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Energy balance-related factors in childhood and adolescence and risk of colorectal cancer expressing different levels of proteins involved in the Warburg-effect

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Abstract

Early-life (childhood to adolescence) energy balance-related factors (height, energy restriction, BMI) have been associated with adult colorectal cancer (CRC) risk. Warburg-effect activation via PI3K/Akt-signaling might explain this link. We investigated whether early-life energy balance-related factors were associated with risk of Warburg-subtypes in CRC. We used immunohistochemistry for six proteins involved in the Warburg-effect (LDHA, GLUT1, MCT4, PKM2, P53, and PTEN) on tissue microarrays of 2399 incident CRC cases from the prospective Netherlands Cohort Study (NLCS). 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 height, energy restriction proxies (exposure to Dutch Hunger Winter; Second World War [WWII]; Economic Depression) and adolescent BMI with Warburg-subtypes in CRC. Height was positively associated with colon cancer in men, regardless of Warburg-subtypes, and with Warburg-low colon and Warburg-moderate rectal cancer in women. Energy restriction during the Dutch Hunger Winter was inversely associated with colon cancer in men, regardless of Warburg-subtypes. In women, energy restriction during the Hunger Winter and WWII was inversely associated with Warburg-low colon cancer, whereas energy restriction during the Economic Depression was positively associated with Warburg-high colon cancer. Adolescent BMI was positively associated with Warburg-high colon cancer in men, and Warburg-moderate rectal cancer in women. In conclusion, the Warburg-effect seems to be involved in associations of adolescent BMI with colon cancer in men, and of energy restriction during the Economic Depression with colon cancer in women. Further research is needed to validate these results.

Abbreviations: BMI, body mass index; CI, confidence interval; CRC, colorectal cancer; FFPE, formalin-fixed paraffin-embedded; HR, hazard ratio; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; IHC, immunohistochemistry; NLCS, Netherlands Cohort Study; TMA, tissue microarray; WWII, World War II.

KEYWORDS
colorectal cancer, early-life energy balance, etiological heterogeneity, prospective cohort study, Warburg-effect

What's new?

Early-life energy balance-related factors, such as height, energy restriction and body mass index (BMI), are associated with colorectal cancer (CRC) risk later in life. The mechanisms behind these associations, however, remain unknown. Here, using data from the prospective Netherlands Cohort Study (NLCS), the authors investigated the involvement of the Warburg-effect in associations between early-life energy balance-related factors and CRC risk. Analyses suggest that the Warburg-effect is involved in associations of adolescent BMI and colon cancer risk in men.

1 | INTRODUCTION

Factors related to early-life (childhood to adolescence) energy balance have been reported to have long-term effects on colorectal cancer (CRC) risk. Increased adult-attained height, as a proxy for fetal and early-life (nutritional) exposures, has been associated with an increased risk of CRC.[1,2] Early-life energy restriction seems to decrease risk of adult CRC,[3-5] though positive associations have been reported as well.[6,7] Childhood or adolescent body mass index (BMI) has been associated with an increased risk of CRC.[8] The mechanism(s) behind these long-term effects of early-life energy balance-related factors remain to be elucidated.

A proposed common effect of energy balance-related factors is aberrant signaling of insulin, insulin-like growth factor (IGF)-1, and IGF binding proteins (IGFBP), as well as adipokine (i.e. leptin and adiponectin) signaling.[9-11] These signaling molecules have previously been associated with CRC risk in adults,[12-15] but it is not clear whether aberrant insulin, IGF-1, or adipokine signaling early in life are associated with adult risk of CRC. Nevertheless, it has been proposed that early-life alterations of signaling molecules might persist until adulthood due to an accumulation effect over the years, by epigenetic changes, or by alterations in the gut microbiota.[16-20]

A common downstream effect of insulin, IGF-1, and leptin is the activation of the PI3K/Akt-signaling pathway, whereas adiponectin counteracts activation of this pathway.[21] Besides its well-known oncogenic effects, activation of the PI3K/Akt-signaling pathway has been shown to induce aerobic glycolysis by upregulation of several transporter proteins and (glycolytic) enzymes.[22,23] This metabolic phenotype is often referred to as the "Warburg-effect", named after its discovery by Otto Warburg and colleagues.[24] It has been suggested that the Warburg-effect is a cause rather than an effect of cancer,[25] which is supported by the addition of metabolic reprogramming as one of the emerging hallmarks of cancer.[26]

We have previously shown that adult BMI and clothing-size are associated with colon cancer expressing high levels of proteins involved in the Warburg-effect.[27] However, studies investigating whether the Warburg-effect might be involved in the long-term effects of early-life energy balance-related factors on CRC risk are currently lacking. We therefore aimed to investigate whether early-life energy balance-related factors were associated with risk of CRC tumors expressing different levels of proteins involved in 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 (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, and MCT4) were combined into a sum score, which was divided into three subgroups, representing tumors with a low, moderate, or high likelihood of the presence of the Warburg-effect, hereafter referred to as the Warburg-subtypes (Warburg-low, Warburg-moderate, Warburg-high, respectively).

We hypothesized that associations between early-life energy balance-related factors (height, energy restriction, and adolescent BMI) and adult risk of CRC differ across Warburg-subtypes.

