regression models

After excluding participants with incomplete or inconsistent data on exposure variables or confounders, 3,911 subcohort members and 1,972 colorectal cancer cases were available for analyses (**Fig. 1**). Associations between energy balance–related factors and colorectal cancer risk were investigated stratified on sex, tumor location, and Warburg subtypes. Cox proportional hazards models were used to estimate hazard ratios (HR) and 95% confidence intervals (CI) for the association between colorectal cancer and BMI (according to sex-specific quartiles, and per 5 kg/m^2 increase), clothing size (according to sex-specific quartiles, and per two sizes increase), nonoccupational physical activity (in categories of <30, 30–60, 60–90, >90 minutes per day, and per 30 minutes/day increase), and, for men, occupational physical activity (energy expenditure in categories of <8, 8–12, >12 kJ/minute; sitting time in categories of >6, 2–6, and <2 working hours/day). Standard errors of the HRs were estimated using the Huber–White sandwich estimator to account for additional variance introduced by sampling the subcohort from the total cohort (35). The proportional hazards assumption was tested using the scaled Schoenfeld residuals (36) and by introducing time–covariate interactions into the models.

All multivariable models were adjusted for age, total energy intake (kcal/day), family history of colorectal cancer (yes/no), and alcohol intake (0; 0.1–4; 5–14; >15 g/day). BMI and clothing size models were additionally adjusted for nonoccupational physical activity (minutes/day), and BMI models for height (cm). All physical activity models were additionally adjusted for BMI. Moreover, clothing size and BMI models were mutually adjusted as an indication of fat distribution, where clothing size adjusted for BMI represents a proxy for abdominal fatness, and BMI adjusted for clothing size as a proxy for subcutaneous fatness (33, 37). Potential additional confounders were smoking status (never/former/current), level of education (primary or lower vocational education; secondary or medium vocational education; higher vocational education or university), red meat consumption (g/day), and processed meat consumption (g/day). These potential confounders were included in multivariable models if they introduced a ≥10% change in HRs.

Heterogeneity in associations between risk factors and Warburg subtypes was tested to evaluate differences across tumors expressing different levels of proteins involved in the Warburg effect. This was done using an adapted version of the competing risks procedure in Stata developed for the case-cohort design, as described previously (38, 39).

Sensitivity analyses were performed by excluding the first 2 years of follow-up. Furthermore, analyses were performed for two instead of three Warburg subtypes (Warburg-low: sum score 0–4; Warburg-high: sum score 5–12) to increase power.

All analyses were conducted in Stata Statistical Software: Release 16 (StataCorp.).

Results

Baseline lifestyle characteristics of subcohort members and colon and rectal cancer cases, overall and according to Warburg subtypes, are shown in **Table 1**. Overweight and obesity were more often observed in colorectal cancer cases compared with subcohort members, especially for Warburg-moderate and Warburg-high colorectal cancer cases in men, and for Warburg-low colorectal cancer cases in women. Clothing size, as a proxy for waist circumference, showed similar trends. Male colon cancer cases showed equal levels of high nonoccupational physical activity as subcohort members, but slightly lower levels of occupational energy expenditure and higher levels of occupational sitting time. In contrast, male rectal cancer cases more often showed high levels of nonoccupational physical activity and low occupational sitting time, especially for the Warburg-high subtype. Female cases less often showed high levels of nonoccupational physical activity compared with female subcohort members, especially in the Warburg-low and Warburgh-moderate groups for colon and in the Warburg-high group for rectal cancer. Warburg-high cases generally had the lowest level of education compared with Warburg-low and Warburg-moderate cases, except for men with colon cancer. Furthermore, a family history of colorectal cancer occurred less frequently in the Warburg-high subgroup compared with Warburg-low or Warburg-moderate subgroups for rectal cancer.

Tables 2–5 show multivariable-adjusted Cox regression models for energy balance–related factors in Warburg subtypes, stratified on sex and tumor location. Age-adjusted Cox regression models are shown in Supplementary Tables S6–S9; results were similar to those of multivariable-adjusted models. Age was included as a time-varying covariate in all models, because of violation of the proportional hazards assumption.

Adiposity

Both BMI and clothing size showed a positive association with total colon cancer risk in men (**Tables 2** and **3**), with HR (95% CI) of 1.24 (1.07–1.44) per 5 kg/m^2 increment and of 1.31 (1.14–1.49) per two sizes increment. HRs for the same increments were enhanced for Warburg-moderate and Warburg-high subtypes [Warburg-moderate: HR_{BMI} (95% CI): 1.26 (1.01–1.57); $HR_{clothing}$: 1.45 (1.18–1.78); Warburg-high: HR_{BMI}: 1.39 (1.11–1.75); $HR_{clothing}$: 1.28 (1.06–1.56)], whereas the Warburg-low subtype showed weaker associations. After additional adjustment for clothing size, as a proxy for subcutaneous fatness, a similar association was found for Warburg-high [HR_{5kg/m_2} (95% CI): 1.42 (1.07–1.88)], whereas associations for total and Warburg-moderate colon cancer diminished (Supplementary Table S10). In contrast, adjustment for BMI in clothing size models, as a proxy for abdominal fatness, led to similar associations for Warburg-moderate [$HR_{two\ sizes}$ (95% CI): 1.41 (1.11–1.78)], but weaker associations for Warburg-high colon cancer [$HR_{two\ sizes}$ (95% CI): 1.15 (0.93–1.42)] (Supplementary Table S11). Neither BMI nor clothing size models showed statistically significant heterogeneity between Warburg subtypes.

For rectal cancer, no associations with BMI or clothing size were observed in men (**Table 2** and **3**). After mutual adjustment, neither BMI nor clothing size showed statistically significant associations (Supplementary Table S10 and S11).

In women, BMI was not associated with colon cancer risk (**Table 2**), whereas clothing size showed a weak positive association with colon cancer risk [$HR_{two\ sizes}$ (95% CI): 1.09 (0.95–1.24)] (**Table 3**). This association was stronger for Warburg-low and Warburg-high subtypes [per two sizes: $HR_{Warburg\text{-}low}$ (95% CI): 1.27 (0.96–1.69); $HR_{Warburg\text{-}high}$: 1.20 (1.01–1.42)], whereas the association for Warburg-moderate seemed to be inverse [$HR_{two\ sizes}$ (95% CI): 0.84 (0.69–1.02)]. Statistically significant heterogeneity between Warburg subtypes was observed for clothing size models per two sizes (continuous $P_{heterogeneity} = 0.007$), as well as for models on quartiles of clothing size (categorical $P_{heterogeneity} = 0.018$). Mutual adjustment, as an indication of fat distribution, resulted in inverse associations for BMI (Supplementary Table S10), and stronger associations for clothing size (Supplementary Table S11). However, BMI and clothing size showed high correlation in women (Spearman rank correlation: 0.76).

For rectal cancer, no associations were found for either BMI or clothing size in women (**Table 2** and **3**). Neither BMI nor clothing size showed statistically significant associations after mutual adjustment (Supplementary Tables S10 and S11).

