

1 **Mendelian randomization study of age at menarche and age at**
2 **menopause and the risk of colorectal cancer**

3 **Running title:** Age at menarche/menopause and colorectal cancer
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89 **Abbreviations:**

90 BMI, body mass index; CCFR, Colon Cancer Family Registry; CI, confidence interval;
91 CORECT, Colorectal Cancer Transdisciplinary Consortium; CRC, colorectal cancer; ER β ,
92 estrogen-receptor β ; GECCO, Genetics and Epidemiology of Colorectal Cancer Consortium;
93 GRS, genetic risk scores; GWAS, genome-wide association study; LD, linkage
94 disequilibrium; MHT, menopausal hormone therapy; MR, Mendelian randomization; OR,
95 odds ratio, SNP, single nucleotide polymorphism.

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105 **Abstract**

106 **Background:** Substantial evidence supports an association between use of menopausal
107 hormone therapy and increased colorectal cancer (CRC) risk, indicating a role of exogenous
108 sex hormones in CRC development. However, findings on endogenous estrogen exposure
109 and CRC are inconsistent.

110 **Methods:** We used a Mendelian randomization approach to test for a causal effect of age at
111 menarche and age at menopause as surrogates for endogenous estrogen exposure on CRC
112 risk. Weighted genetic risk scores based on 358 single nucleotide polymorphisms associated
113 with age at menarche and 51 single nucleotide polymorphisms associated with age at
114 menopause were used to estimate the association with CRC risk using logistic regression in
115 12 944 women diagnosed with CRC and 10 741 women without CRC from three consortia.
116 Sensitivity analyses were conducted to address pleiotropy and possible confounding by body
117 mass index.

118 **Results:** Genetic risk scores for age at menarche (odds ratio per year 0.98, 95% confidence
119 interval: 0.95-1.02) and age at menopause (odds ratio 0.98, 95% confidence interval: 0.94-
120 1.01) were not significantly associated with CRC risk. The sensitivity analyses yielded
121 similar results.

122 **Conclusion:** Our study does not support a causal relationship between genetic risk scores for
123 age at menarche and age at menopause and CRC risk.

124

125 **Keywords:** BMI, egger regression, endogenous estrogen, hormonal factors, menstrual,
126 polygenic risk score, reproductive factor, sex hormone, weighted median

127 **BACKGROUND**

128 Colorectal cancer (CRC) is the third most common cancer worldwide and incidence rates are
129 higher in men than in women.¹ The sex-specific difference might be partly attributed to
130 differential exposure to sex-hormones, especially estrogen.² This hypothesis is partially
131 supported by epidemiologic studies as well as a recent meta-analysis out of four randomized
132 controlled trials, eight cohort and eight case-control studies, which have shown that use of
133 exogenous sex hormones in the form of combined estrogen-progestogen menopausal
134 hormone therapy (MHT) is inversely associated with the risk of CRC.^{3,4}

135 Epidemiologic studies examining reproductive factors such as age at menarche and age at
136 menopause with CRC risk have reported inconsistent results.⁵⁻⁷ A meta-analysis
137 summarizing evidence based on 22 studies published by Li et al. did not find a significant
138 association between age at menarche and CRC risk.⁸ In a large prospective cohort of the NIH
139 American Association of Retired Persons (AARP) Diet and Health Study with more than
140 214,000 postmenopausal women, an inverse association between age at menarche and CRC
141 risk was observed for women without a history of MHT use, whereas increasing age at
142 menopause was associated with higher CRC risk.⁹ It is possible that the inconsistency in
143 results is due to recall bias or to improper adjustment for confounders, which are inherent
144 limitations of observational studies. The Mendelian randomization (MR) approach¹⁰ uses
145 genetic variants as instrumental variables to test for the causal effect of an exposure risk
146 factor on an outcome. Since the genetic variants in offspring are randomly distributed at
147 conception independent of environmental factors given parental genotypes, confounding and
148 reverse causation are less likely to occur in MR analyses. For genetic variants to function as
149 valid instrumental variables in MR analyses, three assumptions have to be met: 1) the genetic
150 variants have to be associated with the exposure risk factor; 2) the variants are not associated
151 with any confounding variables of the exposure-outcome association; and 3) the variants are
152 unrelated to the outcome except through the risk factor of interest.^{11, 12} Using variants
153 associated with age at menarche and age at menopause instead of self-reported measure of

