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RESEARCH ARTICLE

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Energy balance-related factors in childhood and adolescence and risk of colorectal cancer based on *KRAS*, *PIK3CA*, and *BRAF* mutations and MMR status

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Abstract

KRAS mutations ($KRAS_{mut}$), $PIK3CA_{mut}$, $BRAF_{mut}$, and deficient DNA mismatch repair (dMMR) have been associated with the Warburg effect. We previously reported differential associations between early-life energy balance-related factors (height, energy restriction, body mass index [BMI]) and colorectal cancer (CRC) subtypes based on the Warburg effect. We now investigated associations of early-life energy balance-related factors and the risk of CRC subgroups based on mutation and MMR status. Data from the Netherlands Cohort Study was used. $KRAS_{mut}$, $PIK3CA_{mut}$, $BRAF_{mut}$, and MMR status were available for 2349 CRC cases, and complete covariate data for 1934 cases and 3911 subcohort members. Multivariable-adjusted Cox regression was used to estimate associations of height, energy restriction proxies (exposure to Dutch Hunger Winter, Second World War, Economic Depression), and early adult BMI (age 20 years) with risk of CRC based on individual molecular features and combinations thereof (all-wild-type+MMR-proficient [pMMR]; any-mutation/dMMR). Height was positively associated with any-mutation/dMMR CRC but not all-wild-type+pMMR CRC, with the exception of rectal cancer in men, and with heterogeneity in associations observed for colon cancer in men (p-heterogeneity = 0.049) and rectal cancer in women (p-heterogeneity = 0.014). Results on early-life energy restriction proxies in relation to the risk of CRC subgroups did not show clear patterns. Early adult BMI was positively, but not significantly, associated with $KRAS_{mut}$ colon cancer in men and with $BRAF_{mut}$ and dMMR colon cancer in women. Our results suggest a role of $KRAS_{mut}$, $PIK3CA_{mut}$, $BRAF_{mut}$, and dMMR in the etiological pathway between height and CRC risk. $KRAS_{mut}$ might potentially play a role in associations of early adult BMI with colon cancer risk in men, and $BRAF_{mut}$ and dMMR in women.

KEYWORDS
colorectal cancer, early-life energy balance, mismatch repair/microsatellite instability, mutations, prospective cohort study

1 | INTRODUCTION

Energy balance-related factors in early life, that is, from childhood to adolescence, have been associated with colorectal cancer (CRC) risk later in life.[1–9] While there are reports that early-life energy restriction decreases CRC risk,[1–3] the opposite has been reported as well.[8,9] Increased adult-attained height, a surrogate measure of fetal and early-life (nutritional) exposures, and increased childhood or early adult body mass index (BMI) have been associated with increased CRC risk.[4,6,7] Mechanisms underlying these long-term effects of early-life energy balance-related factors are currently unknown. We have previously reported differential associations of early-life energy balance-related factors with CRC risk based on expression levels of proteins involved in the Warburg effect.[10]

It has been suggested that the Warburg effect, which is characterized by increased aerobic glycolysis,[11–13] may play an important role in carcinogenesis.[14,15] Mutations in oncogenes such as *KRAS*, *PIK3CA*, and *BRAF* as well as the presence of DNA mismatch repair (MMR) deficiency have been associated with the presence of the Warburg effect.[11,16–19] While mutations in *KRAS*, *PIK3CA*, and *BRAF* (*KRAS* mutations [$KRAS_{mut}$], $PIK3CA_{mut}$, $BRAF_{mut}$, respectively) and MMR deficiency (dMMR), a surrogate of microsatellite instability (MSI), are common molecular characteristics of CRC,[20–22] there are only a few studies investigating associations between early-life energy balance-related factors and risk of CRC in relation to these molecular features.[23–26]

We hypothesized that associations of early-life energy balance-related factors with CRC risk differ between subgroups based on molecular features ($KRAS_{mut}$, $PIK3CA_{mut}$, $BRAF_{mut}$, and dMMR). We investigated whether early-life energy balance-related factors were associated with CRC risk in relation to these molecular features individually as well as combined into an all-wild-type+pMMR subgroup (i.e., cases wild-type for all genes and MMR proficient [pMMR]) and any-mutation/dMMR subgroup (i.e., cases with a mutation in any of the genes and/or dMMR). We believe that combining these molecular features into subgroups has some advantages. First, mutations in *KRAS*, *PIK3CA*, and *BRAF* have all been associated with metabolic reprogramming toward the Warburg effect,[11,16–18] and MMR deficiency has previously been associated with the estimated presence of the Warburg effect.[19] Therefore, combining these molecular features, presumed to be involved in the same metabolic phenotype, results in a cleaner reference group compared to groups based on individual features (e.g., *KRAS*-mutated vs. wild-type). Second, using combination subgroups based on multiple molecular features results in increased statistical power, since most individual molecular features occur in <20% of CRC cases. The all-wild-type+pMMR subgroup was used as a reference group for all other subgroups. Associations with subgroups of individual molecular features and/or with the any-mutation/dMMR subgroup, but not with the all-wild-type+pMMR subgroup, were considered to indicate potential Warburg effect involvement in the etiological pathway between the energy balance-related factor and CRC.

2 | MATERIALS AND METHODS

2.1 | Design and study population

We used data from the large prospective Netherlands Cohort Study (NLCS), which included 120,852 subjects aged 55–69 years at baseline in 1986. All participants completed a mailed, self-administered baseline questionnaire on cancer risk factors.[27] By completing and returning the questionnaire, participants agreed to participate in the study. The NLCS was approved by institutional review boards from Maastricht University and the Netherlands Organization for Applied Scientific Research. Ethical approval was obtained from the Medical Ethical Committee of Maastricht University Medical Center+. The NLCS uses a case-cohort approach for data processing and analysis.[28] Immediately after baseline, 5000 participants were randomly sampled from the full cohort, and accumulated person-years were estimated from this subcohort. Information on the vital status of subcohort members was obtained by biennial active follow-up and by linkage with municipal population registries. Through annual record linkage with the Netherlands Cancer Registry and PALGA, the nationwide Dutch Pathology Registry,[29] incident cancer cases from the full cohort were identified. The completeness of cancer follow-up by the Netherlands Cancer Registry and PALGA was estimated to be over 96%.[30] For the current study, follow-up covered 20.3 years (September 17, 1986, until January 1, 2007). After excluding cases and subcohort members who reported a history of cancer (except skin cancer) at baseline, a total of 4597 incident CRC cases and 4774 subcohort members were available (Supporting Information: Figure S1). As described previously,[31] formalin-fixed paraffin-embedded (FFPE) tissue blocks from the primary tumor and matched normal colon tissue from 3872 CRC cases were requested from participating laboratories as part of the Rainbow-TMA project during 2012–2017.[27] Tissue blocks from 3021 CRC cases were collected from 43 Dutch pathology laboratories (78% retrieval rate) (Supporting Information: Figure S1) and used to extract tumor DNA.

2.2 | Tissue microarray construction and immunohistochemistry

Three 0.6 mm cores were sampled from FFPE blocks of 2694 CRC cases and combined into 78 tissue microarray (TMA) blocks

(Supporting Information: Figure S1), as described previously.[19] From these TMA blocks, 5 µm-thick-sections were cut and stained with Hematoxylin & Eosin (H&E) according to a standard protocol or subjected to immunohistochemistry (IHC) using an automated immunostainer (DAKO Autostainer Link 48). MMR status, as an indicator of the presence or absence of MSI, was assessed using IHC staining for MLH1 and MSH2 as described previously.[19] All TMA sections were scanned using an Aperio scanner (Leica Microsystems) at x40 magnification at the University of Leeds (UK) Scanning Facility or at the Department of Pathology, Aachen University Hospital (Germany).

For quality control, H&E-stained TMA sections combined with pan-cytokeratin stained sections, if necessary, were reviewed to confirm the presence of adenocarcinoma for each core. Requiring at least one core per case, 2497 cases passed quality control (Supporting Information: Figure S1). IHC-stained TMAs for MLH1 and MSH2 were scored according to the protocol published by Richman et al.[32] by three nonpathologists (GF: senior histology technician; KO: PhD student; JJ: PhD student) after appropriate training,[33] as well as by an experienced histopathologist. Tumors with complete loss of either MLH1 or MSH2 expression were classified as dMMR, and those expressing both MLH1 and MSH2 were classified as pMMR. MMR status information was available for 2455 CRC cases (Supporting Information: Figure S1).