Physical activity

Nonoccupational physical activity was not associated with total colon cancer risk in men (**Table 4**). Stratification on Warburg subtypes did not lead to different associations. Energy expenditure at work was associated with a nonsignificant decreased risk of colon cancer (**Table 5**), and similar associations were shown for all Warburg subtypes. Lower occupational sitting time was associated with a statistically significant decreased risk of colon cancer (**Table 5**), with HR (95% CI) for sitting <2 hours/day versus >6 hours/day of 0.69 (0.53–0.91), and statistically significant trend over categories ($P = 0.007$). After stratification on Warburg subtypes, associations were in the same direction but reached

Table 1. Baseline characteristics [mean (SD) or %] of subcohort members and colorectal cancer cases in Warburg subtypes, by sex and tumor location; NLCS, 1986–2006.

		Colon				Rectum			
	Subcohort	**Total**	**Warburg-Low**	**Warburg-Moderate**	**Warburg-High**	**Total**	**Warburg-Low**	**Warburg-Moderate**	**Warburg-High**
Men									
N	1,971	772	215	280	277	227	76	76	75
Overweight/obesity[a] (%)	46.6	51.0	47.4	50.0	54.9	49.8	48.7	51.3	49.3
Clothing size[b]	51.7 (2.7)	52.1 (2.6)	51.8 (2.5)	52.4 (2.7)	52.2 (2.6)	51.8 (2.5)	51.7 (2.7)	51.7 (2.1)	51.9 (2.7)
Nonoccupational physical activity >60 min/day (%)	51.1	51.0	54.9	47.1	52.0	60.8	55.3	55.3	72.0
Occupational energy expenditure (>12 kJ/min)[c]	13.0	12.5	10.8	13.2	13.2	12.0	12.1	11.7	12.1
Occupational sitting time (<2 hours/day)[c]	25.9	24.6	27.4	24.5	22.4	30.4	27.3	26.7	37.9
Age (years)	61.3 (4.2)	61.6 (4.2)	61.8 (4.2)	61.3 (4.1)	61.8 (4.2)	60.7 (3.9)	60.8 (3.6)	61.1 (4.3)	60.2 (3.6)
Total energy intake (kcal/day)	2,164 (500)	2,121 (461)	2,089 (455)	2,164 (494)	2,102 (427)	2,240 (478)	2,263 (448)	2,230 (518)	2,228 (471)
Family history of colorectal cancer (%)	5.4	10.8	10.2	11.1	10.8	9.3	10.5	10.5	6.7
Alcohol consumption (g/day)	15.1 (17.1)	15.1 (15.9)	15.3 (16.1)	15.6 (15.9)	14.5 (15.7)	17.4 (17.5)	16.4 (17.4)	16.9 (16.3)	18.8 (19.0)
Processed meat intake (g/day)	15.9 (16.9)	15.3 (14.7)	15.4 (15.9)	15.7 (14.4)	14.9 (14.0)	17.9 (17.6)	15.6 (11.7)	20.0 (22.6)	18.1 (16.6)
Red meat intake (g/day)	93.8 (41.2)	91.9 (40.0)	88.4 (40.1)	95.7 (40.2)	90.7 (39.7)	94.4 (39.6)	95.9 (38.2)	86.5 (39.1)	100.8 (40.7)
Never cigarette smokers (%)	12.7	12.7	12.6	12.9	12.6	8.8	7.9	11.8	6.7
University or higher vocational education (%)	19.8	23.6	21.4	24.6	24.4	17.3	21.3	15.8	14.9
Women									
N	1,940	655	170	216	269	127	37	51	39
Overweight/obesity[a] (%)	43.6	45.3	48.2	43.5	45.0	50.4	56.8	52.9	41.0
Clothing size[b]	43.4 (2.9)	43.6 (3.3)	44.0 (4.2)	43.1 (2.9)	43.7 (3.0)	43.6 (2.7)	43.6 (2.5)	43.8 (2.8)	43.4 (2.7)
Nonoccupational physical activity >60 min/day (%)	44.9	40.6	38.8	37.5	44.2	43.3	54.1	43.1	33.3
Age (years)	61.4 (4.3)	61.9 (4.1)	62.0 (4.1)	62.0 (4.1)	61.8 (4.1)	61.4 (4.2)	60.5 (4.1)	62.1 (4.2)	61.5 (4.3)
Total energy intake (kcal/day)	1,684 (392)	1,679 (385)	1,661 (353)	1,711 (411)	1,664 (382)	1,684 (346)	1,664 (315)	1,667 (340)	1,724 (386)
Family history of colorectal cancer (%)	6.0	9.9	10.6	8.8	10.4	10.2	10.8	11.8	7.7
Alcohol consumption (g/day)	6.0 (9.5)	5.7 (9.5)	5.7 (10.1)	5.6 (8.8)	5.8 (9.6)	5.6 (8.7)	4.4 (5.7)	6.7 (9.9)	5.3 (9.4)
Processed meat intake (g/day)	10.3 (11.6)	10.0 (11.0)	10.7 (11.4)	10.5 (11.6)	9.0 (10.3)	11.4 (10.5)	11.2 (9.8)	10.1 (7.7)	13.2 (13.8)
Red meat intake (g/day)	81.0 (38.1)	77.4 (34.6)	77.0 (34.2)	77.6 (34.1)	77.4 (35.2)	88.3 (42.5)	100 (49.3)	83.9 (34.7)	83.1 (43.8)
Never cigarette smokers (%)	57.3	57.7	59.4	58.8	55.8	57.5	56.8	49.0	69.2
University or higher vocational education (%)	9.5	10.0	10.7	11.1	8.7	5.6	2.7	10.0	2.6

Abbreviations: NLCS, Netherlands Cohort Study; SD, standard deviation.

[a]BMI ≥ 25.

[b]Based on fewer participants due to extra missings.

[c]Based on fewer participants due to shorter follow-up (17.3 years), only available for men.

Table 2. Multivariable-adjusted HRs[a] and 95% CIs for associations between BMI and Warburg subtypes in colorectal cancer, by sex and tumor location; NLCS, 1986–2006.