2 | MATERIALS AND METHODS

2.1 | Design and study population

The Netherlands Cohort Study (NLCS) is a population-based prospective cohort study initiated in 1986, which included 120 852 subjects aged 55 to 69 years old.[28] At baseline, all participants filled in a mailed, self-administered questionnaire on cancer risk factors. A case-cohort design was used for data processing and analysis.[29] A subcohort ($n = 5000$) was randomly selected immediately after baseline, providing an estimate of accumulated person-years for the total cohort. Vital status information of subcohort members was checked by biennial active follow-up and by linkage with municipal population registries afterward. Only one male subcohort member was lost to follow-up. Incident cancer cases were drawn from the entire cohort via annual record linkage with the Netherlands Cancer Registry and PALGA, the nationwide Dutch Pathology Registry,[30] covering 20.3 years of follow-up (17 September 1986 until 1 January 2007). The completeness of cancer follow-up by the Netherlands Cancer Registry and PALGA was estimated to be over

96%.[31] A total of 4597 incident CRC and 4774 subcohort members were available after excluding prevalent cancer cases (except skin cancer) at baseline, as described previously.[27]

Formalin-fixed paraffin-embedded (FFPE) CRC tissue blocks from 3872 CRC cases were collected as part of the Rainbow-TMA project from 2012 to 2017.[32] CRC cases were selected based on 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 3021 CRC cases were successfully collected from 43 pathology laboratories throughout the Netherlands (78% retrieval rate).

For tissue microarray (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, Hungary). In total, 78 TMAs were constructed comprising 2694 CRC cases.

2.2 | Immunohistochemistry

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

H&E-stained TMA sections were reviewed, in combination with pan-cytokeratin stained sections if necessary, to confirm the presence of adenocarcinoma for each core. Requiring at least one core with adenocarcinoma per case, 2497 cases passed quality control. Scoring of IHC-stained TMAs was performed by three nonpathologists (G.E. Fazzi: senior histology technician; K. Offermans: PhD-student; J.C.A. Jenniskens: PhD-student; Table S3 shows the contribution of each assessor) after appropriate training, as described previously.[33] IHC scoring protocols for all markers are described in the Supporting Information Methods and shown in Figure S1. Kappa values on inter- and intra-observer scoring agreement are shown in Table S4. Complete IHC protein expression information for all six proteins was available for 2399 cases.

Establishment of Warburg-subtypes from core-level protein expression has previously been described in detail.[27] In short, (i) core-based scores from multiple assessors were combined into a combination score if the same score was assigned by at least two assessors; (ii) scoring discrepancies were resolved by consensus agreement of two nonpathologist assessors or by an experienced pathologist; (iii) final scores from all available cores per case were averaged and rounded to the nearest scoring category; (iv) the average scores per case were categorized as low, moderate, or high expression (Table S4 shows cut-offs per protein); (v) a pathway-based sum score (range: 0-12) was used to combine expression levels of all six proteins; (vi) Warburg-subtypes were established by taking tertiles from the sum score. Cases with sum scores 0 to 3 were classed as "Warburg-low" (n = 698, 29.1%), sum scores 4 to 5 as "Warburg-moderate" (n = 859, 35.8%), and sum scores 6 to 12 as "Warburg-high" (n = 842, 35.1%).

2.3 | Early-life energy balance-related factors

Proxy variables were used to assess exposure to energy restriction during childhood to adolescence, as previously described[16,34]: (i) place of residence during the Dutch Hunger Winter (1944-1945); (ii) place of residence in 1942, reflecting World War II (WWII; 1940-1944); and (iii) employment status of the father during the Dutch Economic Depression (1932-1940). Living in a city in the western part of the Netherlands during the Hunger Winter indicated severe energy restriction, with caloric intake of 400 to 800 kcal per day at the height of the famine.[35,36] Living in a Dutch city in 1942 (WWII) with more than 40 000 inhabitants was used as an indicator for energy restriction. Unemployment of the father during the Economic Depression was used as an indicator of lack of variation in the food pattern, though sufficient calories were available. Participants of the NLCS were 12 to 28 years old during the Hunger Winter, 8 to 28 years old during WWII, and 0 to 23 years old during the Economic Depression.

Height was self-reported at baseline (cm), adolescent BMI was calculated by using self-reported weight at age 20 years and height at baseline (kg/m^2).

2.4 | Cox regression models

After excluding participants with incomplete or inconsistent data on exposure variables or confounders, 3911 subcohort members and 1972 CRC cases were available for analyses. Cox proportional hazards models were used to estimate hazard ratios (HRs) and 95% confidence intervals (CIs) for the association between CRC and early-life energy restriction measures (place of residence during the Hunger Winter; place of residence during WWII; employment status of the father during the Economic Depression), height (according to sex-specific quartiles, and per 5 cm increase), and adolescent BMI (according to sex-specific quartiles, and per 5 kg/m^2 increase). All associations were investigated stratified on sex, tumor location, and Warburg subtypes. 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.[37] The proportional hazards assumption was tested using the scaled Schoenfeld residuals[38] and by introducing time-covariate interactions into the models.

All multivariable models were adjusted for age, family history of CRC (yes/no), alcohol intake (0; 0.1-4; 5-14; >15 g/day), energy intake at baseline (kcal/day), and nonoccupational physical activity (minutes/day). Models on early-life energy restriction and adult-attained height

TABLE 1 Characteristics [mean (SD) or %] of subcohort members and CRC cases; NLCS, 1986-2006