154 the risk factors themselves, this approach can help to avoid issues of confounding, recall bias
155 and reverse causation. Evidence from MHT use suggests that more exposure to estrogen
156 reduces risk of CRC.^{3,4,13} Estrogen exerts its effects in colon cells predominantly through the
157 nuclear receptor estrogen-receptor β (ER β),^{14,15} which has mainly anti-proliferative effects¹⁶
158 and its expression is inversely related to cancer stage, tumor extent and mortality.^{17,18} Longer
159 use of MHT appears to be associated with high ER β expression in tumors.¹⁹ If similar
160 mechanisms hold then longer endogenous estrogen exposure (through earlier age at
161 menarche and/or later age at menopause) would reduce CRC risk as well. To test this
162 hypothesis, we conducted a MR analysis using summary data for single nucleotide
163 polymorphisms (SNPs) known to be associated with age at menarche and age at menopause
164 from prior studies as well as using individual level data of three consortia.

165 **MATERIALS AND METHODS**

166 **Study population.** Epidemiological and genetic data were derived from 26 studies
167 participating in three large consortia of CRC, the Genetics and Epidemiology of Colorectal
168 Cancer Consortium (GECCO)²⁰ (5 386 cases and 5 696 controls), the Colon Cancer Family
169 Registry (CCFR)²¹ (1 678 cases and 1 188 controls) and the Colorectal Cancer
170 Transdisciplinary (CORECT) Consortium²² (5 880 cases and 3 857 controls) (see
171 Supplementary Tables 1 and 2). Different centers of CCFR participated as individual studies
172 in GECCO and/or CORECT, and therefore were analyzed as such. Any participant overlap
173 between the three consortia was excluded. In total, 12 944 female colorectal cancer cases and
174 10 741 female controls, both of European ancestry were included. All participants provided
175 written, informed consent and studies were approved by their respective institutional review
176 boards. Women with incident invasive colorectal adenocarcinoma (International
177 Classification of Disease Code, 9th revision (ICD-9), codes 153-154) were included as cases.
178 Data on demographic factors and lifestyle were collected using in-person interviews or self-
179 completed questionnaires. Data harmonization was done centrally as previously described.²³

180 Self-reported data for age at menarche and age at menopause have not been harmonized and
181 therefore could not be used.

182 **Genotype data and imputation.** Genotype information was available for all included
183 studies. Details on genotyping, quality assurance and imputation are included in the
184 Supplementary Information. In short, SNPs were excluded based on call rate (<98%
185 GECCO; <95% in CORECT), Hardy-Weinberg equilibrium in controls ($P < 1 \times 10^{-4}$) or low
186 minor allele frequency ($\leq 1\%$). Participants received a value of 0, 1, or 2 for carrying 0 (wild-
187 type homozygous), 1 (heterozygous) or 2 (homozygous for the risk allele) alleles associated
188 with higher age at menarche/age at menopause for each SNP. For imputed SNPs, participants
189 were assigned continuous values between 0 and 2.

190 **Calculation of genetic risk scores.** Two recent genome-wide association studies
191 conducted by the REPROGEN consortium (Reproductive Genetics Consortium) involving
192 up to ~370 000 women for the study on age at menarche and up to 69 360 women for the
193 study on age at menopause identified 389 genetic variants associated with age at menarche²⁴
194 and 54 SNPs associated with age at natural menopause²⁵ at a genome-wide significance level
195 (i.e., $P < 5 \times 10^{-8}$). Of the reported 389 genetic variants for age at menarche, 12 variants on sex
196 chromosomes (which were not available in our datasets) were excluded. For further 42
197 missing variants, we used proxy SNPs in strong linkage disequilibrium ($R^2 > 0.8$, median
198 $R^2 = 0.985$, R^2 range: 0.83-1.00) with effect alleles harmonized to reflect increase in age at
199 menarche. Seven variants were excluded because no proxy was found. We checked for
200 correlations between individual SNPs and excluded 12 correlated SNPs ($R^2 > 0.01$) (always
201 the SNP with higher P -value for association with age at menarche was dropped). So data
202 were available for 358 SNPs for this analysis (mean imputation quality score=0.97)
203 (Supplementary Table 5). For age at menopause, three correlated SNPs ($R^2 > 0.01$) were
204 excluded. All remaining 51 SNPs for age at menopause were available in the datasets (mean
205 imputation quality score=0.98) (Supplementary Table 6). The genotype data described were

206 used to construct genetic risk scores (GRS) as instrumental variables for age at menarche and
207 age at menopause, respectively.