	Median[b]	Person-years at risk	Total		Warburg-low		Warburg-moderate		Warburg-high		
			n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	*P*-het
BMI quartiles (kg/m^2)											
Men – colon											
<23.4	22.2	7,993	174	1.00 (ref.)	57	1.00 (ref.)	57	1.00 (ref.)	60	1.00 (ref.)	
23.4–24.9	24.2	8,343	199	1.08 (0.84–1.38)	55	0.91 (0.60–1.36)	81	1.34 (0.92–1.94)	63	1.00 (0.68–1.46)	
25.0–26.6	25.7	7,683	203	1.18 (0.91–1.52)	52	0.91 (0.60–1.38)	63	1.13 (0.76–1.68)	88	1.49 (1.03–2.17)	
>26.6	27.8	7,003	196	1.34 (1.03–1.73)	51	1.05 (0.69–1.59)	79	1.64 (1.12–2.41)	66	1.32 (0.89–1.96)	0.073
P-trend				0.021		0.860		0.035		0.038	
Continuous per 5 kg/m^2		31,022	772	1.24 (1.07–1.44)	215	1.05 (0.82–1.35)	280	1.26 (1.01–1.57)	277	1.39 (1.11–1.75)	0.192
Men – rectum											
<23.4	22.2	7,993	56	1.00 (ref.)	20	1.00 (ref.)	20	1.00 (ref.)	16	1.00 (ref.)	
23.4–24.9	24.2	8,343	53	0.87 (0.58–1.31)	14	0.67 (0.33–1.37)	17	0.77 (0.39–1.51)	22	1.24 (0.63–2.42)	
25.0–26.6	25.7	7,683	69	1.26 (0.85–1.86)	25	1.35 (0.71–2.56)	26	1.34 (0.72–2.47)	18	1.09 (0.54–2.18)	
>26.6	27.8	7,003	49	1.01 (0.66–1.54)	17	1.05 (0.52–2.12)	13	0.74 (0.36–1.51)	19	1.27 (0.62–2.60)	0.499
P-trend				0.507		0.437		0.902		0.634	
Continuous per 5 kg/m^2		31,022	227	1.08 (0.86–1.35)	76	1.13 (0.77–1.67)	76	1.04 (0.72–1.49)	75	1.06 (0.74–1.52)	0.933
Women – colon											
<22.8	21.5	9,014	186	1.00 (ref.)	54	1.00 (ref.)	64	1.00 (ref.)	68	1.00 (ref.)	
22.8–24.7	23.8	8,914	147	0.80 (0.62–1.03)	27	0.50 (0.31–0.82)	51	0.79 (0.53–1.18)	69	1.03 (0.72–1.48)	
24.8–27.0	25.7	8,141	160	0.98 (0.76–1.27)	41	0.86 (0.55–1.34)	53	0.93 (0.63–1.37)	66	1.12 (0.78–1.63)	
>27.0	29.2	8,158	162	1.02 (0.79–1.33)	48	1.05 (0.68–1.62)	48	0.86 (0.57–1.30)	66	1.15 (0.78–1.68)	0.246
P-trend				0.595		0.535		0.628		0.424	
Continuous per 5 kg/m^2		34,228	655	1.05 (0.93–1.19)	170	1.12 (0.91–1.39)	216	0.94 (0.77–1.14)	269	1.09 (0.92–1.30)	0.372
Women – rectum											
<22.8	21.5	9,014	35	1.00 (ref.)	11	1.00 (ref.)	12	1.00 (ref.)	12	1.00 (ref.)	
22.8–24.7	23.8	8,914	26	0.73 (0.43–1.24)	4	0.34 (0.11–1.10)	11	0.93 (0.40–2.17)	11	0.88 (0.37–2.08)	
24.8–27.0	25.7	8,141	31	0.93 (0.55–1.57)	10	0.91 (0.37–2.22)	15	1.44 (0.63–3.32)	6	0.51 (0.19–1.37)	
>27.0	29.2	8,158	35	1.06 (0.64–1.77)	12	1.07 (0.46–2.47)	13	1.30 (0.54–3.13)	10	0.85 (0.35–2.07)	0.985
P-trend				0.652		0.561		0.395		0.513	
Continuous per 5 kg/m^2		34,228	127	1.10 (0.89–1.38)	37	1.12 (0.75–1.66)	51	1.30 (0.94–1.80)	39	0.90 (0.59–1.33)	0.428

Abbreviations: BMI, body mass index; CI, confidence interval; HR, hazard ratio; NLCS, Netherlands Cohort Study; *P*-het, *P*-heterogeneity.

[a]HRs were adjusted for age (years; continuous), nonoccupational physical activity (minutes/day; continuous), height (cm; continuous), total energy intake (kcal/day; continuous), family history of colorectal cancer (yes; no), alcohol consumption (0; 0.1–4; 5–14; >15 g/day), processed meat intake (g/day; continuous), and red meat intake (g/day; continuous). Age was included as a time-varying covariate.

[b]Median BMI per quartile based on the subcohort.

Table 3. Multivariable-adjusted HRs[a] and 95% CIs for associations between lower body clothing size and Warburg subtypes in colorectal cancer, by sex and tumor location; NLCS, 1986–2006.

			Total		Warburg-low		Warburg-moderate		Warburg-high		
	Median[b]	Person-years at risk	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	*P*-het
Clothing size											
Men – colon											
≤50	50	10,903	220	1.00 (ref.)	67	1.00 (ref.)	74	1.00 (ref.)	79	1.00 (ref.)	
52	52	9,750	257	1.30 (1.04–1.63)	80	1.35 (0.94–1.92)	89	1.34 (0.95–1.88)	88	1.23 (0.88–1.72)	
54	54	5,156	135	1.30 (0.99–1.70)	29	0.92 (0.57–1.47)	46	1.32 (0.88–1.98)	60	1.61 (1.10–2.37)	
≥56	56	2,618	90	1.74 (1.27–2.38)	24	1.56 (0.94–2.60)	35	1.98 (1.26–3.11)	31	1.66 (1.04–2.65)	0.344
P-trend				0.001		0.299		0.006		0.006	
Continuous per two sizes		28,428	702	1.31 (1.14–1.49)	200	1.18 (0.96–1.47)	244	1.45 (1.18–1.78)	258	1.28 (1.06–1.56)	0.292
Men – rectum											
≤50	50	10,903	77	1.00 (ref.)	28	1.00 (ref.)	24	1.00 (ref.)	25	1.00 (ref.)	
52	52	9,750	69	1.01 (0.71–1.43)	20	0.81 (0.45–1.46)	29	1.36 (0.77–2.40)	20	0.89 (0.49–1.63)	
54	54	5,156	46	1.31 (0.88–1.95)	17	1.30 (0.69–2.43)	15	1.40 (0.71–2.78)	14	1.22 (0.62–2.42)	
≥56	56	2,619	17	0.97 (0.55–1.70)	6	0.89 (0.36–2.21)	3	0.58 (0.17–1.96)	8	1.41 (0.62–3.24)	0.872
P-trend				0.488		0.773		0.958		0.384	
Continuous per two sizes		28,428	209	1.02 (0.84–1.25)	71	0.91 (0.65–1.29)	71	1.04 (0.78–1.39)	67	1.13 (0.81–1.59)	0.690
Women – colon											
≤40	40	6,574	126	1.00 (ref.)	37	1.00 (ref.)	42	1.00 (ref.)	47	1.00 (ref.)	
42	42	8,582	162	0.97 (0.73–1.28)	32	0.64 (0.39–1.06)	67	1.20 (0.79–1.83)	63	1.02 (0.68–1.54)	
44	44	9,270	167	0.89 (0.68–1.17)	37	0.65 (0.40–1.06)	54	0.85 (0.55–1.31)	76	1.12 (0.75–1.66)	
≥46	46	9,454	188	1.00 (0.76–1.32)	61	1.08 (0.69–1.67)	48	0.74 (0.47–1.15)	79	1.18 (0.79–1.76)	0.018
P-trend				0.907		0.493		0.044		0.350	
Continuous per two sizes		33,880	643	1.09 (0.95–1.24)	167	1.27 (0.96–1.69)	211	0.84 (0.69–1.02)	265	1.20 (1.01–1.42)	0.007
Women – rectum											
≤40	40	6,574	21	1.00 (ref.)	3	1.00 (ref.)	9	1.00 (ref.)	9	1.00 (ref.)	
42	42	8,582	29	0.99 (0.55–1.80)	11	2.56 (0.69–9.47)	12	0.98 (0.40–2.39)	6	0.48 (0.16–1.44)	
44	44	9,270	36	1.14 (0.65–2.00)	14	3.30 (0.98–11.16)	10	0.75 (0.29–1.91)	12	0.86 (0.35–2.08)	
≥46	46	9,454	40	1.19 (0.68–2.08)	9	1.88 (0.53–6.70)	19	1.37 (0.59–3.21)	12	0.81 (0.32–2.04)	0.546
P-trend				0.442		0.426		0.507		0.983	
Continuous per two sizes		33,880	126	1.04 (0.84–1.27)	37	1.06 (0.74–1.52)	50	1.04 (0.75–1.45)	39	1.02 (0.71–1.47)	0.998

Abbreviations: CI, confidence interval; HR, hazard ratio; NLCS, Netherlands Cohort Study; *P*-het, *P*-heterogeneity.