2.3 | DNA isolation and mutation detection

Two 20 µm-thick-sections were cut from primary tumor containing FFPE blocks for DNA extraction. Sections were deparaffinized manually with Buffer ATL (Cat. No. 939011; Qiagen), Proteinase K (Cat. No. 19131; Qiagen), and Deparaffinization Solution (Cat. No. 19093; Qiagen), using an adapted version of the manufacturer's protocol (Supporting Information: Methods). For DNA isolation, the QIAsymphony® DSP DNA Mini Kit (Cat. No. 937236; Qiagen) and the QIAsymphony® (Qiagen) instrument were used according to the manufacturer's protocol (Tissue_HC_200 protocol). The Quantus™ Fluorometer (Promega) with a QuantiFluor® dsDNA system (Promega) was used to determine double-stranded DNA concentrations. Mutations in tumor DNA were analyzed at the Institut für Immunologie und Genetik. The ColoCarta panel (Agena Bioscience) was used to screen for more than 32 mutations in six genes (*BRAF*, *HRAS*, *KRAS*, *MET*, *NRAS*, *PIK3CA*; see Supporting Information: -Table S1 for specific mutations), using Matrix Assisted Laser Desorption Ionization-Time of Flight mass spectrometry (cut-offs: *Z*-score ≥ 4.00; spectrum quality ≥ 0.750; typer peak probability ≥ 0.850; primer extension rate cut-off ≥ 0.200). A mutation frequency of ≥7.5% for any of the alleles was considered evidence of a mutation in the corresponding gene. A failed reaction at a single nucleotide position resulted in missing data for the corresponding gene status only if the reactions at all other positions were wild-type. Information on *KRAS*, *PIK3CA*, and *BRAF* mutation status were complete for 2349 CRC cases (Supporting Information: Figure S1).

The following subgroups were used for statistical analyses: (I) all-wild-type+pMMR—cases wild-type for all genes (*KRAS*, *BRAF*, and *PIK3CA*) and MMR-proficient; (II) any-mutation/dMMR—cases with a mutation in any of the genes (*KRAS*, *BRAF*, and *PIK3CA*) and/or dMMR; (III) $KRAS_{mut}$—cases with a (nonexclusive) *KRAS* mutation; (IV) $BRAF_{mut}$; (V) $PIK3CA_{mut}$; (VI) dMMR. Note: subgroups based on individual mutations and MMR status can overlap since multiple mutations and/or MMR deficiency can occur within the same tumor. Frequencies of molecular features and of co-mutations within this cohort have been published previously.[34]

2.4 | Energy balance-related factors

Three proxy variables were used to assess exposure to energy restriction during childhood, as described in more detail previously[35,36]: (I) place of residence during the Dutch Hunger Winter (1944–1945): living in a city in the western part of the Netherlands during the Hunger Winter indicated severe energy restriction, with a caloric intake of 400–800 kcal/day at the height of the famine[37,38]; (II) place of residence in 1942, reflecting World War II (WWII; 1940–1944): living in a Dutch city in 1942 with more than 40,000 inhabitants was used as an indicator for energy restriction; (III) employment status of the father during the Dutch Economic Depression (1932–1940): unemployment of the father during the Economic Depression indicated a lack of variation in the food pattern, though sufficient calories were available. Participants of the NLCS were 12–28-year-old during the Hunger Winter, 8–28-year-old during WWII, and 0–23-year-old during the Economic Depression. Height was self-reported at baseline (cm), and early adult BMI (age 20 years) was calculated by using a self-reported weight at age 20 years and height at baseline (kg/m^2).

2.5 | Cox regression models

After excluding participants with incomplete or inconsistent data on exposure variables or confounders, 3911 subcohort members and 1934 CRC cases were available for analyses (Supporting Information: Figure S1). Associations between early-life energy balance-related factors and CRC subgroups based on molecular features were investigated stratified on sex and tumor location. Cox proportional hazard models were used to estimate Hazard ratios (HRs) and 95% confidence intervals (CIs) for the associations between subgroups of CRC and early-life energy restriction proxies (place of residence during the Hunger Winter; place of residence during WWII; the employment status of the father during the Economic Depression), height (according to sex-specific quartiles, and per 5 cm increase), and early adult BMI (according to sex-specific quartiles, and per 5 kg/m^2 increase). Standard errors of the HRs were estimated using the Huber–White sandwich estimator to account for additional variance introduced by sampling from the cohort.[39] The proportional hazard assumption was tested using the scaled

Schoenfeld residuals[40] and by introducing time-covariate interactions into the models. Cases with rectosigmoid cancer were excluded from analyses.

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), non-occupational physical activity (min/day), red meat consumption (g/day), and processed meat consumption (g/day). Models on energy restriction and adult-attained height were additionally adjusted for BMI at baseline (kg/m^2). Models on adolescent BMI were additionally adjusted for height (cm). These confounders were based on previous literature in the field.[5–7]

Heterogeneity in associations between early-life energy balance-related factors and CRC subgroups based on molecular features was evaluated using an adapted version of the competing risks procedure in Stata developed for the case-cohort design, as described previously.[41,42] All subgroups were compared pairwise with the all-wild-type+pMMR subgroup, which was the reference group for heterogeneity tests of all subgroups.

All analyses were conducted in Stata Statistical Software: Release 15 (StataCorp., 2017).

3 | RESULTS

3.1 | Cohort characteristics in subgroups based on molecular features

Information on early-life energy balance-related factors of CRC cases, overall and according to subgroups based on molecular features, are shown in Table 1. Both men and women in the any-mutation/dMMR subgroup were taller compared to those in the all-wild-type+pMMR subgroup, with the exception of men with rectal cancer. Among male colon cancer cases, those with a *BRAF*$_{mut}$ or dMMR tumor were the tallest, whereas no clear difference was observed among female colon cancer cases. In general, cases in the any-mutation/dMMR subgroup were more often exposed to energy restriction early in life, with the exception of men with colon cancer, whereas cases in the all-wild-type+pMMR subgroup were more often exposed to energy restriction. Cases in the any-mutation/dMMR subgroup were more often overweight at age 20 years, with the exception of men with rectal cancer. Among male colon cancer cases, those with a *KRAS*$_{mut}$ or *PIK3CA*$_{mut}$ tumor were most frequently overweight at the age of 20 years, whereas among female colon cancer cases this seemed to be the case for *PIK3CA*$_{mut}$ and dMMR tumors.

3.2 | Cox regression analyses

Multivariable-adjusted Cox regression models on early-life energy balance-related factors and risk of CRC in subgroups based on mutation and MMR status are shown in Tables 2–5. Age-adjusted Cox regression models are shown in Supporting Information: Tables S2–S5. Age was included as a time-varying covariate in all models, because of violation of the proportional hazards assumption.

3.2.1 | Adult-attained height

Results on associations between adult-attained height and risk of CRC in subgroups based on molecular features, by tumor location and sex, are shown in Table 2. In men, height was positively associated with the risk of overall colon cancer, and especially with the any-mutation/dMMR subgroup [HR$_{5\,cm}$ (95% CI): 1.17 (1.08–1.27), p-trend$_{quartiles}$: 0.001]. Although in men positive associations were observed between height and all subgroups of individual molecular features in colon cancer, associations were strongest for *BRAF*$_{mut}$ [HR$_{5\,cm}$ (95% CI): 1.23 (1.06–1.43), p-trend$_{quartiles}$: 0.025] and dMMR colon cancer [HR$_{5cm}$ (95% CI): 1.24 (1.03–1.48), p-trend$_{quartiles}$: 0.057]. Compared to the all-wild-type+pMMR subgroup, statistically significant heterogeneity was observed for any-mutation/dMMR (p-heterogeneity = 0.049) and *BRAF*$_{mut}$ (p-heterogeneity = 0.049) colon cancer in men. No associations were observed between height and overall rectal cancer in men, and stratification in subgroups of combinations of molecular features did not lead to clear associations. It seems, however, that height was positively associated with *KRAS*$_{mut}$ rectal cancer in men, but this association did not reach statistical significance. In women, borderline significant and significant positive associations were observed for height and risk of overall colon and rectal cancer, respectively. These positive associations were observed for the any-mutation/dMMR subgroups [colon: HR$_{5cm}$ (95% CI): 1.09 (0.99–1.19), p-trend$_{quartiles}$: 0.069; rectum: 1.38 (1.12–1.70), p-trend$_{quartiles}$: 0.010], but not for the all-wild-type +pMMR subgroups. For rectal cancer, this positive association was observed for the *KRAS*$_{mut}$ subgroup as well [HR$_{5\,cm}$: 1.40 (1.11–1.77), p-trend$_{quartiles}$: 0.012]. For colon cancer, positive associations were observed for all subgroups of individual molecular features, but none reached statistical significance. For rectal cancer in women, statistically significant heterogeneity was observed for any-mutation/dMMR (p-heterogeneity = 0.014) and *KRAS*$_{mut}$ (p-heterogeneity = 0.017) subgroups compared to the all-wild-type+pMMR subgroup (Table 2).