208 The GRS for the k^{th} women is calculated by the sum of the number of risk increasing alleles
209 carried (G) (imputed allele doses) for each SNP weighted by the reported beta-coefficient (β)
210 for association with age at menarche and age at menopause.^{24, 25}

211 Age at menarche

$$\text{GRS}_k = \sum_{n=1}^{358} \beta_n G_{kn}$$

212

213 Age at menopause

$$\text{GRS}_k = \sum_{n=1}^{51} \beta_n G_{kn}$$

214

215 As risk scores themselves do not have meaningful units, we scaled the risk scores in terms of
216 age in years. In this way resulting odds ratios (OR) can be interpreted as the relative change
217 in CRC risk per year older age at menarche/age at menopause. Scaling was done by dividing
218 the GRS by regression coefficients of a linear regression of GRS on self-reported age at
219 menarche (beta= 0.57) or age at menopause (beta=0.98). These regression coefficients were
220 obtained using the DACHS (Darmkrebs: Chancen der Verhütung durch Screening) study
221 (853 control women) and the WHI (Womens' Health Initiative) study (1 492 control women)
222 for which self-reported data on age at menarche were available and regression coefficients of
223 the two studies were combined using meta-analysis.

224 An additional GRS as a surrogate for total lifetime exposure to endogenous estrogen was
225 calculated as the sum of the scaled risk scores for age at menarche and age at menopause.

$$\text{GRS}_{\text{time period}} = \text{GRS}_{\text{age at menarc he}} + \text{GRS}_{\text{age at menopause}}$$

226

227 For this analysis, risk scores for age at menarche were calculated as the sum of the number
228 of alleles associated with decreasing age at menarche and the number of alleles associated
229 with increasing age at menopause. So the GRS for time period of estrogen exposure reflects
230 higher risk for longer exposure to endogenous estrogen. For this score, four SNPs
231 (rs3136269, rs11031040, rs537244, rs4303811) were excluded due to high linkage
232 disequilibrium ($R^2 > 0.01$) between age at menarche and age at menopause SNPs.

233

234 **Statistical analysis.**

235 *Validating MR assumptions*

236 The first assumption of MR regarding instrumental variable strength (i.e. association
237 between the genetic variants and the exposure risk factor) was verified by calculating the F-
238 statistic out of $F = (R^2(n-K-1))/((1-R^2)K)$, where R^2 refers to the variance explained by the
239 instrumental variable,^{24, 25} K indicates the number of instrumental variables and n stands for
240 the sample size.²⁶ An F-statistic > 10 suggests that the genetic instrument is sufficiently
241 strong.²⁷ To evaluate the second assumption of MR (i.e. no association between genetic
242 variants and potential confounders), we tested associations between GRS for age at
243 menarche/age at menopause and the following risk factors for CRC: smoking status
244 (ever/never), family history of CRC, education/educational level, ever aspirin/NSAID use (at
245 least once per month for more than one year), body mass index (BMI) (continuous),
246 menopausal hormone therapy (estrogen/progestin combined and estrogen alone), using linear
247 regression for continuous variables and logistic/multinomial logistic regression for
248 categorical variables in a subset of the studies with available data (n=6 285 controls). To
249 address the third assumption of MR i.e. to assess the presence of pleiotropy, we applied the
250 MR-Egger method.²⁸ MR-Egger relies on the InSIDE assumption (Instrument Strength
251 Independent of Direct Effect), which is the assumption that the pleiotropic effects of the
252 genetic variants are not correlated with the effects of genetic variants on the risk factors.
253 MR-Egger uses an inverse-variance weighted estimator and by plotting the SNP's effect on

254 the exposure against its effect on the outcome, the intercept term of MR-Egger provides a
255 test for directional pleiotropy, i.e. the average effect of pleiotropy is non-zero, across all
256 genetic variants used. If the average pleiotropic effect of all variants is zero and the InSIDE
257 assumption is satisfied, pleiotropy is “balanced” and will not be detected. If the intercept
258 differs from zero, it suggests horizontal pleiotropy, which means that some genetic variants
259 affect the outcome through a pathway different from the exposure of interest. For visual
260 inspection of pleiotropy, we used funnel plots of each SNPs ratio estimate against its
261 precision (1/standard error of the ratio estimate).^{29, 30} Any deviation from symmetry would
262 suggest pleiotropy.