		Colon				Rectum			
	Subcohort	Total	Warburg-Low	Warburg-Moderate	Warburg-High	Total	Warburg-Low	Warburg-Moderate	Warburg-High
Men									
N[a]	1971	772	215	280	277	227	76	76	75
Age at baseline (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)
Height (cm)	176.6 (6.7)	177.3 (6.8)	177.1 (6.6)	177.5 (6.8)	177.3 (6.9)	176.9 (6.6)	177.7 (7.0)	176.4 (5.5)	176.7 (7.0)
Hunger Winter (living in Western city %)	21.4	16.3	16.1	18.3	14.4	15.9	15.8	15.8	16.0
WWII (living in city %)	49.7	46.5	49.7	47.9	42.4	45.6	40.3	46.6	50.0
Economic depression (father unemployed %)	10.8	10.6	13.3	9.6	9.7	10.5	10.8	9.3	11.4
Overweight at age 20 years (%)	7.6	8.5	7.8	8.5	9.1	6.0	4.4	5.9	7.9
Weight change since age 20 years (kg)	10.3 (9.3)	11.1 (9.5)	11.5 (9.3)	11.4 (9.7)	10.6 (9.6)	9.7 (9.2)	10.4 (8.9)	9.0 (8.8)	9.7 (10.0)
Women									
N[a]	1940	655	170	216	269	127	37	51	39
Age at baseline (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)
Height (cm)	165.3 (6.1)	166.0 (6.2)	166.5 (5.8)	165.8 (6.3)	165.8 (6.3)	166.5 (6.4)	166.9 (6.0)	166.8 (6.2)	165.8 (7.1)
Hunger Winter (living in Western city %)	27.8	24.6	22.3	23.7	26.8	25.0	22.2	31.4	18.9
WWII (living in city %)	52.3	49.1	42.5	50.6	52.4	51.1	56.7	46.3	52.2
Economic depression (father unemployed %)	11.3	11.8	9.3	8.8	15.7	9.8	5.7	10.0	13.2
Overweight at age 20 years (%)	7.3	7.9	5.1	6.2	11.3	7.8	5.9	13.0	2.9
Weight change since age 20 years (kg)	9.9 (9.9)	10.1 (10.4)	10.8 (10.7)	9.6 (10.3)	10.1 (10.2)	9.7 (10.0)	10.4 (9.7)	9.5 (10.9)	9.4 (9.4)

Abbreviations: SD, standard deviation; CRC, colorectal cancer; NLCS, Netherlands Cohort Study; WWII, World War II.

[a]Total number based on the most complete variable (height). Numbers of other variables might not add up to the same total because of missing values.

TABLE 2 Multivariable-adjusted HRs[a] and 95% CIs for associations between adult-attained height and CRC, stratified on sex, tumor location, and Warburg-subtypes; 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
Quartiles of height (cm)											
Men—colon											
<173	170	8935	196	1.00 (ref.)	55	1.00 (ref.)	69	1.00 (ref.)	72	1.00 (ref.)	
173-176	175	7680	191	1.19 (0.93-1.52)	52	1.14 (0.75-1.73)	71	1.24 (0.86-1.79)	68	1.18 (0.82-1.71)	
177-181	179	7097	178	1.20 (0.93-1.55)	53	1.29 (0.85-1.95)	66	1.23 (0.85-1.79)	59	1.11 (0.76-1.62)	
>181	185	7310	207	1.46 (1.14-1.87)	55	1.39 (0.92-2.09)	74	1.43 (1.00-2.05)	78	1.54 (1.07-2.22)	.978
P-trend				.004		.092		.064		.035	
Per 5 cm		31 022	772	1.12 (1.05-1.19)	215	1.09 (0.98-1.21)	280	1.13 (1.03-1.24)	277	1.13 (1.02-1.24)	.736
Men—rectum											
<173	170	8935	61	1.00 (ref.)	17	1.00 (ref.)	23	1.00 (ref.)	21	1.00 (ref.)	
173-176	175	7680	51	0.93 (0.62-1.40)	16	1.06 (0.53-2.13)	18	0.87 (0.45-1.68)	17	0.90 (0.46-1.75)	
177-181	179	7097	66	1.35 (0.92-1.98)	24	1.69 (0.88-3.22)	21	1.17 (0.63-2.19)	21	1.26 (0.65-2.42)	
>181	185	7310	49	0.97 (0.64-1.48)	19	1.30 (0.65-2.59)	14	0.76 (0.38-1.51)	16	0.94 (0.46-1.90)	.927
P-trend				.609		.232		.673		.881	
Per 5 cm		31 022	227	1.04 (0.93-1.15)	76	1.07 (0.90-1.28)	76	1.01 (0.87-1.18)	75	1.03 (0.85-1.25)	.749
Women—colon											
<162	158	8764	140	1.00 (ref.)	30	1.00 (ref.)	51	1.00 (ref.)	59	1.00 (ref.)	
162-165	164	9216	185	1.26 (0.97-1.63)	54	1.75 (1.09-2.81)	57	1.04 (0.70-1.55)	74	1.20 (0.83-1.74)	
166-169	168	7771	152	1.21 (0.92-1.59)	34	1.30 (0.78-2.19)	58	1.22 (0.81-1.83)	60	1.15 (0.77-1.71)	
>169	172	8477	178	1.34 (1.03-1.75)	52	1.92 (1.19-3.11)	50	0.97 (0.64-1.47)	76	1.38 (0.94-2.01)	.315
P-trend				.056		.033		.918		.137	
Per 5 cm		34 228	655	1.09 (1.01-1.17)	170	1.16 (1.02-1.32)	216	1.05 (0.93-1.18)	269	1.08 (0.96-1.20)	.649
Women—rectum											
<162	158	8764	28	1.00 (ref.)	8	1.00 (ref.)	9	1.00 (ref.)	11	1.00 (ref.)	
162-165	164	9216	23	0.78 (0.45-1.37)	7	0.86 (0.31-2.35)	7	0.77 (0.28-2.08)	9	0.73 (0.30-1.78)	
166-169	168	7771	38	1.52 (0.91-2.55)	11	1.60 (0.64-4.03)	21	2.70 (1.20-6.10)	6	0.60 (0.22-1.64)	
>169	172	8477	38	1.42 (0.84-2.41)	11	1.50 (0.60-3.78)	14	1.72 (0.70-4.23)	13	1.16 (0.49-2.74)	.935
P-trend				.047		.224		.037		.778	
Per 5 cm		34 228	127	1.16 (0.99-1.37)	37	1.21 (0.92-1.59)	51	1.22 (0.95-1.55)	39	1.08 (0.79-1.47)	.880