3.2.2 | Proxies for early-life energy restriction

Results on associations between energy restriction proxies and risk of CRC in subgroups based on molecular features, by tumor location and sex, are shown in Tables 3 and 4. Living in a western part of the Netherlands during the Dutch Hunger Winter (1944–1945) was associated with a decreased risk of overall colon and rectal cancer in men (Table 3). For colon cancer, inverse associations were observed for both the any-mutation/dMMR subgroup [HR$_{western\ rural}$ (95% CI): 0.70 (0.49–0.99); HR$_{western\ city}$: 0.59 (0.43–0.80)] and the all-wild-type+pMMR subgroup [HR$_{western\ rural}$ (95% CI): 0.63 (0.41–0.97); HR$_{western\ city}$: 0.71 (0.51–1.00)]. Although statistically significant

TABLE 1 Characteristics (mean [SD] or %) of CRC cases in subgroups based on mutation and MMR status, by sex and tumor location; NLCS, 1986–2006

	Total	Wild type + pMMR[c]	Any-mutation/ dMMR[d]	*KRAS*$_{mut}$	*PIK3CA*$_{mut}$[e]	*BRAF*$_{mut}$[e]	dMMR[e]
Men—Colon							
N[a]	754	309	445	256	150	105	70
Age (years)	61.6 (4.2)	61.2 (4.2)	61.9 (4.2)	62.0 (4.2)	61.5 (4.3)	62.2 (4.1)	62.7 (4.1)
Height (cm)	177.4 (6.8)	176.8 (6.4)	177.8 (7.1)	177.3 (6.9)	177.3 (7.3)	178.3 (7.1)	178.2 (7.3)
Hunger Winter (living in Western city, %)	16.6	18.7	15.1	15.9	16.2	11.7	9.0
WWII (living in city %)	46.1	50.7	42.9	42.9	40.5	45.1	34.8
Economic depression (father unemployed, %)	10.9	12.8	9.6	10.9	8.3	7.1	7.6
Overweight[b] at age 20 years (%)	8.5	7.7	9.0	10.3	9.7	6.0	6.8
Weight change since age 20 years (kg)	11.4 (9.7)	11.4 (10.1)	11.4 (9.4)	11.0 (9.5)	12.0 (9.8)	11.3 (9.6)	12.5 (10.6)
Men—Rectum							
N[a]	224	135	89	68	30	8	1
Age (years)	60.8 (3.9)	60.4 (4.0)	61.4 (3.9)	61.7 (3.9)			
Height (cm)	177.0 (6.7)	177.0 (6.5)	177.0 (6.9)	178.0 (6.4)			
Hunger Winter (living in Western city, %)	16.1	14.8	18.0	14.7			
WWII (living in city, %)	46.7	45.4	48.7	45.6			
Economic depression (father unemployed, %)	10.2	11.7	8.0	7.4			
Overweight[b] at age 20 years (%)	5.6	5.9	5.2	5.1			
Weight change since age 20 years (kg)	9.7 (9.3)	9.3 (8.9)	10.5 (9.9)	10.8 (10.0)			
Women—Colon							
N[a]	630	179	451	222	116	173	131
Age (years)	62.0 (4.1)	61.1 (3.9)	62.3 (4.1)	62.2 (4.1)	62.2 (4.2)	62.6 (4.1)	62.3 (4.0)
Height (cm)	165.9 (6.2)	165.7 (5.9)	166.0 (6.3)	166.0 (6.8)	165.7 (7.1)	165.9 (5.9)	165.8 (5.8)
Hunger Winter (living in Western city, %)	24.4	20.7	25.8	24.4	24.4	28.2	28.7
WWII (living in city, %)	48.2	47.6	48.5	46.3	53.3	48.6	52.8
Economic depression (father unemployed, %)	11.8	12.1	11.6	14.2	17.3	10.2	11.1
Overweight[b] at age 20 years (%)	7.7	4.4	9.0	10.8	11.4	9.0	11.5
Weight change since age 20 years (kg)	10.1 (10.3)	10.5 (10.4)	9.9 (10.3)	11.0 (10.5)	9.4 (10.5)	9.1 (10.0)	8.3 (11.2)
Women—Rectum							
N[a]	131	64	67	55	15	6	2
Age (years)	61.5 (4.2)	60.9 (4.3)	62.0 (4.0)	61.7 (4.0)			
Height (cm)	166.5 (6.5)	165.3 (6.9)	167.6 (5.9)	167.8 (6.2)			

(Continues)

TABLE 1 (Continued)

	Total	Wild type + pMMR[c]	Any-mutation/ dMMR[d]	$KRAS_{mut}$	$PIK3CA_{mut}$[e]	$BRAF_{mut}$[e]	dMMR[e]
Hunger Winter (living in Western city %)	25.0	23.4	26.6	22.6			
WWII (living in city, %)	50.0	42.9	57.5	54.1			
Economic depression (father unemployed, %)	9.5	4.8	13.9	14.8			
Overweight[b] at age 20 years (%)	7.6	6.8	8.3	8.0			
Weight change since age 20 years (kg)	9.5 (9.9)	9.5 (10.7)	9.5 (9.1)	10.1 (9.1)			

Abbreviations: CRC, colorectal cancer; dMMR, deficient mismatch repair; mut, mutated; NLCS, Netherlands Cohort Study; pMMR, proficient mismatch repair; SD, standard deviation; 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.

[b]Body mass index ≥25.

[c]This group excludes cases with mutations in any of the genes (*KRAS*, *BRAF*, or *PIK3CA*), as well as MMR-deficient cases.

[d]This group includes cases with mutations in any of the genes (*KRAS*, *BRAF*, or *PIK3CA*) and/or cases that are dMMR.

[e]Analyses for subgroups with <50 cases were not performed.

inverse associations were observed with all subgroups of individual molecular features in colon cancer, associations were strongest for $BRAF_{mut}$ [$HR_{western\ rural}$ (95% CI): 0.44 (0.20–0.98); $HR_{western\ city}$: 0.44 (0.23–0.84)] and dMMR [$HR_{western\ rural}$ (95% CI): 0.57 (0.24–1.35); $HR_{western\ city}$: 0.34 (0.14–0.83)] tumors (Table 4). For rectal cancer, inverse associations were observed for all subgroups of (combinations of) molecular features (Tables 3 and 4), but only the association with the all-wild-type+pMMR subgroup was statistically significant [$HR_{western\ rural}$ (95% CI): 0.83 (0.49–1.40); $HR_{western\ city}$: 0.55 (0.33–0.92)]. In women, the inverse association between exposure to the Hunger Winter and overall colon cancer risk was borderline significant (Table 3). This association was statistically significant for the all-wild-type+pMMR subgroup [$HR_{western\ rural}$ (95% CI): 0.68 (0.41–1.12); $HR_{western\ city}$: 0.64 (0.43–0.95)], whereas no association was observed for the any-mutation/dMMR subgroup (Table 3). No clear associations were observed for subgroups of individual molecular features in colon cancer in women (Table 4). Furthermore, no clear associations were observed between exposure to the Hunger Winter and the risk of overall rectal cancer in women, and stratification in subgroups based on individual molecular features or combinations thereof did not lead to clear associations (Tables 3 and 4).