[a]HRs were adjusted for age (years; continuous), nonoccupational physical activity (minutes/day; continuous), total energy intake (kcal/day; continuous), family history of colorectal cancer (yes; no), alcohol consumption (0; 0.1–4; 5–14; >15 g/day), processed meat intake (g/day; continuous), and red meat intake (g/day; continuous). Age was included as a time-varying covariate.

[b]Median clothing size per category based on the subcohort.

Table 4. Multivariable-adjusted HRs[a] and 95% CIs for associations between nonoccupational physical activity and Warburg subtypes in colorectal cancer, by sex and tumor location; NLCS, 1986–2006.

	Median[b]	Person-years at risk	Total		Warburg-low		Warburg-moderate		Warburg-high		*P*-het
			n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	
Nonoccupational physical activity (min/day)											
Men – colon											
≤30	21.4	4,997	131	1.10 (0.85–1.43)	36	1.28 (0.81–2.00)	48	0.97 (0.67–1.41)	47	1.13 (0.76–1.68)	
31–60	42.9	10,100	247	1.00 (ref.)	61	1.00 (ref.)	100	1.00 (ref.)	86	1.00 (ref.)	
61–90	73.6	6,001	164	1.16 (0.90–1.48)	53	1.54 (1.02–2.32)	51	0.88 (0.60–1.28)	60	1.21 (0.85–1.75)	
>90	130.0	9,925	230	0.96 (0.77–1.20)	65	1.11 (0.76–1.62)	81	0.83 (0.61–1.15)	84	1.01 (0.72–1.40)	0.611
P-trend				0.505		0.917		0.270		0.812	
Continuous per 30 min/day		31,022	772	0.99 (0.95–1.03)	215	0.99 (0.93–1.06)	280	0.98 (0.92–1.05)	277	0.99 (0.93–1.05)	0.987
Men – rectum											
≤30	21.4	4,997	19	0.57 (0.33–0.97)	10	0.90 (0.42–1.93)	9	0.77 (0.35–1.71)	0	—	
31–60	42.9	10,100	70	1.00 (ref.)	24	1.00 (ref.)	25	1.00 (ref.)	21	1.00 (ref.)	
61–90	73.6	6,001	58	1.41 (0.96–2.05)	21	1.51 (0.83–2.75)	14	0.96 (0.48–1.91)	23	1.85 (1.00–3.43)	
>90	130.0	9,925	80	1.20 (0.85–1.69)	21	0.90 (0.50–1.65)	28	1.16 (0.66–2.04)	31	1.60 (0.90–2.86)	0.099
P-trend				0.004		0.882		0.332		<0.001	
Continuous per 30 min/day		31,022	227	1.05 (0.99–1.10)	76	0.97 (0.88–1.06)	76	1.05 (0.96–1.16)	75	1.10 (1.02–1.19)	0.089
Women – colon											
≤30	19.3	7,756	177	1.17 (0.92–1.49)	51	1.31 (0.87–1.96)	62	1.23 (0.85–1.78)	64	1.04 (0.73–1.48)	
31–60	42.9	10,923	212	1.00 (ref.)	53	1.00 (ref.)	73	1.00 (ref.)	86	1.00 (ref.)	
61–90	75.0	8,000	148	0.94 (0.73–1.20)	37	0.94 (0.61–1.47)	46	0.84 (0.56–1.25)	65	1.02 (0.72–1.44)	
>90	115.7	7,550	118	0.81 (0.62–1.05)	29	0.80 (0.50–1.28)	35	0.69 (0.45–1.06)	54	0.91 (0.63–1.32)	0.704
P-trend				0.008		0.042		0.006		0.574	
Continuous per 30 min/day		34,228	655	0.96 (0.90–1.02)	170	0.96 (0.86–1.08)	216	0.87 (0.78–0.96)	269	1.02 (0.94–1.11)	0.050
Women – rectum											
≤30	19.3	7,756	30	0.98 (0.60–1.61)	6	0.77 (0.27–2.17)	13	1.12 (0.53–2.36)	11	1.00 (0.45–2.24)	
31–60	42.9	10,923	42	1.00 (ref.)	11	1.00 (ref.)	16	1.00 (ref.)	15	1.00 (ref.)	
61–90	75.0	8,000	33	1.05 (0.65–1.68)	13	1.53 (0.69–3.43)	13	1.09 (0.51–2.32)	7	0.64 (0.26–1.58)	
>90	115.7	7,550	22	0.73 (0.42–1.25)	7	0.83 (0.30–2.24)	9	0.83 (0.36–1.92)	6	0.56 (0.21–1.48)	0.919
P-trend				0.352		0.649		0.569		0.143	
Continuous per 30 min/day		34,228	127	1.01 (0.89–1.14)	37	1.06 (0.86–1.30)	51	0.91 (0.77–1.08)	39	1.05 (0.84–1.32)	0.329

Abbreviations: CI, confidence interval; HR, hazard ratio; NLCS, Netherlands Cohort Study; *P*-het, *P*-heterogeneity.

[a]HRs were adjusted for age (years; continuous), BMI (kg/m^2), total energy intake (kcal/day; continuous), family history of colorectal cancer (yes; no), alcohol consumption (0; 0.1–4; 5–14; >15 g/day), processed meat intake (g/day; continuous), and red meat intake (g/day; continuous). Age was included as a time-varying covariate.

[b]Median daily minutes of physical activity per category based on the subcohort.

Table 5. Multivariable-adjusted HRs[a] and 95% CIs for associations between occupational physical activity and Warburg subtypes in colorectal cancer in men, by tumor location, NLCS 1986–2003.