263 *Estimation of Causal Effect*

264 GRS based analyses

265 We examined the association between GRS and CRC risk using logistic regression models
266 adjusted for study, age as well as principal components (PCs) of genetic ancestry (three PCs
267 were used for GECCO and 10 PCs were used for CORECT), to account for potential
268 population stratification. Summary results for GECCO and CORECT were derived using
269 fixed-effects meta-analysis assuming that the included studies share a common effect size.
270 As there are indications for differential associations of age at menarche with CRC risk
271 according to menopausal hormone use,^{9, 31} stratified analyses by menopausal hormone
272 therapy were performed (only in GECCO where harmonized data were available). Additional
273 stratified analyses were performed according to menopausal status (for age at menarche),
274 combined menopausal estrogen or progesterone therapy, estrogen therapy alone, and BMI
275 categories in kg/m² (BMI<18.5: underweight, 18.5–24.9: normal weight, 25-30: overweight,
276 >30: obese) (only for age at menopause). Due to reported differences in risk between colon
277 and rectal cancer associated with hormone use,^{32, 33} we also conducted site-specific analyses
278 for 4 037 female colon cancer cases and 1 184 rectal cancer cases in GECCO. Power
279 calculations were conducted to estimate the magnitude of effects detectable with our study
280 size assuming 5% alpha level and an R² of 0.069 for age at menarche and R² of 0.057 for age

281 at menopause, which corresponds to the variance in age at menarche/age at menopause
282 explained by the SNPs used for this analyses.³⁴

283 It is known that BMI in childhood is strongly associated with age at menarche³⁵ and that
284 some SNPs associated with age at menarche could also have pleiotropic effects e.g. are
285 related to BMI as well.²⁴ To address this issue and thereby also account for violations of the
286 third MR assumption, we conducted further BMI-specific sensitivity analyses. We adjusted
287 for BMI in the logistic regression analysis using a weighted GRS for BMI comprising 77
288 SNPs previously reported to be associated with BMI at a genome-wide significance level in
289 European subjects.³⁶ For the second sensitivity analysis, we identified age at menarche SNPs
290 showing pleiotropy by testing the association of these SNPs with BMI in a subset of our
291 sample (n=5 832 cases/6 285 controls) and found 29 SNPs to be associated with BMI at
292 nominal significance (P value<0.05). Two further SNPs overlapped with reported BMI-
293 SNPs³⁶ and eleven more SNPs were in high linkage disequilibrium ($R^2>0.1$) with BMI SNPs.
294 A restricted GRS for age at menarche excluding the 42 BMI-associated SNPs (n=316 SNPs)
295 was constructed and then assessed for association with CRC risk. For the analysis of lifetime
296 estrogen-exposure we also generated a restricted risk score excluding the same 42 BMI-
297 associated SNPs.

298 Two-sample MR analyses

299 We performed two-sample MR analyses as sensitivity analyses using published summary
300 statistics for SNP-exposure associations (age at menarche²⁴/age at menopause²⁵), SNP
301 outcome associations were estimated in GECCO/CORECT (Supplementary Tables 8 and 9
302 for age at menarche/menopause-SNPs respectively). We applied the weighted median
303 estimator approach, which is robust against violations due to pleiotropic SNPs even when up
304 to 50% of the genetic instruments are invalid.³⁷ For this approach, we used SNP-exposure
305 and SNP-outcome associations to build ratio estimates for each SNP. These estimates were
306 ordered and weighted by the inverse of their variance. Bootstrapped standard errors were
307 calculated and used for construction of 95% confidence intervals (CI). Furthermore, we

308 assessed the slope of MR-Egger regression (see section on validation of MR assumptions)
309 which yields a pleiotropy-adjusted estimate of the true causal effect.²⁸

310 All analyses were conducted using R version 3.2.2 (R Foundation for Statistical Computing,
311 Vienna, Austria). For supplementary figures, we used the R packages “Mendelian
312 Randomization”³⁸ and “ggplot2”.