Place of residence during WWII (1942) was not associated with overall colon cancer in men (Table 3). However, living in an urban area during WWII showed a borderline significant inverse association with any-mutation/dMMR colon cancer [$HR_{urban\ area}$ (95% CI): 0.77 (0.60–1.00)], but not with all-wild-type+pMMR colon cancer (p-heterogeneity = 0.041). Even though inverse associations were observed for all subgroups of individual molecular features, only the association with $KRAS_{mut}$ colon cancer reached statistical significance in men [$HR_{urban\ area}$ (95% CI): 0.72 (0.53–0.99)] (Table 4). Compared to the all-wild-type+pMMR subgroup, associations with $KRAS_{mut}$ and $PIK3CA_{mut}$ were statistically different (p-heterogeneity = 0.049 and 0.045, respectively). Place of residence during WWII was not associated with risk of rectal cancer in men, nor with colon or rectal cancer in women, and stratification in subgroups did not lead to clear associations (Tables 3 and 4).

Lastly, the employment status of the father during the Dutch Economic Depression (1932–1940) was not associated with overall colon or rectal cancer risk, neither in men nor in women (Table 4). Stratification in subgroups of (combinations of) molecular features did not lead to clear associations (Tables 4 and 5).

3.2.3 | Early adult BMI

Results on associations between early adult BMI (age 20 years) and risk of CRC in subgroups based on molecular features, by tumor location and sex, are shown in Table 5. BMI at the age of 20 years was not associated with risk of overall colon or rectal cancer in men, and stratification in subgroups based on (combinations of) molecular features only led to a nonsignificant positive association with $KRAS_{mut}$ colon cancer [$HR_{5\ kg/m2}$ (95% CI): 1.30 (0.94–1.80); $p\text{-trend}_{quartiles}$: 0.198]. No clear associations were observed for overall colon or rectal cancer in women either. However, stratification in subgroups led to borderline significant positive associations for $BRAF_{mut}$ [$HR_{5\ kg/m^2}$ (95% CI): 1.29 (0.97–1.70), $p\text{-trend}_{quartiles}$: 0.189] and dMMR [$HR_{5\ kg/m^2}$ (95% CI): 1.36 (0.98–1.90), $p\text{-trend}_{quartiles}$: 0.231] colon cancer in women, but not for other subgroups of individual molecular features or combinations thereof. For rectal cancer, stratification in subgroups based on (combinations of) molecular features did not lead to clear associations (Table 5).

TABLE 2 Multivariable-adjusted HRs[a] and 95% CIs for associations between height and CRC in subgroups based on mutation and MMR status, by sex and tumor location; NLCS, 1986–2006

		Total		Wild type + pMMR[b]		Any-mutation/dMMR[c]		
	Person-years at risk	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	*p*-het
Height quartiles (cm): Range (median)								
Men—Colon								
<173 (170)	8935	191	1.00 (ref.)	77	1.00 (ref.)	114	1.00 (ref.)	
173–176 (175)	7680	182	1.17 (0.91–1.50)	84	1.32 (0.93–1.88)	98	1.06 (0.78–1.44)	
177–181 (179)	7097	175	1.22 (0.95–1.57)	78	1.35 (0.94–1.92)	97	1.13 (0.83–1.54)	
>181 (185)	7310	206	1.49 (1.16–1.91)	70	1.26 (0.88–1.81)	136	1.64 (1.23–2.21)	0.049
p-trend			0.002		0.178		0.001	
per 5 cm	31,022	754	1.13 (1.05–1.20)	309	1.06 (0.97–1.16)	445	1.17 (1.08–1.27)	0.049
Men—Rectum								
<173 (170)	8935	61	1.00 (ref.)	39	1.00 (ref.)	22	1.00 (ref.)	
173–176 (175)	7680	46	0.84 (0.56–1.27)	26	0.73 (0.44–1.23)	20	1.04 (0.55–1.96)	
177–181 (179)	7097	67	1.36 (0.93–2.00)	41	1.27 (0.79–2.05)	26	1.52 (0.83–2.78)	
>181 (185)	7310	50	0.99 (0.66–1.50)	29	0.85 (0.51–1.42)	21	1.27 (0.67–2.40)	0.855
p-trend			0.464		0.943		0.264	
per 5 cm	31,022	224	1.04 (0.93–1.15)	135	1.03 (0.91–1.17)	89	1.05 (0.88–1.24)	0.836
Women—Colon								
<162 (158)	8764	140	1.00 (ref.)	39	1.00 (ref.)	101	1.00 (ref.)	
162–165 (164)	9216	173	1.17 (0.90–1.52)	54	1.31 (0.85–2.02)	119	1.12 (0.83–1.52)	
166–169 (168)	7771	147	1.17 (0.89–1.54)	45	1.31 (0.83–2.07)	102	1.12 (0.82–1.54)	
>169 (172)	8477	170	1.27 (0.97–1.66)	41	1.08 (0.67–1.73)	129	1.34 (0.99–1.82)	0.459
p-trend			0.107		0.794		0.069	
per 5 cm	34,228	630	1.07 (0.99–1.15)	179	1.02 (0.90–1.15)	451	1.09 (0.99–1.19)	0.413
Women—Rectum								
<162 (158)	8764	29	1.00 (ref.)	18	1.00 (ref.)	11	1.00 (ref.)	
162–165 (164)	9216	24	0.79 (0.45–1.37)	11	0.59 (0.28–1.23)	13	1.12 (0.49–2.56)	
166–169 (168)	7771	38	1.46 (0.88–2.44)	20	1.25 (0.65–2.42)	18	1.81 (0.83–3.94)	
>169 (172)	8477	40	1.44 (0.86–2.41)	15	0.88 (0.43–1.80)	25	2.37 (1.11–5.06)	0.285
p-trend			0.047		0.788		0.010	
per 5 cm	34,228	131	1.15 (0.98–1.35)	64	0.95 (0.75–1.21)	67	1.38 (1.12–1.70)	0.014

		KRAS$_{mut}$		*PIK3CA*$_{mut}$[d]		*BRAF*$_{mut}$[d]		dMMR[d]	
	Person-years at risk	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)
Height quartiles (cm): Range (median)									
Men—Colon									
<173 (170)	8935	74	1.00 (ref.)	40	1.00 (ref.)	24	1.00 (ref.)	16	1.00 (ref.)
173–176 (175)	7680	56	0.63–1.36)	32	1.00 (0.61–1.63)	24	1.21 (0.67–2.18)	16	1.26 (0.62–2.59)
177–181 (179)	7097	55	0.98 (0.67–1.44)	33	1.11 (0.68–1.80)	22	1.22 (0.67–2.24)	16	1.39 (0.68–2.82)
>181 (185)	7310	71	1.34 (0.93–1.94)	45	1.56 (0.98–2.47)	35	1.95 (1.12–3.39)	22	1.92 (0.98–3.73)
p-trend			0.140		0.063		0.025		0.057
per 5 cm	31,022	256	1.14 (1.03–1.26)	150	1.11 (0.97–1.27)	105	1.23 (1.06–1.43)*	70	1.24 (1.03–1.48)

(Continues)

TABLE 2 (Continued)