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

[a]Hazard Ratios were adjusted for age (years; continuous), total energy intake (kcal/day; continuous), family history of CRC (yes/no), alcohol consumption (0; 0.1-4; 5-14; >15 g/day), BMI at baseline (kg/m^2; continuous), nonoccupational physical activity (minutes/day; continuous), processed meat intake (g/day; continuous), red meat intake (g/day; continuous). Age was included as a time-varying covariate.

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

TABLE 3 Multivariable-adjusted HRs and 95% CIs for associations between early-life energy restriction and CRC, stratified on sex, tumor location, and Warburg-subtypes; NLCS, 1986-2006

	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)
Place of residence during the Dutch Hunger Winter (1944-1945)									
Men—colon	30 174	754		211		273		270	
Non-west	15 188	446	1.00 (ref.)	124	1.00 (ref.)	169	1.00 (ref.)	153	1.00 (ref.)
Western rural	4129	78	0.69 (0.51-0.92)	22	0.68 (0.42-1.11)	24	0.56 (0.35-0.89)	32	0.83 (0.55-1.25)
Western city	6524	123	0.63 (0.50-0.81)	34	0.62 (0.41-0.94)	50	0.69 (0.49-0.97)	39	0.58 (0.40-0.85)
P-heterogeneity									.606
Men—rectum	30 174	227		76		76		75	
Non-west	15 188	141	1.00 (ref.)	44	1.00 (ref.)	49	1.00 (ref.)	48	1.00 (ref.)
Western rural	4129	27	0.72 (0.46-1.12)	11	0.94 (0.47-1.88)	10	0.75 (0.37-1.51)	6	0.48 (0.20-1.13)
Western city	6524	36	0.60 (0.40-0.89)	12	0.64 (0.33-1.23)	12	0.57 (0.30-1.09)	12	0.60 (0.31-1.15)
P-heterogeneity									.983
Women—colon	33 722	646		166		215		265	
Non-west	18 083	373	1.00 (ref.)	108	1.00 (ref.)	125	1.00 (ref.)	140	1.00 (ref.)
Western rural	4851	81	0.81 (0.61-1.08)	16	0.57 (0.33-0.99)	27	0.79 (0.50-1.24)	38	1.02 (0.69-1.50)
Western city	9234	159	0.83 (0.67-1.03)	37	0.68 (0.46-1.00)	51	0.81 (0.57-1.14)	71	0.97 (0.71-1.32)
P-heterogeneity									.238
Women—rectum	33 722	124		36		51		37	
Non-west	18 083	74	1.00 (ref.)	23	1.00 (ref.)	25	1.00 (ref.)	26	1.00 (ref.)
Western rural	4851	11	0.59 (0.31-1.13)	4	0.71 (0.23-2.20)	5	0.73 (0.28-1.93)	2	0.32 (0.08-1.39)
Western city	9234	31	0.86 (0.55-1.34)	8	0.71 (0.31-1.62)	16	1.25 (0.66-2.36)	7	0.59 (0.24-1.44)
P-heterogeneity									.803
Place of residence during World War II (1942)									
Men—colon	23 793	572		167		209		196	
Rural area	11 327	287	1.00 (ref.)	78	1.00 (ref.)	104	1.00 (ref.)	105	1.00 (ref.)
Urban area	11 713	266	0.90 (0.73-1.11)	83	1.00 (0.71-1.41)	100	0.96 (0.71-1.30)	83	0.77 (0.56-1.05)
P-heterogeneity									.614
Men—rectum	23 973	182		62		58		62	
Rural area	11 327	95	1.00 (ref.)	37	1.00 (ref.)	29	1.00 (ref.)	29	1.00 (ref.)
Urban area	11 713	83	0.88 (0.63-1.22)	25	0.65 (0.37-1.11)	27	0.95 (0.54-1.66)	31	1.13 (0.66-1.96)
P-heterogeneity									.381
Women—colon	26 164	505		139		158		208	
Rural area	11 882	243	1.00 (ref.)	78	1.00 (ref.)	74	1.00 (ref.)	91	1.00 (ref.)
Urban area	13 562	248	0.90 (0.73-1.11)	59	0.66 (0.46-0.94)	80	0.97 (0.68-1.37)	109	1.05 (0.78-1.43)
P-heterogeneity									.111
Women—rectum	26 164	94		30		41		23	
Rural area	11 882	42	1.00 (ref.)	13	1.00 (ref.)	19	1.00 (ref.)	10	1.00 (ref.)
Urban area	13 562	48	1.02 (0.66-1.58)	17	1.20 (0.57-2.54)	19	0.87 (0.45-1.65)	12	1.17 (0.47-2.93)
P-heterogeneity									.592
Employment of the father during the Dutch Economic Depression (1932-1940)									
Men—colon	29 841	743		203		271		269	
Employed	26 697	664	1.00 (ref.)	176	1.00 (ref.)	245	1.00 (ref.)	243	1.00 (ref.)