313 **RESULTS**

314 The assessment of the MR assumptions indicated that our instrumental variables for age at
315 menarche and for age at menopause were both strong instruments (F-statistic for age at
316 menarche=1 755, $R^2=0.069^{24}$; F-statistic for age at menopause=1 431, $R^2=0.057^{25}$. Secondly,
317 we did not find significant associations between the GRS for age at menarche and CRC risk
318 factors, including smoking, family history of cancer, education, aspirin/NSAID use,
319 estrogen/progestin therapy, estrogen alone therapy, with the exception of BMI, which
320 showed a significant association (Supplementary Table 3). Similarly, there was no
321 association of the GRS for age at menopause with any of the tested risk factors
322 (Supplementary Table 3). Table 1 shows the results of the MR analyses for age at menarche,
323 age at menopause and lifetime estrogen exposure with CRC risk. Yearly increment in GRS
324 for age at menarche was associated with CRC risk with an OR of 0.98 (95% CI: 0.95-1.02).
325 Sensitivity analyses adjusting for BMI using a GRS yielded similar results for age at
326 menarche (OR 0.99 per year, 95% CI: 0.95-1.02). Further sensitivity analysis using restricted
327 risk scores for age at menarche (by excluding 42 BMI-associated SNPs) showed similar
328 effect sizes for the association between age at menarche and CRC risk (OR 0.99 per year,
329 95% CI: 0.95-1.03). We also did not find evidence to support a causal association with risk
330 of CRC for age at menopause (OR 0.98 per year, 95% CI: 0.94-1.01) or for lifetime estrogen
331 exposure (OR 0.99 per year, 95% CI: 0.97-1.02) using GRS-based analyses.

332 Results of the weighted median estimator approach also did not indicate causal association
333 between age at menarche (OR per year 1.00, 95% CI: 0.90-1.11), age at menopause (OR per

334 year 1.00, 95% CI: 0.95-1.05) or lifetime estrogen-exposure (OR per year 1.00, 95% CI:
335 0.95-1.05) with CRC risk.

336 The pleiotropy adjusted MR estimate (OR) derived from the slope of Egger regression was
337 0.99 (95% CI: 0.83-1.17) for age at menarche, 1.02 (95% CI: 0.94-1.10) for age at
338 menopause and 0.97 (95% CI: 0.91-1.01) for lifetime estrogen-exposure (details of results
339 per GECCO and CORECT consortium in Supplementary Table 7). The intercept term from
340 MR-Egger regression was centered at the origin for age at menarche (intercept term -0.0008,
341 95% CI: -0.007-0.005, *P* value 0.80) and age at menopause (intercept term -0.009, 95% CI: -
342 0.023-0.006, *P* value 0.23) suggesting absence of strong directional pleiotropy
343 (Supplementary Figure 1 and 2). Also the funnel plots for age at menarche and age at
344 menopause appear to be generally symmetrical and therefore do not suggest presence of
345 pleiotropy (Supplementary Figure 3 and 4). Table 2 shows stratified analyses for the
346 association of GRS for age at menarche and CRC risk by menopausal status, combined
347 estrogen/progesterone therapy and estrogen alone therapy as well as cancer site based on
348 GECCO data. None of these factors modified substantially the association between age at
349 menarche and CRC risk. The site specific analysis showed no evidence for a difference in
350 association between colon cancer (OR 0.97 per year, 95% CI: 0.91-1.02) and rectal cancer
351 (OR 1.04 per year, 95% CI: 0.95-1.14). The analyses for age at menopause stratified by
352 combined estrogen/progesterone therapy, estrogen alone therapy, BMI and cancer site did not
353 yield evidence for effect heterogeneity (Table 3). Power calculation shows that our study had
354 > 80 % power to detect an OR of 0.85 per standard deviation change in exposure variable
355 (1.5 years for age at menarche, 4.8 years for age at menopause) but only around 50% power
356 for an OR of 0.90 (Supplementary Table 4).

357 **DISCUSSION**

358 In this large MR study we aimed to clarify the inconsistent findings from observational
359 studies regarding the association of age at menarche and age at menopause with CRC risk
360 and thereby the role of endogenous estrogen exposure. We investigated the association of

361 GRS for age at menarche and age at menopause as surrogates for endogenous estrogen
362 exposure on CRC risk. Our results do not support an association between GRS for age at
363 menarche and age at menopause and CRC risk, which under MR assumptions can be
364 interpreted as absence of a causal effect.