	Person-years at risk	$KRAS_{mut}$		$PIK3CA_{mut}$[d]		$BRAF_{mut}$[d]		dMMR[d]	
		n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)
Men—Rectum									
<173 (170)	8935	13	1.00 (ref.)						
173–176 (175)	7680	16	1.42 (0.67–3.03)						
177–181 (179)	7097	21	2.01 (0.97–4.17)						
>181 (185)	7310	18	1.81 (0.85–3.86)						
p-trend			0.063						
per 5 cm	31,022	68	1.14 (0.95–1.36)						
Women—Colon									
<162 (158)	8764	53	1.00 (ref.)	29	1.00 (ref.)	37	1.00 (ref.)	27	1.00 (ref.)
162–165 (164)	9216	56	1.03 (0.69–1.54)	25	0.83 (0.48–1.45)	44	1.10 (0.70–1.76)	41	1.42 (0.85–2.36)
166–169 (168)	7771	49	1.07 (0.70–1.63)	30	1.17 (0.68–2.01)	43	1.24 (0.77–2.00)	28	1.10 (0.62–1.93)
>169 (172)	8477	64	1.33 (0.89–1.97)	32	1.17 (0.69–1.97)	49	1.34 (0.84–2.14)	35	1.31 (0.75–2.27)
p-trend			0.164		0.355		0.191		0.567
per 5 cm	34,228	222	1.09 (0.95–1.23)	116	1.05 (0.88–1.25)	173	1.08 (0.95–1.23)	131	1.07 (0.92–1.24)
Women—Rectum									
<162 (158)	8764	9	1.00 (ref.)						
162–165 (164)	9216	10	1.07 (0.43–2.69)						
166–169 (168)	7771	15	1.87 (0.80–4.38)						
>169 (172)	8477	21	2.43 (1.07–5.53)						
p-trend			0.012						
per 5 cm	34,228	55	1.40 (1.11–1.77)*						

Abbreviations: BMI, body mass index; CIs, confidence intervals; CRC, colorectal cancer; dMMR, deficient mismatch repair; H*p*Rs, hazard ratios; $KRAS_{mut}$, *KRAS* mutations; NLCS, Netherlands Cohort Study; *p*-het, *p*-heterogeneity; pMMR, proficient mismatch repair.

[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 (min/day; continuous), processed meat intake (g/day; continuous), red meat intake (g/day; continuous). Age was included as a time-varying covariate.

[b]This group excludes cases with mutations in any of the genes (*KRAS*, *BRAF*, or *PIK3CA*), as well as MMR-deficient cases.

[c]This group includes cases with mutations in any of the genes (*KRAS*, *BRAF*, or *PIK3CA*) and/or cases that are MMR-deficient.

[d]Analyses for subgroups with <50 cases were not performed.

*Statistically significant *p*-heterogeneity; $BRAF_{mut}$ men colon: $p = 0.049$; $KRAS_{mut}$ women rectum: $p = 0.017$ (reference group: wild-type for *KRAS*, *PIK3CA*, *BRAF*, and pMMR).

4 | DISCUSSION

$KRAS_{mut}$, $PIK3CA_{mut}$, $BRAF_{mut}$, and dMMR have all been associated with the Warburg effect.[11,16–19] We previously reported differential associations between early-life energy balance-related factors and CRC subtypes based on the expression of proteins involved in the Warburg effect.[10] Using data from a large prospective cohort study, we now investigated associations between early-life energy balance-related factors and the risk of CRC subgroups based on $KRAS_{mut}$, $PIK3CA_{mut}$, $BRAF_{mut}$, and MMR status. Associations between early-life energy balance-related factors and risk of CRC varied by the above mentioned molecular features, as well as by sex and tumor location. Height was positively associated with any-mutation/dMMR CRC but not all-wild-type+pMMR CRC, with the exception of men with rectal cancer, and this difference reached statistically significant heterogeneity in men with colon cancer and women with rectal cancer. Results on early-life energy restriction proxies, reflecting exposure to energy restriction during the Dutch Hunger Winter, WWII, and the Dutch Economic Depression, in relation to the risk of CRC subgroups did not show clear patterns. A high early adult BMI (age 20 years) was (nonsignificantly) associated with an increased risk of $KRAS_{mut}$ colon cancer in men and of $BRAF_{mut}$ and dMMR colon cancer in women.

In the current study, we combined CRC cases into subgroups based on common molecular features ($KRAS_{mut}$, $PIK3CA_{mut}$, $BRAF_{mut}$, dMMR) and studied potential etiological differences between these

TABLE 3 Multivariable-adjusted HRs[a] and 95% CIs for associations between early-life energy restriction and CRC in subgroups based on mutation and MMR status, by sex and tumor location; NLCS, 1986–2006

		Total		Wild-type + pMMR[b]		Any-mutation/dMMR[c]		
	Person-years at risk	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	*p*-het
Place of residence during the Dutch Hunger Winter (1944–1945)								
Men—Colon	30,174	736		300		436		
Non-west	15,188	442	1.00 (ref.)	182	1.00 (ref.)	260	1.00 (ref.)	
Western rural	4129	75	0.67 (0.50–0.90)	29	0.63 (0.41–0.97)	46	0.70 (0.49–0.99)	
Western city	6524	122	0.64 (0.50–0.81)	56	0.71 (0.51–1.00)	66	0.59 (0.43–0.80)	0.546
Men—Rectum	30,174	224		135		89		
Non-west	15,188	138	1.00 (ref.)	84	1.00 (ref.)	54	1.00 (ref.)	
Western rural	4129	28	0.76 (0.49–1.17)	19	0.83 (0.49–1.40)	9	0.64 (0.31–1.32)	
Western city	6524	36	0.61 (0.41–0.90)	20	0.55 (0.33–0.92)	16	0.70 (0.39–1.25)	0.781
Women—Colon	33,722	620		174		446		
Non-west	18,083	360	1.00 (ref.)	107	1.00 (ref.)	253	1.00 (ref.)	
Western rural	4851	76	0.79 (0.59–1.06)	20	0.68 (0.41–1.12)	56	0.84 (0.60–1.16)	
Western city	9234	151	0.82 (0.65–1.02)	36	0.64 (0.43–0.95)	115	0.90 (0.70–1.15)	0.369
Women—Rectum	33,722	128		64		64		
Non-west	18,083	76	1.00 (ref.)	37	1.00 (ref.)	39	1.00 (ref.)	
Western rural	4851	12	0.63 (0.34–1.18)	6	0.66 (0.27–1.58)	6	0.61 (0.25–1.48)	
Western city	9234	32	0.87 (0.56–1.34)	15	0.83 (0.45–1.54)	17	0.90 (0.49–1.65)	0.986
Place of residence during World War II (1942)								
Men—Colon	23,793	560		231		329		
Rural area	11,327	283	1.00 (ref.)	105	1.00 (ref.)	178	1.00 (ref.)	
Urban area	11,713	258	0.89 (0.72–1.09)	117	1.08 (0.80–1.45)	141	0.77 (0.60–1.00)	0.041
Men—Rectum	23,973	184		108		76		
Rural area	11,327	95	1.00 (ref.)	57	1.00 (ref.)	38	1.00 (ref.)	
Urban area	11,713	86	0.89 (0.64–1.23)	49	0.85 (0.56–1.29)	37	0.94 (0.58–1.53)	0.689
Women—Colon	26,164	483		126		357		
Rural area	11,882	235	1.00 (ref.)	62	1.00 (ref.)	173	1.00 (ref.)	
Urban area	13,562	233	0.88 (0.71–1.09)	60	0.87 (0.59–1.27)	173	0.88 (0.69–1.13)	0.829
Women—Rectum	26,164	96		49		47		
Rural area	11,882	44	1.00 (ref.)	25	1.00 (ref.)	19	1.00 (ref.)	
Urban area	13,562	48	0.98 (0.63–1.51)	21	0.75 (0.40–1.38)	27	1.29 (0.71–2.35)	0.216
Employment of the father during the Dutch Economic Depression (1932–1940)								
Men—Colon	29,841	724		296		428		
Employed	26,697	645	1.00 (ref.)	258	1.00 (ref.)	387	1.00 (ref.)	
Unemployed	3145	79	0.97 (0.73–1.30)	38	1.16 (0.79–1.70)	41	0.84 (0.58–1.22)	0.180
Men—Rectum	29,841	216		128		88		
Employed	26,697	194	1.00 (ref.)	113	1.00 (ref.)	81	1.00 (ref.)	
Unemployed	3145	22	0.96 (0.60–1.53)	15	1.16 (0.66–2.05)	7	0.69 (0.31–1.54)	0.757

(Continues)

TABLE 3 (Continued)

	Person-years at risk	Total		Wild-type + pMMR[b]		Any-mutation/dMMR[c]		*p*-het
		n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	
Women—Colon	32,597	604		174		430		
Employed	29,046	533	1.00 (ref.)	153	1.00 (ref.)	380	1.00 (ref.)	
Unemployed	3552	71	1.09 (0.81–1.47)	21	1.13 (0.69–1.86)	50	1.07 (0.76–1.51)	0.904
Women—Rectum	32,597	127		62		65		
Employed	29,046	115	1.00 (ref.)	59	1.00 (ref.)	56	1.00 (ref.)	
Unemployed	3552	12	0.83 (0.45–1.53)	3	0.40 (0.12–1.30)	9	1.29 (0.63–2.62)	0.892

Abbreviations: BMI, body mass index; CIs, confidence intervals; CRC, colorectal cancer; dMMR, deficient mismatch repair; HRs, hazard ratios; NLCS, Netherlands Cohort Study; *p*-het, *p*-heterogeneity; pMMR, proficient mismatch repair.