	Person-years at risk	Total		Warburg-low		Warburg-moderate		Warburg-high		
		n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	*P*-het
Colon										
Energy expenditure	25,073	574		157		212		205		
<8 kJ/min	15,144	364	1.00 (ref.)	100	1.00 (ref.)	135	1.00 (ref.)	129	1.00 (ref.)	
8–12 kJ/min	6,368	138	0.88 (0.70–1.12)	40	0.97 (0.65–1.46)	49	0.83 (0.58–1.19)	49	0.87 (0.60–1.25)	
>12 kJ/min	3,561	72	0.79 (0.58–1.08)	17	0.75 (0.43–1.29)	28	0.78 (0.50–1.22)	27	0.83 (0.52–1.33)	0.972
P-trend			0.107		0.355		0.202		0.356	
Sitting time	25,073	574		157		212		205		
>6 hours/day	6,511	184	1.00 (ref.)	53	1.00 (ref.)	71	1.00 (ref.)	60	1.00 (ref.)	
2–6 hours/day	11,617	249	0.72 (0.57–0.92)	61	0.62 (0.42–0.92)	89	0.66 (0.47–0.93)	99	0.90 (0.63–1.28)	
<2 hours/day	6,944	141	0.69 (0.53–0.91)	43	0.78 (0.50–1.21)	52	0.64 (0.43–0.94)	46	0.69 (0.45–1.06)	0.617
P-trend			0.007		0.264		0.025		0.087	
Rectum										
Energy expenditure	25,073	184		66		60		58		
<8 kJ/min	15,144	106	1.00 (ref.)	42	1.00 (ref.)	34	1.00 (ref.)	30	1.00 (ref.)	
8–12 kJ/min	6,368	56	1.33 (0.94–1.90)	16	1.00 (0.55–1.79)	19	1.40 (0.76–2.57)	21	1.69 (0.95–3.00)	
>12 kJ/min	3,561	22	0.88 (0.54–1.44)	8	0.80 (0.36–1.77)	7	0.90 (0.38–2.12)	7	0.94 (0.41–2.14)	0.734
P-trend			0.841		0.630		0.799		0.523	
Sitting time	25,073	184		66		60		58		
>6 hours/day	6,511	57	1.00 (ref.)	24	1.00 (ref.)	16	1.00 (ref.)	17	1.00 (ref.)	
2–6 hours/day	11,617	71	0.67 (0.46–0.97)	24	0.53 (0.29–0.95)	28	0.92 (0.49–1.75)	19	0.61 (0.32–1.18)	
<2 hours/day	6,944	56	0.92 (0.62–1.37)	18	0.72 (0.39–1.35)	16	0.93 (0.45–1.92)	22	1.19 (0.63–2.23)	0.414
P-trend			0.714		0.308		0.841		0.568	

Abbreviations: CI, confidence interval; HR, hazard ratio; NLCS, Netherlands Cohort Study.

[a]HRs were adjusted for age (years; continuous), BMI (kg/m^2), total energy intake (kcal/day; continuous), family history of colorectal cancer (yes; no), alcohol consumption (0; 0.1–4; 5–14; >15 g/day), processed meat intake (g/day; continuous), and red meat intake (g/day; continuous). Age was included as a time-varying covariate.

statistical significance only for the Warburg-moderate group [HR (95% CI): 0.64 (0.43–0.94); $P_{trendcategories} = 0.025$). Tests for heterogeneity did not reach statistical significance for any of these exposures.

For rectal cancer, nonoccupational physical activity showed a positive association in men (**Table 4**), with HR (95% CI) of 1.05 (0.99–1.10) per 30 minutes/day and a statistically significant trend over categories ($P_{trendcategories} = 0.004$). The association was stronger for the Warburg-high subtype [$HR_{30min/day}$ (95% CI): 1.10 (1.02–1.19)], with a statistically significant trend over categories ($P_{trendcategories} < 0.001$). Heterogeneity between Warburg subtypes was not statistically significant. For occupational physical activity, no clear associations were found with rectal cancer risk (**Table 5**).

In women, nonoccupational physical activity was associated with decreased colon cancer risk (**Table 4**), with HR (95% CI) of 0.96 (0.90–1.02) per 30 minutes/day, and a statistically significant trend over categories ($P_{trendcategories} = 0.008$). After stratification on Warburg subtypes, a similar association was found for Warburg-low [$HR_{30min/day}$ (95% CI): 0.96 (0.86–1.08); $P_{trendcategories} = 0.042$], a stronger effect for Warburg-moderate [$HR_{30min/day}$ (95% CI): 0.87 (0.78–0.96); $P_{trendcategories} = 0.006$), and no effect for Warburg-high. A statistically significant difference in associations per 30 minutes/day increase was found between Warburg subtypes ($P_{heterogeneity} = 0.050$), but not for categorical models.

For rectal cancer, no statistically significant associations for nonoccupational physical activity were found in women (**Table 4**).

Sensitivity analyses

Sensitivity analyses excluding the first 2 years of follow-up did not lead to essential changes. Furthermore, analyses with two instead of three Warburg subtypes generally led to similar conclusions. Associations that were found for the Warburg-moderate subtype (e.g., occupational sitting time with colon cancer in men) when using three Warburg subtypes, resulted in similar associations for Warburg-low and Warburg-high subtypes when two Warburg subtypes were used.

Discussion

The role of metabolic reprogramming, in particular the Warburg effect, in cancer development is becoming increasingly recognized (22, 23). We investigated whether the associations between energy balance-related factors and colorectal cancer risk differ between tumors expressing low versus high levels of proteins involved in the Warburg effect. In this prospective cohort study, we found positive associations for BMI and clothing size with risk of Warburg-moderate and Warburg-high colon cancer in men. In women, clothing size showed positive associations with Warburg-low and Warburg-high colon cancer. Nonoccupational physical activity was inversely associated with Warburg-low and Warburg-moderate colon cancer in women, and occupational sitting time was inversely associated with Warburg-moderate colon cancer in men. In contrast, nonoccupational physical activity was positively associated with Warburg-high rectal cancer in men. Statistically significant heterogeneity between Warburg subtypes was found for clothing size and nonoccupational physical activity and risk of colon cancer in women, whereas differences found in men did not show statistically significant heterogeneity.

Previous studies classifying colorectal cancer into so-called metabolic subtypes (40) or specifically glycolysis-related subtypes (41) focused mainly on prognosis. Up to now, there are no studies relating etiologic research to metabolic/glycolysis/Warburg subtypes in colorectal cancer. However, studies have investigated the relationship between proposed precursors (i.e., leptin, adiponectin, insulin, IGF-1) of the Warburg effect in relation to colorectal cancer development (12–14). It has been suggested that leptin, adiponectin, insulin, and IGF-1 can influence PI3K/Akt signaling (16), leading to the metabolic switch towards the Warburg effect (17, 18). Leptin, insulin, and IGF-1 have been associated with increased risk of colorectal cancer (12–14), whereas adiponectin has been associated with a decreased colorectal cancer risk (14). However, results of these associations are rather inconsistent, which is likely caused by differences in study design (12, 14), because it is difficult to differentiate between cause and effect with circulating biomarkers, especially when biomarker levels in the serum are measured at the time of cancer diagnosis (e.g., case–control design). The current study, however, has a prospective cohort design, where the proposed mechanism is measured in tumor tissue instead of serum, hereby adding further insights in the link between energy balance and colorectal cancer risk.

The observed differences in associations between energy balance–related factors and Warburg subtypes in colorectal cancer, further varying by sex and tumor location, suggest that various carcinogenic mechanisms may explain the link between energy balance and colorectal cancer.

Adiposity

The current results suggest a role of the Warburg effect in colon cancer risk enhancement resulting from increased adiposity in both men and women (associations Warburg-high), but an additional mechanism might be involved in women (associations Warburg-low). A possible explanation for this additional mechanism in women might be related to sex hormones. Gunter and colleagues (42) have previously reported a positive association between estradiol (an endogenous estrogen) levels and colorectal cancer risk in postmenopausal women, potentially through promotion of cancer cell proliferation by estradiol, as well as for hyperinsulinemia and IGF-1 with colorectal cancer risk. They proposed that at least two pathways are related to the obesity-colorectal cancer link in postmenopausal women, one involving estradiol and one involving hyperinsulinemia and IGF-1 signaling. These proposed pathways might support the current results, where the pathway involving insulin and IGF-1 reflects the associations with the Warburg-high subtype. The associations we found for the Warburg-low subtype, which are thus not linked to the Warburg effect, might be explained by aberrant signaling of sex hormones.