365 In line with the findings of our study, several prospective studies and one meta-analysis
366 reported no association^{6, 8, 39} between self-reported age at menarche and CRC risk, although
367 some studies found an inverse association.^{5, 9} Two more recent prospective studies reported
368 inverse associations with CRC risk for age at menarche only among never users of any MHT.
369 In never users of hormone therapy, Zervoudakis et al. reported a hazard ratio of 0.73 (95%
370 CI: 0.57-0.94) for age at menarche (>15 vs. 11-12 years) in association with risk of CRC⁹
371 and Murphy et al. found a hazard ratio of 0.72 (95% CI: 0.54-0.96) for age at menarche (>15
372 vs. 11-12 years).³¹ We therefore assessed the association of GRS for age at menarche with
373 CRC risk stratified by ever/never use of MHT, separately for combined estrogen-
374 progesterone therapy and for estrogen monotherapy. No difference in the association
375 according to either combined estrogen-progesterone therapy or estrogen-alone therapy was
376 found.

377 Higher BMI in childhood is associated with earlier age at menarche^{24, 35} and also with a
378 higher risk for CRC.⁴⁰ Therefore we conducted BMI-specific sensitivity analyses to account
379 for violations of the MR assumptions by confounding and pleiotropy. Due to a strong
380 association between childhood/adolescent BMI and adult BMI⁴¹ and also a high concordance
381 between adolescent and adult BMI-SNPs,⁴² we accounted for adult BMI in sensitivity
382 analyses. The effect sizes observed in the sensitivity analysis (by excluding BMI-associated
383 SNPs and by adjustment using a GRS for BMI) were slightly smaller compared to results of
384 the main analysis, which suggests that some of the effect was confounded by BMI. Our
385 restricted risk-score might not be totally BMI-unrelated, considering that Day et al.²⁴ found
386 age at menarche variants that appear unrelated to BMI at a nominal level in their sample to
387 be still BMI associated collectively ($P=4.2 \times 10^{-9}$). Because of this strong interrelationship
388 between age at menarche and BMI it is difficult to separate the SNPs into BMI-related and

389 BMI-unrelated variants. So our adjustment of the analysis by GRS-BMI might more
390 effectively control for BMI. Thus, any observed inverse relationship between age at
391 menarche and CRC risk in previous observational studies could have been due to inadequate
392 control of confounding by higher BMI in childhood.

393 For age at menopause, results of observational studies on the association with CRC risk have
394 also been inconclusive. The large NIH-AARP study observed a statistically significant
395 elevated risk for higher age at menopause in postmenopausal women (≥ 55 vs < 40 ; HR 1.50,
396 95% CI: 1.23, 1.83),⁹ whereas most other studies reported null associations.^{6, 31, 39}
397 Corresponding to the lack of association with age at menarche and age at menopause, the
398 GRS for the reproductive period as indicator for the lifetime estrogen exposure was also not
399 significantly associated to CRC risk. Additional adjustment of that analysis by education,
400 family history of CRC, ever regular aspirin use, MHT usage, BMI and smoking did not
401 substantially change the results (Supplementary Table 10). This is compatible with the
402 observation of no association between the reproductive period (≥ 36 years vs. ≤ 30 years)
403 and CRC risk in a prospective observational study conducted in Japan⁴³. The GRS for
404 lifetime for endogenous estrogen exposure, which we constructed, does not account for other
405 factors like parity or breast feeding, which influence overall estrogen exposure, however,
406 these factors have not been associated with CRC risk.

407 Results of prospective studies that investigated the association between serum levels of
408 endogenous estrogens and CRC risk have also been inconsistent. One study reported a
409 positive association between circulating estradiol and CRC risk,⁴⁴ another study observed an
410 inverse relationship⁴⁵ while most other studies found no associations.⁴⁶⁻⁴⁸ There are reports
411 that earlier age at menarche is associated with higher estrogen levels.^{49, 50} So estrogen levels
412 could also be a possible link between age at menarche and CRC risk. Due to the inconsistent
413 results of these reports, further studies are needed to clarify these associations.