[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 (min/day; continuous), processed meat intake (g/day; continuous), red meat intake (g/day; continuous). Age was included as a time-varying covariate.

[b]This group excludes cases with mutations in any of the genes (*KRAS*, *BRAF*, or *PIK3CA*), as well as MMR deficient cases.

[c]This group includes cases with mutations in any of the genes (*KRAS*, *BRAF*, or *PIK3CA*) and/or cases that are MMR deficient.

subgroups. We believe that combining these molecular features into subgroups has some advantages. First, mutations in *KRAS*, *PIK3CA*, and *BRAF* have all been associated with metabolic reprogramming toward the Warburg effect,[11,16–18] and MMR deficiency has previously been associated with the estimated presence of the Warburg effect.[19] Therefore, combining these molecular features, presumed to be involved in the same metabolic phenotype, results in a cleaner reference group compared to groups based on individual features (e.g., *KRAS*-mutated vs. wild-type). We observed that co-occurrence of $KRAS_{mut}$ and $PIK3CA_{mut}$ and of $BRAF_{mut}$ and dMMR was relatively common in the current cohort. Thus, by using the all-wild-type + pMMR subgroup as the reference for all subgroups of individual molecular features, this reference group is less heterogeneous regarding the Warburg effect compared to, for example, the *KRAS*-wild-type group, which still contains a large number of cases with a *PIK3CA* mutation. Second, using combination subgroups based on multiple molecular features results in increased statistical power, since most individual molecular features occurred in <20% of CRC cases (e.g., MMR deficiency: 10.7%).

Up to now, only a very limited number of studies have investigated associations between early-life energy balance-related factors and the risk of CRC in relation to specific (individual) molecular features.[23–26] Our results are concordant with those of a pooled analysis of the NLCS, using 7.3 years of follow-up, and the Melbourne Collaborative Cohort study, which showed stronger associations between height and $BRAF_{mut}$ compared to $BRAF_{wt}$ CRC in both cohorts, and stronger associations for MSI compared to microsatellite stable CRC, again in both cohorts.[25] However, our results on height are not in line with those of Brändstedt et al.[23,24] who did not find clear (differences between) associations based on $KRAS_{mut}$, $BRAF_{mut}$, or MMR status, which could have been related to limited statistical power. With respect to associations between early adult BMI and CRC in relation to MSI status, results of the current study are not in line with a previous case-control study,[26] which showed the strongest association with MSI low CRC (men and women combined), whereas we observed stronger associations with dMMR colon cancer in women. This difference could be related to the difference in study design (cohort vs. case-control) or by the difference in stratification on tumor location and sex. To the best of our knowledge, studies on early-life energy restriction and the risk of CRC subgroups based on molecular features are currently lacking.

The current results suggest a role of the molecular features ($KRAS_{mut}$, $PIK3CA_{mut}$, $BRAF_{mut}$, and dMMR) in the etiological pathway of height with CRC. Regarding colon cancer, in women, all individual molecular features seemed equally involved in this etiological pathway, whereas in men it seems that especially *BRAF*mut and MMR deficiency are involved in this etiological pathway (note: *BRAF*mut and MMR deficiency often co-occur). Regarding rectal cancer, $KRAS_{mut}$ seem to be involved in this etiological pathway, both in men and women. However, it should be noted that the majority of mutations in rectal cancer were observed in *KRAS*, the other molecular features considered here could thus not be investigated for rectal cancer in the current study due to limited power. The molecular features investigated in the current study have all been associated with the Warburg effect.[11,16–19] Since we hypothesized that associations with the any-mutation/dMMR subgroup or subgroups of individual molecular features indicate a higher likelihood of Warburg effect involvement, the current results would indicate a potential role of the Warburg effect in the etiological pathway between height and CRC in both men and women. Previously, we investigated associations between early-life energy balance-related factors and the risk of Warburg subtypes in CRC, based on the immunohistochemical expression of proteins involved in the Warburg effect.[10] In contrast to the current results, those of our previous study did not indicate a role of the Warburg effect in the etiological pathway between height and CRC risk.

For energy restriction, the current results do not give a clear indication of whether or not the studied molecular features ($KRAS_{mut}$,

TABLE 4 Multivariable-adjusted HRs[a] and 95% CIs for associations between early-life energy restriction and CRC for individual mutations and MMR status, by sex and tumor location; NLCS, 1986–2006

		KRAS$_{mut}$		*PIK3CA*$_{mut}$[b]		*BRAF*$_{mut}$[b]		dMMR[b]	
	Person-years at risk	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	251		148		103		67	
Non-west	15,188	149	1.00 (ref.)	92	1.00 (ref.)	62	1.00 (ref.)	42	1.00 (ref.)
Western rural	4129	29	0.78 (0.50–1.20)	16	0.69 (0.39–1.21)	7	0.44 (0.20–0.98)	6	0.57 (0.24–1.35)
Western city	6524	40	0.61 (0.42–0.90)	24	0.62 (0.39–0.99)	12	0.44 (0.23–0.84)	6	0.34 (0.14–0.83)
Men—Rectum	30,174	68							
Non-west	15,188	45	1.00 (ref.)						
Western rural	4129	8	0.70 (0.32–1.53)						
Western city	6524	10	0.52 (0.26–1.06)						
Women—Colon	33,722	221		115		170		129	
Non-west	18,083	130	1.00 (ref.)	65	1.00 (ref.)	90	1.00 (ref.)	72	1.00 (ref.)
Western rural	4851	24	0.68 (0.43–1.09)	15	0.89 (0.49–1.62)	25	1.05 (0.65–1.69)	14	0.76 (0.41–1.38)
Western city	9234	54	0.81 (0.57–1.14)	28	0.86 (0.54–1.37)	48	1.05 (0.72–1.52)	37	1.03 (0.68–1.57)
Women—Rectum	33,722	53							
Non-west	18,083	33	1.00 (ref.)						
Western rural	4851	6	0.70 (0.28–1.72)						
Western city	9234	12	0.76 (0.37–1.54)						
Place of residence during World War II (1942)									
Men—Colon	23,793	196		116		71		46	
Rural area	11,327	110	1.00 (ref.)	66	1.00 (ref.)	35	1.00 (ref.)	26	1.00 (ref.)
Urban area	11,713	84	0.72 (0.53–0.99)*	47	0.73 (0.49–1.09)*	32	0.89 (0.53–1.48)	16	0.66 (0.34–1.29)
Men—Rectum	23,973	57							
Rural area	11,327	31	1.00 (ref.)						
Urban area	11,713	26	0.81 (0.46–1.42)						
Women—Colon	26,164	175		90		138		106	
Rural area	11,882	87	1.00 (ref.)	41	1.00 (ref.)	67	1.00 (ref.)	49	1.00 (ref.)
Urban area	13,562	81	0.83 (0.60–1.15)	48	1.08 (0.69–1.69)	67	0.88 (0.61–1.27)	56	0.99 (0.66–1.50)
Women—Rectum	26,164	37							
Rural area	11,882	16	1.00 (ref.)						
Urban area	13,562	20	1.18 (0.61–2.28)						
Employment of the father during the Dutch Economic Depression (1932–1940)									
Men—Colon	29,841	247		144		99		66	
Employed	26,697	220	1.00 (ref.)	132	1.00 (ref.)	92	1.00 (ref.)	61	1.00 (ref.)
Unemployed	3145	27	0.95 (0.61–1.48)	12	0.73 (0.39–1.35)	7	0.62 (0.28–1.36)	5	0.67 (0.26–1.71)
Men—Rectum	29,841	68							
Employed	26,697	63	1.00 (ref.)						
Unemployed	3145	5	0.62 (0.24–1.56)						
Women—Colon	32,597	212		110		167		126	

(Continues)

TABLE 4 (Continued)

		$KRAS_{mut}$		$PIK3CA_{mut}$[b]		$BRAF_{mut}$[b]		dMMR[b]	
	Person-years at risk	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)		
Employed	29,046	182	1.00 (ref.)	91	1.00 (ref.)	150	1.00 (ref.)	112	1.00 (ref.)
Unemployed	3552	30	1.33 (0.87–2.04)	19	1.62 (0.95–2.78)	17	0.91 (0.53–1.56)	14	1.05 (0.57–1.90)
Women—Rectum	32,597	54							
Employed	29,046	46	1.00 (ref.)						
Unemployed	3552	8	1.43 (0.67–3.04)						

Abbreviations: BMI, body mass index; CIs, confidence intervals; CRC, colorectal cancer; dMMR, mismatch repair deficiency; HRs, hazard ratios; KRASmut, KRAS mutations; mut, mutated; NLCS, Netherlands Cohort Study.