Mutually adjusted BMI and clothing size models might give some further insight in the associations between adiposity and Warburg subtypes. Although we do not have data on visceral adipose tissue (VAT) or subcutaneous adipose tissue (SAT), it has been shown that waist circumference could be used as a surrogate of VAT, especially when BMI is added to the model, and that BMI better predicts SAT in men, especially when adjusting for waist circumference (37). The difference we found between Warburg-moderate and Warburg-high subtypes in men with colon cancer in mutually adjusted models of BMI and clothing size might be explained by differences in fat distribution. VAT seems to be an indicator of adiponectin levels, whereas SAT influences leptin levels (43). The role of adiponectin in the enhancement of the Warburg effect is less established compared with that of leptin (5). This suggests that adiponectin might not be associated with the Warburg effect, which might possibly explain why clothing size associations with Warburg-high colon cancer were attenuated after adjustment for BMI (proxy VAT), whereas BMI associations did not change after adjustment for clothing size (proxy SAT), and vice versa

for Warburg-moderate. Though we should be careful with drawing any conclusions because we only have proxy measures for VAT and SAT, we believe these differences are interesting and studies with well-defined data on fat distribution are required to further investigate this. For women, we refrain from interpreting results from mutually adjusted BMI and clothing size models, since the changes in HRs might be caused by multicollinearity (for comparison, the Spearman rank correlation was 0.53 for men and 0.76 for women).

Physical activity

Interestingly, both higher levels of nonoccupational physical activity in women and less occupational sitting time in men were inversely associated with Warburg-low and -moderate colon cancer. These observations might indicate that the Warburg effect is not involved, at least not to a great extent, in the relation between measures of physical activity and colon cancer. Potentially, some of the proteins of our Warburg panel are involved in the association, but not all. Very few studies have investigated whether any of these proteins are involved in the etiologic link between physical activity and colon cancer. Shirvani and colleagues (44) found increased levels of the tumor-suppressor P53 in mice after exercise training. However, Slattery and colleagues (45) did not find a difference in associations of physical activity for tumors with and without a P53 mutation in a case–control study. Further research, preferably in large prospective cohorts, is required to examine which markers might be involved in this link. In women, inverse associations for nonoccupational physical activity were found for Warburg-low colon cancer as well, indicating that the Warburg effect is not involved. A possible explanation for this association might be the decreased exposure to fecal carcinogens of the colonical mucosal surface, due to a shorter bowel transit time (4). This is further underlined by the results of Song and colleagues (46), who found a reduction in transit time for highly physically active women, but not men.

We observed a positive association for physical activity and risk of Warburg-high rectal cancer in men. Positive, though nonsignificant, associations have previously been reported for rectal cancer within the NLCS (34), as well as other studies (47–49), but results from meta-analyses suggest that physical activity is not related to rectal cancer in men (2, 4). For now, we do not have an explanation for this counterintuitive finding. Further research is necessary to see whether this relation will be observed in other populations.

Strengths and limitations

A major strength of this study is the large prospective population-based cohort design with long follow-up (20.3 years) and availability of tumor material from a large number of incident colorectal cancer cases. This enabled us to combine extensive epidemiologic lifestyle data with molecular pathologic profiling of tumor tissue for a large number of incident colorectal cancer cases. However, despite the large sample size, the number of cases in final statistical analyses was limited for some groups (especially rectal cancer) due to heterogeneity in sex and tumor location. In particular, the absence of clear differences in associations between Warburg subtypes in rectal cancer might be caused by a lack of statistical power. Other large prospective cohort studies with availability of tumor specimens might help to further investigate the mechanisms for rectal cancer, as well as confirming our findings for colon cancer. Furthermore, due to the heterogeneity based on sex and tumor location, multiple testing might have been a problem in the current study. Even though our analyses were hypothesis-driven, this is a common issue in MPE studies (50). It is therefore important that our analyses will be replicated in large (MPE) studies. Another common problem in MPE studies is selection bias based on referral hospital (50). However, for the current study, FFPE blocks of incident cancer cases within the NLCS were collected from 43 hospitals throughout the Netherlands, both academic and peripheral, minimizing the risk of selection bias. A third problem with MPE studies is the usage of TMAs instead of full sections. Even though the TMA-technique enables large-throughput analyses at reduced costs (51), it may not provide a full picture of the tumor. Still, in the construction of TMAs, cores were sampled in different regions to capture potential tumor heterogeneity. In addition, IHC scoring on these TMA sections is often performed by non-pathologists (e.g., PhD students or technicians), because the number of cores are often very large in MPE studies, making it impossible for a pathologist to score all available material. However, we have previously shown that non-pathologists can produce reproducible IHC-scoring results, similar to those of a pathologist, after sufficient training by an experienced pathologist (29).

To enable replication of the current results, we used a transparent way of making subtypes by using a simple sum score of six proteins involved in the Warburg effect. We acknowledge that this also entails some disadvantages, as it probably does not reflect all factors involved in the Warburg effect. For example, a case classified as Warburg-low might still show high expression for one of the proteins, whereas a case classified as Warburg-high might show high expression in only half of the proteins. This might be the reason why several associations were found for either Warburg-low and Warburg-moderate, or Warburg-high and Warburg-moderate. By using a Warburg-moderate group here, we were able to compare the more extreme cases and reduce misclassification in Warburg-low and Warburg-high subtypes. In addition, the six proteins used for the sum score and subsequent Warburg subtypes are a selection of the total pathway, and might not capture the full picture. However, capturing the complete pathway is nearly impossible considering time and budgetary constraints. We aimed to capture the Warburg effect by incorporating proteins from different levels of the pathway in this sum score (i.e., from upstream regulators, glucose import, glycolysis, to lactate secretion), attempting to provide a comprehensive view of the Warburg effect.

Because we were the first to study the associations between energy balance–related factors and risk of Warburg subtypes in colorectal cancer, our results should be interpreted with caution because validation of the current findings is needed.

Conclusion

In this large prospective cohort study, we found that associations of energy balance–related factors and colorectal cancer risk differed between Warburg subtypes, further varying by sex and tumor location. The Warburg effect seems to be involved in associations between adiposity and risk of colon cancer, both in men and women, though additional mechanisms are probably at play in women as well. The link between physical activity and colon cancer is probably explained by mechanisms other than the Warburg effect. Further research is needed to reproduce these results, and investigate possible additional mechanisms.

Authors' Disclosures

P.A. van den Brandt reports grants from Dutch Cancer Society during the conduct of the study. No disclosures were reported by the other authors.

Authors' Contributions

J.C.A. Jenniskens: Conceptualization, formal analysis, writing–original draft. **K. Offermans:** Conceptualization, writing–original draft. **C.C.J.M. Simons:** Conceptualization, writing–review and editing. **I. Samarska:** Writing–review and editing. **G.E. Fazzi:** Writing–review and editing. **K.M. Smits:** Writing–review and editing. **L.J. Schouten:** Writing–review and editing. **M.P. Weijenberg:** Writing–review and editing. **H.I. Grabsch:** Conceptualization, supervision, funding acquisition, writing–original draft. **P.A. van den Brandt:** Conceptualization, supervision, funding acquisition, methodology, writing–original draft, data acquisition.