414 On the other hand, observational studies reported that exogenous estrogen exposure by MHT
415 mainly in the form of combined estrogen-progestogen was associated with a reduced risk for
416 CRC.^{13, 51} The Womens's Health Initiative Clinical Trial (WHI-CT) reported no effect of

417 estrogen-alone therapy,^{52, 53} and a significant risk reduction for the association of estrogen
418 plus progestin vs. placebo and CRC risk,³³ which was suggested to have resulted from
419 diagnostic delay instead of true risk reduction⁵⁴. However, a recent meta-analysis which
420 summarized results of four clinical trials including WHI-CT and 16 observational studies
421 concluded that there is consistent evidence to support a protective effect of MHT on CRC
422 risk.⁴

423 Thus, it appears that exogenous and endogenous estrogens, which also vary in absolute
424 amount of estrogen, may play different roles in the development of CRC, presumably by
425 different mechanisms, which are not well understood. Estrogen acts in colon cells
426 predominantly through ER β ,^{14, 15} which exerts proapoptotic and anti-proliferative effects in
427 the colon¹⁶ and its expression is reduced in tumor tissue.^{17, 18} According to an in vivo study,
428 estrogen treatment was associated with an increase in expression of ER β in colon tissue,⁵⁵
429 supporting a mechanism by which MHT may affect CRC risk. There is also some evidence
430 that the protective effect of MHT on CRC risk may vary by the expression status of ER β .
431 Two studies found that the magnitude of risk reduction by MHT was different between
432 colorectal tumors with higher and with lower expression of ER β .^{56, 57} It is possible that the
433 effect of endogenous estrogens on CRC risk may be modulated by ER β expression as well.
434 Therefore, larger studies with data on expression of ER β in colon tissue are warranted to
435 assess whether the association of age at menarche/age at menopause and CRC risk differs by
436 ER β expression status.

437 In this MR study, we aimed to use proxies for start and endpoint of endogenous estrogen
438 exposure in women, specifically age at menarche/age at menopause, which themselves are
439 complex traits influenced by many variants with only small effects on the trait. Although we
440 did not see large pleiotropic effects using Egger regression, there might have been residual
441 pleiotropy, which is difficult to exclude. Previous studies of age at menarche performed LD
442 score regression using 123 SNPs associated with age at menarche and found, amongst others,
443 genetic correlations with BMI, adult height or Type 2 diabetes. Residual pleiotropy related to

444 adult height or Type 2 diabetes, which have been reported to be associated with higher risk
445 for CRC as well, cannot be fully excluded.⁵⁸ In addition, Day et al. reported genetic
446 correlations for the 54 age at menopause SNPs with adult obesity and other growth-related
447 traits. The top menopause-SNPs were also associated with fasting glucose and were enriched
448 in DNA repair pathways, yielding further sources of residual pleiotropy.²⁵ Residual
449 pleiotropy is a general limitation of MR, especially when exploring complex traits. When
450 considering the recently published hypothesis of an omnigenic model of complex traits,
451 coined „network pleiotropy“, essentially any regulatory variant in a trait-relevant cell-type
452 can have some effect on the trait.⁵⁹ This is because specific cell-types have specific
453 regulatory networks, where any single variant could affect trait relevant genes, „core genes“,
454 mediated through the same regulatory networks. So also for GWAS findings, it is highly
455 likely that some genetic variants exhibit horizontal pleiotropy.⁶⁰ As the selected age at
456 menarche/age at menopause SNPs might also contribute tiny effects on further traits through
457 network-pleiotropy, we cannot fully rule out pleiotropy. These limitations should be kept in
458 mind and methods to explore the impact of such effects should be developed. That said, our
459 sensitivity analyses especially Egger regression, did not indicate large pleiotropic effects.

460 The second assumption of MR is that the IV is not associated with confounding factors of
461 the observational association between age at menarche/menopause and CRC risk. We were
462 able to exclude most risk factors for CRC (smoking, family history of CRC, education,
463 aspirin use, MHT) as confounding variables except for BMI, which was accounted for using
464 several sensitivity analyses. In addition, substantial overlap between datasets used for
465 estimating SNP-exposure and SNP-outcome associations would bias results in the direction
466 of the observational estimate. There was some overlap between the studies (see
467 Supplementary Information on participant overlap) but unlikely to have substantially
468 influenced the results.

469 Strengths of our study include the large sample size, the availability of centrally harmonized
470 data and the robustness of the instrumental variables. Power calculations showed that our
471 study has limited power to detect weak effects. Therefore, we cannot exclude a weak

472 association of CRC with age at menarche or age at menopause. In summary, in our large MR
473 study, evidence is limited for causal associations between age at menarche/age at menopause
474 and CRC risk.