[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 (min/day; continuous), processed meat intake (g/day; continuous), red meat intake (g/day; continuous). Age was included as a time-varying covariate.

[b]Analyses for subgroups with <50 cases were not performed.

*Statistically significant *p*-heterogeneity; WWII $KRAS_{mut}$ men colon: $p = 0.049$; WWII $PIK3CA_{mut}$ men colon: $p = 0.045$ (reference group: wild-type for *KRAS*, *PIK3CA*, and *BRAF*, and dMMR).

$PIK3CA_{mut}$, $BRAF_{mut}$, and dMMR) are involved in the etiological pathway with CRC risk, since results varied across the three proxy measures. Neither our results in men nor in women suggest a role of molecular features in the etiological pathway between exposure to energy restriction during the Hunger Winter and CRC risk. In contrast, our results suggest a potential role of these molecular features, in particular, $KRAS_{mut}$ and/or $PIK3CA_{mut}$, in the etiological pathway between exposure to energy restriction during WWII and colon cancer risk in men. It should be noted that *KRAS* and *PIK3CA* were often co-mutated. Other analyses of the WWII and Economic Depression proxies with CRC risk did not show clear associations. Therefore, the current results on energy restriction do not indicate a role of the Warburg effect in the etiological pathway between energy restriction during the Hunger Winter or Economic Depression and risk of CRC in men or women but do indicate a possible role of the Warburg effect in the etiological pathway between energy restriction experienced during WWII and risk of colon cancer in men. This is largely in line with the results of our previous study on Warburg subtypes in CRC.[10] In our previous study, we observed inverse associations between energy restriction during the Hunger Winter and CRC regardless of Warburg subtypes in men, and with Warburg-low colon cancer in women, which does not indicate a role of the Warburg effect. However, we previously observed a (nonsignificant) inverse association between energy restriction during WWII and specifically Warburg-high colon cancer in men, which suggests a role of the Warburg effect.

As previously described,[10] the variation of early adult BMI across participants from the NLCS is limited, possibly explaining the lack of statistically significant associations between early adult BMI and CRC risk. While this, to a certain extent, may have prevented us from detecting the involvement of molecular features ($KRAS_{mut}$, $PIK3CA_{mut}$, $BRAF_{mut}$, and dMMR) in the association between early adult BMI and CRC risk in men and women, $BRAF_{mut}$ and/or MMR deficiency seemed to play a role in the risk enhancement observed for colon cancer in women with a high early adult BMI, whereas $KRAS_{mut}$ may be involved in the risk enhancement for colon cancer in men. The Warburg effect might thus potentially play a role in the etiological pathway between early adult BMI and colon cancer. These results are partly in line with our previous study on Warburg subtypes in CRC,[10] where we observed a positive association between early adult BMI and specifically Warburg-high colon cancer in men, and with Warburg-moderate rectal cancer in women.

All in all, the results of the current study are only partly in line with those of our previous study on Warburg subtypes in CRC assessed by IHC. As mentioned, the molecular features that were considered in the current study have all been associated with the Warburg effect.[11,16–19] However, these molecular features are additionally known for their involvement in numerous diverse (oncogenic) cellular pathways for cell growth, differentiation, proliferation, and survival.[20–22] These molecular features might thus not always be a good reflection of the Warburg effect. In addition, while being wild-type for the genes currently studied as well as MMR proficient, tumors of cases in the all-wild-type+pMMR subgroup might still be characterized by other molecular features that were not assessed in the current study. These molecular features may possibly also be associated with the Warburg effect, potentially reducing any contrast between the all-wild-type+pMMR group and other groups. Nevertheless, the combination of molecular features into the all-wild-type+pMMR and any-mutation/dMMR subgroups seems to be a straightforward way of subtyping CRC cases.

Strengths of the current study are the prospective cohort design with long follow-up (20.3 years) and the availability of DNA from FFPE tumor material from a large number of incident CRC cases. A limitation of the current study is that we did not have a validation cohort available to confirm our results. Therefore, replication of the current results in additional large prospective cohorts is needed. In addition, despite the large sample size, the number of cases in final statistical analyses was limited for some groups (especially rectal

TABLE 5 Multivariable-adjusted HRs[a] and 95% CIs for associations between adolescent BMI and CRC in subgroups based on mutation and MMR status, by sex and tumor location; NLCS, 1986–2006

		Total		Wild-type + pMMR[a]		Any-mutation/dMMR[b]		
	Person-years at risk	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	*p*-het
Quartiles of BMI at age 20 years (kg/m^2): Range (median)								
Men—Colon								
<20.2 (19.2)	6103	155	1.00 (ref.)	64	1.00 (ref.)	91	1.00 (ref.)	
20.2–21.6 (21.0)	6458	148	0.93 (0.70–1.22)	51	0.76 (0.51–1.14)	97	1.05 (0.76–1.46)	
21.7–23.3 (22.4)	6308	151	1.00 (0.75–1.32)	67	1.04 (0.70–1.53)	84	0.97 (0.69–1.37)	
>23.3 (24.3)	6012	173	1.24 (0.94–1.63)	77	1.27 (0.87–1.86)	96	1.21 (0.86–1.69)	0.253
p-trend			0.112		0.099		0.369	
per 5 kg/m^2	24,881	627	1.04 (0.85–1.27)	259	0.94 (0.71–1.25)	368	1.12 (0.87–1.43)	0.497
Men—Rectum								
<20.2 (19.2)	6103	41	1.00 (ref.)	25	1.00 (ref.)	16	1.00 (ref.)	
20.2–21.6 (21.0)	6458	62	1.44 (0.94–2.20)	32	1.18 (0.68–2.05)	30	1.83 (0.98–3.44)	
21.7–23.3 (22.4)	6308	45	1.09 (0.68–1.74)	31	1.19 (0.66–2.12)	14	0.91 (0.42–1.93)	
>23.3 (24.3)	6012	47	1.19 (0.75–1.89)	30	1.19 (0.66–2.15)	17	1.15 (0.57–2.32)	0.293
p-trend			0.808		0.583		0.713	
per 5 kg/m^2	24,881	195	1.12 (0.85–1.48)	118	1.13 (0.79–1.60)	77	1.09 (0.72–1.66)	0.703
Women—Colon								
<19.6 (18.4)	7795	142	1.00 (ref.)	44	1.00 (ref.)	98	1.00 (ref.)	
19.6–21.2 (20.5)	7731	143	1.03 (0.78–1.36)	41	0.94 (0.59–1.49)	102	1.08 (0.79–1.48)	
21.3–23.0 (22.0)	7964	147	1.04 (0.79–1.38)	44	0.98 (0.63–1.54)	103	1.08 (0.78–1.48)	
>23.0 (24.2)	7683	137	1.07 (0.81–1.42)	29	0.67 (0.40–1.11)	108	1.27 (0.92–1.75)	0.284
p-trend			0.645		0.168		0.171	
per 5 kg/m^2	31,173	569	1.07 (0.91–1.26)	158	0.89 (0.69–1.16)	411	1.15 (0.96–1.39)	0.149
Women—Rectum								
<19.6 (18.4)	7795	23	1.00 (ref.)	10	1.00 (ref.)	13	1.00 (ref.)	
19.6–21.2 (20.5)	7731	37	1.71 (0.98–2.98)	20	2.03 (0.92–4.47)	17	1.47 (0.68–3.20)	
21.3–23.0 (22.0)	7964	29	1.30 (0.73–2.31)	15	1.43 (0.62–3.31)	14	1.22 (0.55–2.67)	
>23.0 (24.2)	7683	30	1.44 (0.80–2.60)	14	1.37 (0.59–3.22)	16	1.57 (0.71–2.48)	0.875
p-trend			0.408		0.757		0.359	
per 5 kg/m^2	31,173	119	1.15 (0.87–1.52)	59	1.03 (0.70–1.52)	60	1.27 (0.86–1.90)	0.847