(Continues)

TABLE 3 (Continued)

	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)
Unemployed	3145	79	0.95 (0.71-1.26)	27	1.23 (0.79-1.91)	26	0.85 (0.55-1.33)	26	0.84 (0.54-1.30)
P-heterogeneity									.214
Men—rectum	29 841	219		74		75		70	
Employed	26 697	196	1.00 (ref.)	66	1.00 (ref.)	68	1.00 (ref.)	62	1.00 (ref.)
Unemployed	3145	23	0.99 (0.63-1.57)	8	1.01 (0.47-2.15)	7	0.86 (0.40-1.89)	8	1.09 (0.51-2.34)
P-heterogeneity									.756
Women—colon	32 597	627		161		204		262	
Employed	29 046	553	1.00 (ref.)	146	1.00 (ref.)	186	1.00 (ref.)	221	1.00 (ref.)
Unemployed	3552	74	1.09 (0.81-1.47)	15	0.85 (0.48-1.49)	18	0.79 (0.47-1.32)	41	1.51 (1.03-2.20)
P-heterogeneity									.872
Women—rectum	32 597	123		35		50		38	
Employed	29 046	111	1.00 (ref.)	33	1.00 (ref.)	45	1.00 (ref.)	33	1.00 (ref.)
Unemployed	3552	12	0.85 (0.46-1.57)	2	0.41 (0.09-1.80)	5	0.88 (0.35-2.21)	5	1.29 (0.51-3.27)
P-heterogeneity									.936

Note: Hazard Ratios were adjusted for age (years; continuous), total energy intake (kcal/day; continuous), family history of CRC (yes/no), alcohol consumption (0; 0.1-4; 5-14; >15 g/day), BMI at baseline (kg/m^2; continuous), nonoccupational physical activity (minutes/day; continuous), processed meat intake (g/day; continuous), red meat intake (g/day; continuous). Age was included as a time-varying covariate.
Abbreviations: HR, hazard ratio; CI, confidence interval; CRC, colorectal cancer; NLCS, Netherlands Cohort Study; *P*-het, *P*-heterogeneity.

were additionally adjusted for BMI at baseline (kg/m^2). Models on adolescent BMI were additionally adjusted for height (cm). Potential additional confounders were weight change since adolescence (kg), 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.[39,40] In sensitivity analyses, we used two instead of three Warburg-subtypes (Warburg-low: sum score 0-4; Warburg-high: sum score 5-12) to increase power. Since our analyses were hypothesis-driven and exposures reflect different aspects of (early-life) energy balance, we did not correct for multiple testing. All analyses were conducted in Stata Statistical Software: Release 16 (StataCorp., College Station, TX).

3 | RESULTS

Descriptive results on early-life energy balance-related factors are shown in Table 1, stratified on sex, tumor location, and Warburg subtypes. First, cases were taller compared to subcohort members. Cases with a Warburg-low tumor were generally tallest, except for male colon cancer cases, which showed similar mean height across Warburg subtypes. Second, cases were generally less often exposed to early-life energy restriction compared to subcohort members. This was especially the case for male colon cancer cases with Warburg-high tumors, and for female colon cancer cases with Warburg-low tumors. Third, cases were more often overweight at age 20 years compared to subcohort members, except for male rectal cancer cases. This was especially the case for Warburg-high tumors, except for rectal cancer in women, where Warburg-moderate tumors showed the highest percentage of overweight cases.

Multivariable-adjusted Cox regression models on early-life energy balance-related factors are shown in Tables 2 to 4. Age-adjusted Cox regression models are shown in Tables S5 to S7. All models were stratified on sex, tumor location, and Warburg-subtypes, and included age as a time-varying covariate because of violation of the proportional hazards assumption.

3.1 | Adult-attained height

In men, height was positively associated with risk of colon cancer [$HR_{5\ cm}$ (95% CI): 1.12 (1.05-1.19); $P\text{-trend}_{quartiles} = .004$], but not rectal cancer (Table 2). After stratification, similar associations were found across all Warburg subtypes.

In women, height was positively associated with risk of both colon [$HR_{5\ cm}$ (95% CI): 1.09 (1.01-1.17); $P\text{-trend}_{quartiles} = .056$] and rectal cancer [$HR_{5\ cm}$ (95% CI): 1.16 (0.99-1.37); $P\text{-trend}_{quartiles} = .047$] (Table 2). After stratification on Warburg-subtypes, height was positively associated with Warburg-low colon cancer [$HR_{5\ cm}$ (95% CI): 1.16 (1.02-1.32); $P\text{-trend}_{quartiles} = .033$]. For rectal cancer, positive