Acknowledgments

This project was funded by The Dutch Cancer Society (KWF 11044, to P.A. van den Brandt).

The authors would like to thank the participants of the Netherlands Cohort Study (NLCS), the Netherlands Cancer Registry, and the Dutch Pathology Registry. They are grateful to Ron Alofs and Harry van Montfort for data management and programming assistance; Jaleesa van der Meer, Edith van den Boezem, and Peter Moerkerk for TMA construction; and Jakob Kather (University Hospital Aachen, Germany) for scanning of slides.

The Rainbow-TMA consortium was financially supported by BBMRI-NL, a Research Infrastructure financed by the Dutch government (NWO 184.021.007, to P.A. van den Brandt), and Maastricht University Medical Center, University Medical Center Utrecht, and Radboud University Medical Centre, the Netherlands. The authors would like to thank all investigators from the Rainbow-TMA consortium project group [P.A. van den Brandt, A. zur Hausen, H.I. Grabsch, M. van Engeland, L.J. Schouten, J. Beckervordersandforth (Maastricht University Medical Center, Maastricht, the Netherlands); P.H.M. Peeters, P.J. van Diest, H.B. Bueno de Mesquita (University Medical Center Utrecht, Utrecht, the Netherlands); J. van Krieken, I. Nagtegaal, B. Siebers, B. Kiemeney (Radboud University Medical Center, Nijmegen, the Netherlands); F.J. van Kemenade, C. Steegers, D. Boomsma, G.A. Meijer (VU University Medical Center, Amsterdam, the Netherlands); F.J. van Kemenade, B. Stricker (Erasmus University Medical Center, Rotterdam, the Netherlands); L. Overbeek, A. Gijsbers (PALGA, the Nationwide Histopathology and Cytopathology Data Network and Archive, Houten, the Netherlands)] and collaborating pathologists [Among others: A. de Bruïne (VieCuri Medical Center, Venlo); J.C. Beckervordersandforth (Maastricht University Medical Center, Maastricht); J. van Krieken, I. Nagtegaal (Radboud University Medical Center, Nijmegen); W. Timens (University Medical Center Groningen, Groningen); F.J. van Kemenade (Erasmus University Medical Center, Rotterdam); M.C.H. Hogenes (Laboratory for Pathology OostNederland, Hengelo); P.J. van Diest (University Medical Center Utrecht, Utrecht); R.E. Kibbelaar (Pathology Friesland, Leeuwarden); A.F. Hamel (Stichting Samenwerkende Ziekenhuizen Oost-Groningen, Winschoten); A.T.M.G. Tiebosch (Martini Hospital, Groningen); C. Meijers (Reinier de Graaf Gasthuis/S.S.D. Z., Delft); R. Natté (Haga Hospital Leyenburg, The Hague); G.A. Meijer (VU University Medical Center, Amsterdam); J.J.T.H. Roelofs (Academic Medical Center, Amsterdam); R.F. Hoedemaeker (Pathology Laboratory Pathan, Rotterdam); S. Sastrowijoto (Orbis Medical Center, Sittard); M. Nap (Atrium Medical Center, Heerlen); H.T. Shirango (Deventer Hospital, Deventer); H. Doornewaard (Gelre Hospital, Apeldoorn); J.E. Boers (Isala Hospital, Zwolle); J.C. van der Linden (Jeroen Bosch Hospital, Den Bosch); G. Burger (Symbiant Pathology Center, Alkmaar); R.W. Rouse (Meander Medical Center, Amersfoort); P.C. de Bruin (St. Antonius Hospital, Nieuwegein); P. Drillenburg (Onze Lieve Vrouwe Gasthuis, Amsterdam); C. van Krimpen (Kennemer Gasthuis, Haarlem); J.F. Graadt van Roggen (Diaconessenhuis, Leiden); S.A.J. Loyson (Bronovo Hospital, The Hague); J.D. Rupa (Laurentius Hospital, Roermond); H. Kliffen (Maasstad Hospital, Rotterdam); H.M. Hazelbag (Medical Center Haaglanden, The Hague); K. Schelfout (Stichting Pathologisch en Cytologisch Laboratorium West-Brabant, Bergen op Zoom); J. Stavast (Laboratorium Klinische Pathologie Centraal Brabant, Tilburg); I. van Lijnschoten (PAMM laboratory for Pathology and Medical Microbiology, Eindhoven); K. Duthoi (Amphia Hospital, Breda)].

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Received May 28, 2021; revised September 23, 2021; accepted December 10, 2021; published first December 20, 2021.

References

1. Moghaddam AA, Woodward M, Huxley R. Obesity and risk of colorectal cancer: a meta-analysis of 31 studies with 70,000 events. Cancer Epidemiol Biomarkers Prev 2007;16:2533–47.
2. Robsahm TE, Aagnes B, Hjartåker A, Langseth H, Bray FI, Larsen IK. Body mass index, physical activity, and colorectal cancer by anatomical subsites: a systematic review and meta-analysis of cohort studies. Eur J Cancer Prev 2013;22:492–505.
3. Wolin KY, Yan Y, Colditz GA, Lee I. Physical activity and colon cancer prevention: a meta-analysis. Br J Cancer 2009;100:611–6.
4. Samad A, Taylor R, Marshall T, Chapman MA. A meta-analysis of the association of physical activity with reduced risk of colorectal cancer. Colorectal Dis 2005;7:204–13.
5. Park J, Morley TS, Kim M, Clegg DJ, Scherer PE. Obesity and cancer—mechanisms underlying tumour progression and recurrence. Nat Rev Endocrinol 2014;10:455–65.
6. Hopkins BD, Goncalves MD, Cantley LC. Obesity and cancer mechanisms: cancer metabolism J Clin Oncol 2016;34:4277–83.
7. Friedenreich CM, Ryder-Burbidge C, McNeil JJMO. Physical activity, obesity and sedentary behavior in cancer etiology: epidemiologic evidence and biologic mechanisms. Mol Oncol 2021;15:790–800.
8. Fasshauer M, Blüher M. Adipokines in health and disease. Trends Pharmacol Sci 2015;36:461–70.
9. Thomas RJ, Kenfield SA, Jimenez A. Exercise-induced biochemical changes and their potential influence on cancer: a scientific review. Br J Sports Med 2017;51:640–4.
10. Yu N, Ruan Y, Gao X, Sun J. Systematic review and meta-analysis of randomized, controlled trials on the effect of exercise on serum leptin and adiponectin in overweight and obese individuals. Horm Metab Res 2017;49:164–73.
11. Renehan AG, Frystyk J, Flyvbjerg A. Obesity and cancer risk: the role of the insulin–IGF axis. Trends Endocrinol Metab 2006;17:328–36.
12. Yin S, Bai H, Jing D. Insulin therapy and colorectal cancer risk among type 2 diabetes mellitus patients: a systemic review and meta-analysis. Diagn Pathol 2014;9:91.
13. Renehan AG, Zwahlen M, Minder C, T O'Dwyer S, Shalet SM, Egger M. Insulin-like growth factor (IGF)-I, IGF binding protein-3, and cancer risk: systematic review and meta-regression analysis. Lancet 2004;363:1346–53.
14. Joshi RK, Kim WJ, Lee S-A. Association between obesity-related adipokines and colorectal cancer: a case-control study and meta-analysis. World J Gastroenterol 2014;20:7941–9.
15. Yoon YS, Kwon AR, Lee YK, Oh SW. Circulating adipokines and risk of obesity related cancers: a systematic review and meta-analysis. Obes Res Clin Pract 2019;13:329–39.
16. Huang XF, Chen JZ. Obesity, the PI3K/Akt signal pathway and colon cancer. Obes Rev 2009;10:610–6.
17. Levine AJ, Puzio-Kuter AM. The control of the metabolic switch in cancers by oncogenes and tumor suppressor genes. Science 2010;330:1340–4.
18. Feron O. Pyruvate into lactate and back: from the Warburg effect to symbiotic energy fuel exchange in cancer cells. Radiother Oncol 2009;92:329–33.
19. Pham D-V, Park P-H. Tumor metabolic reprogramming by adipokines as a critical driver of obesity-associated cancer progression. Int J Mol Sci 2021;22:1444.
20. Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. Nat Rev Cancer 2011;11:85–95.
21. Warburg O. The metabolism of carcinoma cells. Cancer Res 1925;9:148–63.
22. Schwartz L, Supuran CT, Alfarouk KO. The Warburg effect and the hallmarks of cancer. Anticancer Agents Med Chem 2017;17:164–70.
23. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646–74.
24. van den Brandt PA, Goldbohm RA, Veer PVT, Volovics A, Hermus RJ, Sturmans F. A large-scale prospective cohort study on diet and cancer in The Netherlands. J Clin Epidemiol 1990;43:285–95.
25. Prentice RL. A case-cohort design for epidemiologic cohort studies and disease prevention trials. Biometrika 1986;73:1–11.
26. van den Brandt PA, Schouten LJ, Goldbohm RA, Dorant E, Hunen PM. Development of a record linkage protocol for use in the Dutch Cancer Registry for Epidemiological Research. Int J Epidemiol 1990;19:553–8.