		KRAS$_{mut}$		*PIK3CA*$_{mut}$[c]		*BRAF*$_{mut}$[c]		dMMR[c]	
	Person-years at risk	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)
Quartiles of BMI at age 20 years (kg/m^2): Range (median)									
Men—Colon									
<20.2 (19.2)	6103	50	1.00 (ref.)	32	1.00 (ref.)	19	1.00 (ref.)	19	1.00 (ref.)
20.2–21.6 (21.0)	6458	50	0.96 (0.63–1.47)	33	0.98 (0.59–1.62)	25	1.39 (0.75–2.58)	16	0.89 (0.45–1.76)
21.7–23.3 (22.4)	6308	57	1.16 (0.76–1.77)	23	0.72 (0.41–1.25)	19	1.13 (0.57–2.23)	9	0.52 (0.23–1.17)
>23.3 (24.3)	6012	57	1.26 (0.82–1.94)	35	1.18 (0.71–1.97)	21	1.36 (0.70–2.66)	15	0.94 (0.46–1.93)
p-trend			0.198		0.774		0.515		0.581
per 5 kg/m^2	24,881	214	1.30 (0.94–1.80)	123	1.08 (0.73–1.60)	84	1.00 (0.66–1.53)	59	0.74 (0.43–1.27)

(Continues)

TABLE 5 (Continued)

	Person-years at risk	$KRAS_{mut}$		$PIK3CA_{mut}$[c]		$BRAF_{mut}$[c]		dMMR[c]	
		n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)	n_{cases}	HR (95% CI)
Men—Rectum									
<20.2 (19.2)	6103	11	1.00 (ref.)						
20.2–21.6 (21.0)	6458	24	2.15 (1.04–4.47)						
21.7–23.3 (22.4)	6308	12	1.19 (0.50-2.83)						
>23.3 (24.3)	6012	12	1.32 (0.57–3.05)						
p-trend			0.965						
per 5 kg/m^2	24,881	59	1.21 (0.75–1.95)						
Women—Colon									
<19.6 (18.4)	7795	46	1.00 (ref.)	25	1.00 (ref.)	37	1.00 (ref.)	25	1.00 (ref.)
19.6–21.2 (20.5)	7731	52	1.19 (0.78–1.83)	29	1.24 (0.71–2.17)	35	0.97 (0.59–1.60)	28	1.16 (0.66–2.04)
21.3–23.0 (22.0)	7964	49	1.10 (0.71–1.69)	20	0.85 (0.46–1.56)	43	1.20 (0.74–1.93)	30	1.24 (0.71–2.17)
>23.0 (24.2)	7683	56	1.39 (0.91–2.14)	31	1.45 (0.82–2.54)	41	1.32 (0.81–2.16)	30	1.42 (0.79–2.53)
p-trend			0.192		0.403		0.189		0.231
per 5 kg/m^2	31,173	203	1.17 (0.91–1.50)	105	1.25 (0.91–1.71)	156	1.29 (0.97–1.70)	113	1.36 (0.98–1.90)
Women—Rectum									
<19.6 (18.4)	7795	11	1.00 (ref.)						
19.6–21.2 (20.5)	7731	13	1.32 (0.56–3.10)						
21.3–23.0 (22.0)	7964	12	1.25 (0.53–2.92)						
>23.0 (24.2)	7683	14	1.64 (0.69–3.90)						
p-trend			0.299						
per 5 kg/m^2	31,173	50	1.22 (0.78–1.90)						

Note: *p*-heterogeneity tests for individual molecular features ($KRAS_{mut}$, $PIK3CA_{mut}$, $BRAF_{mut}$, and dMMR) were not statistically significant (reference group for all tests: wild-type for KRAS, PIK3CA, BRAF, and pMMR).

Abbreviations: BMI, body mass index; CIs, confidence intervals; CRC, colorectal cancer; dMMR, deficient mismatch repair; HRs, hazard ratios; $KRAS_{mut}$, *KRAS* mutations; NLCS, Netherlands Cohort Study; *p*-het, *p*-heterogeneity; pMMR, proficient mismatch repairy.

[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 (min/day; continuous), processed meat intake (g/day; continuous), red meat intake (g/day; continuous). Age was included as a time-varying covariate.

[b]This group excludes cases with mutations in any of the genes (*KRAS*, *BRAF*, or *PIK3CA*), as well as MMR-deficient cases.

[c]This group includes cases with mutations in any of the genes (*KRAS*, *BRAF*, or *PIK3CA*) and/or cases that are MMR-deficient.

[d]Analyses for subgroups with <50 cases were not performed.

cancer) due to heterogeneity in sex and tumor location. Furthermore, the use of the MassARRAY technology in the current study to detect mutations has been shown to be suitable for mutation typing in (older) FFPE material.[43] However, even though the ColoCarta panel includes the most known mutations in *KRAS* (99%) and *BRAF* (98%), only 78% of known *PIK3CA* mutations are included.[44] As the most common *PIK3CA* mutations are included,[45] it appears to be unlikely that additional detection of less common mutations would have altered the current results. As for MSI status, the usage of MLH1 and MSH2 immunohistochemical expression as an indicator of MSI status might have led to the misclassification of some of the cases since not all MMR genes were included. However, it has been shown that loss of MLH1 or MSH2 expression was observed in ~90% of MSI cases.[46]

Another strength of the current study is that the NLCS provides the unique opportunity to investigate associations between exposure to (severe) energy restriction early in life and the risk of CRC in relation to common molecular features. Nevertheless, the proxy measures for energy restriction during the Dutch Hunger Winter, WWII, and the Dutch Economic Depression might entail exposure misclassification, since individual data on dietary exposures during those times were not available. However, it has previously been shown that the proxy measure for energy restriction during the

Hunger Winter was reasonably adequate among female subcohort members.[47]

In conclusion, results from this large prospective cohort study provide further insights into the associations between early-life energy balance-related factors and CRC risk according to $KRAS_{mut}$, $PIK3CA_{mut}$, $BRAF_{mut}$, and MMR status. Our results indicate a role of these molecular features in the etiological pathway between height and CRC risk. $BRAF_{mut}$ and/or MMR deficiency seemed to be mainly involved in the association of height with colon cancer in men, whereas $KRAS_{mut}$ seem to be important for rectal cancer in both men and women. Furthermore, $KRAS_{mut}$ might potentially be involved in the etiological pathway between early adult BMI and colon cancer risk in men, whereas $BRAF_{mut}$ and/or MMR deficiency potentially play a role in the etiological pathway between early adult BMI and colon cancer risk in women.

AUTHOR CONTRIBUTIONS

Josien C. A. Jenniskens: Conceptualization; formal analysis and investigation; writing – original draft preparation. **Kelly Offermans**: Conceptualization; writing – original draft preparation. **Colinda C. J. M. Simons**: Conceptualization; writing – original draft preparation; **Heike I. Grabsch**: Conceptualization; writing – original draft preparation; funding acquisition. **Piet A. van den Brandt**: Conceptualization; funding acquisition; methodology; supervision. **Iryna Samarska**: Writing – review and editing. **Gregorio E. Fazzi**: Writing – review and editing. **Jaleesa R. M. van der Meer**: Writing – review and editing. **Kim M. Smits**: Writing – review and editing. **Leo J. Schouten**: Writing – review and editing. **Matty P. Weijenberg**: Writing – review and editing.

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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 remaining authors declare no conflict of interest.

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.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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