TABLE 4 Age-adjusted HRs[a] and 95% CIs for associations between adolescent BMI (age 20 years) and CRC, stratified on sex, tumor location, and Warburg-subtypes; 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
Quartiles BMI at age 20 years (kg/m²)											
Men—colon											
<20.2	19.2	6103	159	1.00 (ref.)	48	1.00 (ref.)	61	1.00 (ref.)	50	1.00 (ref.)	
20.2-21.6	21.0	6458	149	0.91 (0.69-1.20)	48	0.97 (0.63-1.50)	47	0.74 (0.49-1.12)	54	1.05 (0.69-1.59)	
21.7-23.3	22.4	6308	157	1.01 (0.77-1.34)	41	0.88 (0.55-1.39)	62	1.06 (0.71-1.57)	54	1.09 (0.72-1.67)	
>23.3	24.3	6012	181	1.26 (0.96-1.66)	42	0.95 (0.60-1.51)	65	1.21 (0.82-1.79)	74	1.62 (1.08-2.43)	0.301
P-trend				.069		.734		.169		.022	
Per 5 kg/m²		24 881	646	1.06 (0.87-1.29)	179	0.93 (0.67-1.30)	235	1.03 (0.77-1.40)	232	1.19 (0.89-1.59)	0.400
Men—rectum											
<20.2	19.2	6103	40	1.00 (ref.)	15	1.00 (ref.)	14	1.00 (ref.)	11	1.00 (ref.)	
20.2-21.6	21.0	6458	63	1.50 (0.98-2.30)	21	1.34 (0.67-2.68)	20	1.39 (0.69-2.78)	22	1.82 (0.85-3.90)	
21.7-23.3	22.4	6308	46	1.14 (0.71-1.82)	15	1.06 (0.49-2.26)	19	1.33 (0.62-2.87)	12	1.01 (0.43-2.36)	
>23.3	24.3	6012	50	1.29 (0.81-2.05)	17	1.32 (0.63-2.77)	15	1.07 (0.48-2.38)	18	1.51 (0.69-3.31)	0.890
P-trend				.573		.627		.921		.675	
Per 5 kg/m²		24 881	199	1.14 (0.87-1.51)	68	1.26 (0.83-1.89)	68	1.05 (0.65-1.69)	63	1.12 (0.69-1.82)	0.877
Women—colon											
<19.6	18.4	7795	148	1.00 (ref.)	39	1.00 (ref.)	52	1.00 (ref.)	57	1.00 (ref.)	
19.6-21.2	20.5	7731	152	1.05 (0.80-1.38)	41	1.09 (0.68-1.75)	45	0.87 (0.56-1.33)	66	1.21 (0.82-1.78)	
21.3-23.0	22.0	7964	151	1.03 (0.79-1.36)	43	1.16 (0.73-1.84)	55	1.04 (0.69-1.57)	53	0.94 (0.63-1.41)	
>23.0	24.2	7683	141	1.06 (0.80-1.40)	34	1.01 (0.62-1.66)	43	0.87 (0.56-1.35)	64	1.26 (0.85-1.87)	0.577
P-trend				.752		.880		.744		.490	
Per 5 kg/m²		31 173	592	1.07 (0.91-1.26)	157	1.02 (0.79-1.33)	195	0.95 (0.75-1.21)	240	1.21 (0.95-1.53)	0.319
Women—rectum											
<19.6	18.4	7795	22	1.00 (ref.)	8	1.00 (ref.)	7	1.00 (ref.)	7	1.00 (ref.)	
19.6-21.2	20.5	7731	37	1.77 (1.01-3.12)	11	1.45 (0.55-3.83)	13	2.05 (0.78-5.37)	13	1.89 (0.73-4.86)	
21.3-23.0	22.0	7964	28	1.29 (0.72-2.33)	7	0.83 (0.29-2.36)	12	1.85 (0.71-4.87)	9	1.34 (0.48-3.72)	
>23.0	24.2	7683	28	1.39 (0.76-2.54)	8	1.01 (0.37-2.78)	14	2.40 (0.91-6.30)	6	0.93 (0.30-2.91)	0.970
P-trend				.529		.720		.092		0.724	
Per 5 kg/m²		31 173	115	1.15 (0.86-1.53)	34	0.81 (0.46-1.42)	46	1.64 (1.12-2.39)	35	0.97 (0.60-1.58)	0.095

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

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

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

associations were found for the Warburg-low, although not statistically significant [$HR_{5\ cm}$ (95% CI): 1.21 (0.92-1.59); $P\text{-trend}_{quartiles} = .224$], and the Warburg-moderate subtype [$HR_{5\ cm}$ (95% CI): 1.22 (0.95-1.55); $P\text{-trend}_{quartiles} = 0.037$].

3.2 | Proxies for early life energy restriction

Living in the western part of the Netherlands during the Hunger Winter (1944-1945) was associated with a decreased risk of both colon and rectal cancer in men, compared to participants living in a non-western part [colon: $HR_{western\ rural}$ (95% CI): 0.69 (0.51-0.92); $HR_{western\ city}$: 0.63 (0.50-0.81); rectum: $HR_{western\ rural}$: 0.72 (0.46-1.12); $HR_{western\ city}$: 0.60 (0.40-0.89)] (Table 3). After stratification, similar associations were found for all Warburg subtypes. Place of residence during WWII or employment status of the father during the Economic Depression were not associated with adult colon or rectal cancer risk in men (Table 3).

In women, none of the proxy variables for early-life energy restriction showed statistically significant associations with overall colon or rectal cancer risk (Table 3). However, after stratification on Warburg-subtypes, living in the western part of the Netherlands during the Hunger Winter was associated with a lower risk of Warburg-low colon cancer [$HR_{western\ rural}$ (95% CI): 0.57 (0.33-0.99); $HR_{western\ city}$: 0.68 (0.46-1.00)]. Similarly, living in an urban area during WWII was inversely associated with risk of Warburg-low colon cancer [$HR_{urban\ area}$ (95% CI): 0.66 (0.46-0.94)]. In contrast, unemployment of the father during the Economic Depression was associated with a higher risk of Warburg-high colon cancer [HR (95% CI): 1.51 (1.03-2.20)]. For rectal cancer, no clear associations were found after stratification on Warburg-subtypes.

3.3 | Adolescent BMI

A high adolescent BMI was nonsignificantly associated with an increased risk of overall colon cancer in men [highest compared to lowest quartile: HR (95% CI): 1.26 (0.96-1.66); $P\text{-trend}_{quartiles} = .069$] (Table 4). After stratification on Warburg-subtypes, this association was stronger for the Warburg-high subtype [highest compared to lowest quartile: HR (95% CI): 1.62 (1.08-2.43); $P\text{-trend}_{quartiles} = .022$], whereas no associations were found for Warburg-low and Warburg-moderate subtypes. Adolescent BMI was not associated with rectal cancer risk in men.