27. Goldbohm RA, van den Brandt PA, Dorant E. Estimation of the coverage of Dutch municipalities by cancer registries and PALGA based on hospital discharge data; 1994.
28. van den Brandt PA. Molecular pathological epidemiology of lifestyle factors and colorectal and renal cell cancer risk. maastricht pathology 2018. 11th joint meeting of the british division of the international academy of pathology and the pathological society of great Britain & Ireland, 19–22 June 2018. J Pathol 2018; 246:S9.
29. Jenniskens JCA, Offermans K, Samarska I, Fazzi GE, Simons CCJM, Smits KM, et al. Validity and reproducibility of immunohistochemical scoring by trained non-pathologists on tissue microarrays. Cancer Epidemiol Biomarkers Prev 2021;30:1867–74.
30. Resnick MB, Routhier J, Konkin T, Sabo E, Pricolo VE. Epidermal growth factor receptor, c-MET, β-catenin, and p53 expression as prognostic indicators in stage II colon cancer: a tissue microarray study. Clin Cancer Res 2004;10:3069–75.
31. Richman SD, Adams R, Quirke P, Butler R, Hemmings G, Chambers P, et al. Pre-trial inter-laboratory analytical validation of the FOCUS4 personalised therapy trial. J Clin Pathol 2016;69:35–41.
32. Cairns RA. Drivers of the Warburg phenotype. Cancer J 2015;21:56–61.
33. Hughes LA, Schouten LJ, Goldbohm RA, van den Brandt PA, Weijenberg MP. Self-reported clothing size as a proxy measure for body size. Epidemiology 2009; 20:673–6.
34. Simons CCJM, Hughes LA, Van Engeland M, Goldbohm RA, van den Brandt PA, Weijenberg MP. Physical activity, occupational sitting time, and colorectal cancer risk in the Netherlands cohort study. Am J Epidemiol 2013;177:514–30.
35. Lin D, Wei L. The robust inference for the Cox proportional hazards model J Am Stat Assoc 1989;84:1074–8.
36. Schoenfeld D. Partial residuals for the proportional hazards regression model. Biometrika 1982;69:239–41.
37. Janssen I, Heymsfield SB, Allison DB, Kotler DP, Ross R. Body mass index and waist circumference independently contribute to the prediction of nonabdominal, abdominal subcutaneous, and visceral fat. Am J Clin Nutr 2002;75:683–8.
38. De Vogel S, Bongaerts BW, Wouters KA, Kester AD, Schouten LJ, de Goeij AF, et al. Associations of dietary methyl donor intake with MLH1 promoter hypermethylation and related molecular phenotypes in sporadic colorectal cancer. Carcinogenesis 2008;29:1765–73.
39. Wacholder S, Gail MH, Pee D, Brookmeyer R. Alternative variance and efficiency calculations for the case-cohort design. Biometrika 1989;76:117–23.
40. Guinney J, Dienstmann R, Wang X, De Reyniès A, Schlicker A, Soneson C, et al. The consensus molecular subtypes of colorectal cancer. Nat Med 2015; 21:1350–6.
41. Zhu J, Wang S, Bai H, Wang K, Hao J, Zhang J, et al. Identification of five glycolysis-related gene signature and risk score model for colorectal cancer. Front Oncol 2021;11:588811.
42. Gunter MJ, Hoover DR, Yu H, Wassertheil-Smoller S, Rohan TE, Manson JE, et al. Insulin, insulin-like growth factor-I, endogenous estradiol, and risk of colorectal cancer in postmenopausal women. Cancer Res 2008;68:329–37.
43. Park K-G, Park KS, Kim M-J, Kim H-S, Suh Y-S, Ahn JD, et al. Relationship between serum adiponectin and leptin concentrations and body fat distribution. Diabetes Res Clin Pract 2004;63:135–42.
44. Shirvani H, Bazgir B, Rahimi M, Isanejad A, Samadi M, Sobhani V, et al. Interval exercise training affects expression of MCT-1, GLUT-1, PFK-1 and p53 as key metabolic regulators on azoxymethane-induced experimental colon cancer. 2021. Available from: https://doi.org/10.21203/rs.3.rs-151527/v1.
45. Slattery ML, Curtin K, Ma K, Edwards S, Schaffer D, Anderson K, et al. Diet, activity, and lifestyle associations with p53 mutations in colon tumors. Cancer Epidemiol Biomarkers Prev 2002;11:541–8.
46. Song BK, Cho KO, Jo Y, Oh JW, Kim YS. Colon transit time according to physical activity level in adults. J Neurogastroenterol Motil 2012;18:64–9.
47. Friedenreich C, Norat T, Steindorf K, Boutron-Ruault M-C, Pischon T, Mazuir M, et al. Physical activity and risk of colon and rectal cancers: the European prospective investigation into cancer and nutrition. Cancer Epidemiol Biomarkers Prev 2006;15:2398–407.
48. Lee IM, Paffenbarger RS Jr, Hsieh C. Physical activity and risk of developing colorectal cancer among college alumni. J Natl Cancer Inst 1991;83:1324–9.
49. Tavani A, Braga C, La Vecchia C, Conti E, Filiberti R, Montella M, et al. Physical activity and risk of cancers of the colon and rectum: an Italian case-control study. Br J Cancer 1999;79:1912–6.
50. Ogino S, Chan AT, Fuchs CS, Giovannucci E. Molecular pathological epidemiology of colorectal neoplasia: an emerging transdisciplinary and interdisciplinary field. Gut 2011;60:397–411.
51. Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 1998;4:844–7.