In women, adolescent BMI was not associated with either colon or rectal cancer risk (Table 4). After stratification on Warburg-subtypes, a positive association was found with risk of Warburg-moderate rectal cancer [$HR_{5\ kg/m^2}$ (95% CI): 1.64 (1.12-2.39); $P\text{-trend}_{quartiles} = .092$].

3.4 | Tests for heterogeneity

Heterogeneity tests did not show any statistically significant differences between Warburg subtypes for any of the associations.

3.5 | Sensitivity analyses

The use of two instead of three Warburg subtypes generally led to similar conclusions. Associations that were found for the Warburg-moderate subtype when using three Warburg-subtypes resulted in similar associations for Warburg-low and Warburg-high subtypes when two Warburg-subtypes were used (data not shown).

4 | DISCUSSION

In this large prospective cohort study, we investigated the associations of early-life energy balance-related factors with adult risk of Warburg-subtypes in CRC using a molecular pathological epidemiology approach. Height was positively associated with an increased risk of colon cancer in men, irrespective of Warburg-subtypes. In women, height was positively associated with risk of Warburg-low colon cancer, and Warburg-low and Warburg-moderate rectal cancer. Place of residence during the Dutch Hunger Winter, as a proxy for energy restriction, was inversely associated with colon cancer risk in men, regardless of Warburg-subtypes. None of the other energy restriction proxies were associated with colon or rectal cancer risk in men. In women, energy restriction during the Hunger Winter or during WWII showed an inverse association with Warburg-low colon cancer. In contrast, unemployment of the father during the Dutch Economic Depression was positively associated with risk of Warburg-high colon cancer in women. Adolescent BMI was associated with an increased risk of Warburg-high colon cancer in men, and Warburg-moderate rectal cancer in women. None of the heterogeneity tests showed statistically significant differences between Warburg subtypes.

Studies investigating potential etiological differences across CRC cases expressing different levels of the Warburg-effect in the tumor are currently lacking. However, proposed precursors (i.e. insulin, IGF-1, leptin, and adiponectin) of the Warburg-effect have been investigated in relation to CRC risk,[12-15] reporting positive associations for leptin, insulin, and IGF-1, and inverse associations for adiponectin. However, these associations were studied in adults. It has been proposed that early-life alterations in these signaling molecules, for example by exposure to energy balance-related factors, can have long-term detrimental effects.[16-18,41] We were the first, to the best of our knowledge, to investigate whether early-life energy-balance-related factors are associated with CRC subtypes expressing different levels of proteins involved in the Warburg-effect. Even though heterogeneity tests were not statistically significant, we will discuss observed differences between Warburg-subtypes.

4.1 | Height

The current results do not suggest a role of the Warburg-effect in the link between height and CRC. In men, similar associations with height were found for all Warburg-subtypes of colon cancer, suggesting that a pathway other than the Warburg-effect dominates this risk-enhancement. In

women, height was associated with Warburg-low colon cancer and Warburg-moderate rectal cancer, suggesting that the Warburg-effect does not play a role, at least not to a great extent, in women either.

Height is often not considered to be the cause of cancer itself, but rather a marker for various dietary and lifestyle exposures early in life, from conception until puberty, that affect growth.[42] The various early-life exposures associated with height might affect adult CRC risk via different pathways. Apart from the pathway under investigation in the current study, involving IGF-1, insulin, leptin, and/or adiponectin signaling, it has been suggested that increased cancer incidence in tall people might be related to the higher number of cells and cell divisions.[9,43] In particular, it has been suggested that height is associated with intestinal length,[44] potentially resulting in a higher risk of cellular alterations leading to malignancies.[1] Further research is needed to decipher which mechanisms might be involved in the link between height and CRC risk.

4.2 | Energy restriction

In men, exposure to the Dutch Hunger Winter, which was used as an indicator of severe energy restriction early in life, was associated with a decreased risk of colon and rectal cancer, irrespective of Warburg subtypes. In women, exposure to the Dutch Hunger Winter as well as during WWII was inversely associated with Warburg-low colon cancer. This might suggest that energy restriction hinders oncogenic mechanisms other than the Warburg-effect. In contrast, exposure to unemployment of the father during the Dutch Economic Depression was associated with an increased risk of Warburg-high colon cancer in women, suggesting a role of the Warburg-effect in this risk-enhancement.

Previous studies on early-life energy restriction in relation to adult CRC risk show inconsistent results, reporting both inverse and positive associations.[3-7,45] The use of different proxies for early-life energy restriction entails variation in exposure, for example, timing, duration, or severity. This variation in exposure potentially explains the differential associations we observed. First, participants were older during the Hunger Winter and WWII (12-28 and 8-28 years, respectively) compared to the Economic Depression (0-23 years). It has previously been proposed that particularly exposure early in childhood increases risk of CRC.[7] Second, place of residence during the Hunger Winter and WWII are indicators of (severe) energy restriction, whereas unemployment of the father during the Economic Depression indicates a lack of dietary variety.[34] It has been proposed that lack of vitamins or important (micro)nutrients are associated with an increased risk of CRC.[6,7]

The potential involvement of the Warburg-effect in the positive association between energy restriction during the Economic Depression and colon cancer in women might be supported by a study of Elias et al,[18] which showed a long-term effect of early-life energy restriction on the IGF-axis in adult women, with increased plasma levels of IGF-1 and IGFBP-3. As mentioned, IGF-1 is considered a potential instigator of the Warburg-effect in tumor cells.[21-23] Other studies investigating the relationship between energy restriction and the Warburg-effect were performed in adults and entailed rather short-term effects.[10,46]

4.3 | Adolescent BMI

Our results suggest a role of the Warburg-effect in men for the association between high adolescent BMI and increased adult risk of CRC. This effect was only shown for the highest quartile, which included cases with a BMI >23.3 kg/m^2, and was thus the only one encompassing overweight cases according to standards of the World Health Organization, defining a BMI ≥25 kg/m^2 as overweight. The variation of adolescent BMI across cases from the NLCS was thus limited, which might explain the lack of significant associations for example for colon cancer in women, which showed a similar HR per 5 kg/m^2 as men. It would thus be interesting to replicate the current study in a population with more variation in adolescent BMI across individuals, as increased contrasts might give further insights.

In line with the current results, we have previously shown that adult BMI, reported at baseline (age range: 55-69), was associated with Warburg-moderate and Warburg-high colon cancer in men.[27] This suggests that the same pathway, involving the Warburg-effect, is involved in both the long-term and shorter-term effect of a high BMI on colon cancer risk in men.

The positive association we found for adolescent BMI and risk of Warburg-moderate rectal cancer in women suggests that the Warburg-effect is not involved, at least not to a great extent, in this relation. In our previous study on adult BMI,[27] a positive association was found for Warburg-moderate rectal cancer as well, but this association did not reach statistical significance. As proposed previously, a potential mechanism other than the Warburg-effect in the relationship between adiposity and CRC risk in women might be related to sex hormones,[27] though this was investigated in adult women instead of adolescents.[47] Further research is needed to investigate potential mechanisms involved in the link between (adolescent) BMI and rectal cancer in women.

4.4 | Strengths and limitations

A major strength of the current study is the large prospective cohort design with long follow-up (20.3 years). However, heterogeneity in associations based on sex and tumor location limited the number of cases in final statistical analyses. Furthermore, this potentially resulted in chance findings due to multiple testing. Our results should thus be interpreted with caution since validation of the current findings is needed.

Another important strength of the current study is the unique opportunity to investigate exposure to early-life energy restriction in relation to adult CRC risk in a large prospective cohort. Nevertheless, the use of proxy measures for energy restriction during the Dutch Hunger Winter, WWII and the Dutch Economic Depression might have resulted in some exposure misclassification, since individual data on dietary exposures during those times were not available. However,

a previous validation study among female subcohort members concluded that the proxy measure for energy restriction in the Hunger Winter was adequate.[48] In addition, since the timing of exposure to energy restriction might influence its association with cancer risk,[7,45] the wide age-span (0-28 years) of our early-life energy restriction proxies might have affected the current results. An additional limitation for the current study is the retrospectively obtained information on early-life energy-balance-related factors, which could introduce information bias, especially for adolescent weight.

A third important strength of the current study is the availability of tumor material for a large number of incident CRC cases. Still, assessment of protein expression by IHC on TMAs from FFPE tissue might potentially entail some concerns. First, the age of FFPE tissue blocks from CRC resections might be a concern for the reliability of IHC results, though a study by Grillo et al[49] showed that most antigens are well preserved and available for analysis after several decades. Furthermore, we used internal controls when possible as a quality control measure to ensure adequate staining of the tissue. Second, the usage of TMAs instead of full sections might have resulted in misclassification due to the subsampling of the tumor. However, we tried to minimize this problem by sampling the three cores from different regions to capture potential tumor heterogeneity. Third, IHC-scoring by nonpathologists might be conceived as a potential problem. However, we have previously shown that nonpathologists can generate reproducible IHC-scoring results, similar to those of a pathologist, provided they were sufficiently trained by an experienced pathologist.[33] Fourth, the semiquantitative assessment of protein expression entails a margin of error, potentially leading to outcome misclassification. To minimize this issue, we used multiple observers to assess protein expression levels based on IHC-stained tissue.

To enable replication of the current results, we used a relatively 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. 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, and lactate secretion), aiming to provide a comprehensive view of the Warburg-effect. Additional important considerations for the choice of the specific proteins included: (i) the availability and quality of scientific literature on the protein, preferably in relation to the Warburg-effect in CRC; and (ii) whether the protein was already an established marker in the routine diagnostic histopathology lab to ensure reliable IHC staining results. We have previously reported differences in prognosis, independent of well-known prognostic factors, for the current Warburg subtypes.[50]

5 | CONCLUSION

In this large prospective cohort study, we found different associations for early-life energy balance-related factors and CRC risk between Warburg-subtypes defined by expression levels of six key proteins related to the Warburg-effect. The Warburg-effect seems to be involved in the positive association between adolescent BMI and colon cancer risk in men. The Warburg-effect is probably involved neither in the association between height and colon or rectal cancer risk in men or women, nor in the association between adolescent BMI and rectal cancer risk in women. Results on early-life energy restriction proxies in relation to risk of Warburg-subtypes in CRC did not show clear patterns. Further research is needed to validate current results and investigate potential additional mechanisms.

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CONFLICT OF INTEREST

Heike I. Grabsch: Honorarium from Astra Zeneca and BMS for scientific advisory board activities not related to the current study. The other authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The anonymized data that are minimally required to replicate the outcomes of the study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The NLCS was approved by institutional review boards from Maastricht University and the Netherlands Organization for Applied Scientific Research. Participants agreed to be included in the cohort and follow-up by returning the questionnaire they completed.

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SUPPORTING INFORMATION

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