475 **Additional Information**

476 **Ethics approval and consent to participate**

477 All participants provided written, informed consent and studies were approved by their
478 respective institutional review boards.

479 **Availability of data and materials**

480 Genotyping data of the GECCO studies will soon be available at the database of Genotypes
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482 **Conflict of interest:**

483 The authors declare no conflict of interest.

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586 **Authors' contributions**

587 SN conducted the literature review, performed parts of the data analysis, interpreted the data
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Table 1: Association between age at menarche/age at menopause and CRC risk using MR analyses and sensitivity analyses											
		GRS-based analyses						2-sample MR ^f			
		MR estimate ^{a,b}		Adjusted by GRS-BMI ^{a,c}		Restricted risk score ^{a,d}		MR-Egger ^{b,e}		Weighted Median Estimator ^b	
Variable (per year)	Cases/Controls	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
Age at menarche	12 944/10 741	0.98	0.95-1.02	0.99	0.95-1.02	0.99	0.95-1.03	0.99	0.83-1.17	1.00	0.90-1.11
Age at menopause	12 944/10 741	0.98	0.94-1.01	NA	NA	NA	NA	1.02	0.94-1.10	1.00	0.95-1.05
Time period of hormone exposure	12 944/10 741	0.99	0.97-1.02	0.99	0.97-1.02	0.99	0.97-1.01	0.97	0.93-1.01	1.00	0.95-1.05

Abbreviations: BMI, body mass index; CCFR, colon cancer family registry; CI, confidence interval; CORECT, Colorectal Transdisciplinary study; GECCO, Genetics and Epidemiology of Colorectal Cancer Consortium; GRS, genetic risk score; MR, Mendelian randomization; NA, not applicable; OR, odds ratio per year, SNPs, single nucleotide polymorphisms; se, standard error.

^a Logistic regression model adjusted for age, study and principal components of genetic ancestry;

^b Meta-analyzed estimate of GECCO/CORECT datasets (CCFR centers participated in GECCO or CORECT and were analyzed as such);

^c additionally adjusted for a GRS for BMI out of 77 reported SNPs for BMI³³;

^d 42 BMI-associated SNPs were excluded from the age at menarche and time period of estrogen -exposure – risk scores;

^e estimate derived from the slope of MR-Egger;

^f estimates derived using summary statistics; se for calculation of CI obtained via bootstrapping.

Table 2. Association of genetically predicted age at menarche with CRC risk according to subgroups, CCFR, GECCO Consortium					
Subgroup	N (cases/controls)	OR^a	95% CI	P value	Heterogeneity P value^b
All	5832/6285	0.97	0.92-1.02	0.20	
Menopausal status					
Premenopausal	545/621	0.95	0.80-1.12	0.54	0.94
Postmenopausal	5263/5647	0.97	0.92-1.02	0.23	
Menopausal hormone therapy combined					
No	3266/3490	0.96	0.90-1.03	0.24	0.69
yes	593/807	0.98	0.85-1.14	0.82	
Estrogen monotherapy					
No	3120/3214	0.94	0.88-1.01	0.11	0.17
yes	716/1088	1.04	0.91-1.19	0.55	
Site					
Colon	4037/6285	0.97	0.91-1.02	0.26	0.25 ^c
Rectum	1184/6285	1.04	0.95-1.14	0.36	

Abbreviations: CCFR, colon cancer family registry; CI, confidence interval; GRS, genetic risk score; OR, odds ratio per year.

^a all analyses adjusted for age, sex, study and principal components of genetic ancestry;

^b P value calculated using likelihood ratio tests comparing the model with and without interaction term;

^c P value for heterogeneity was obtained in case-only analysis of colon vs. rectal cancer.

Table 3. Association of genetically predicted age at menopause and CRC risk according to subgroups, CCFR, GECCO Consortium					
	N (cases/controls)	OR^a	95% CI	P value	Heterogeneity P value^b
All	5832/6285	0.99	0.95-1.03	0.56	
Combined estrogen-progesterone therapy					
No	3266/3490	0.99	0.93-1.05	0.78	0.75
Yes	593/807	0.96	0.85-1.10	0.58	
Estrogen monotherapy					
No	3120/3214	0.99	0.93-1.05	0.27	0.76
Yes	716/1088	1.01	0.89-1.14	0.90	
BMI					
Normal weight	2146/2665	1.00	0.93-1.07	0.97	0.50
Overweight	1866/1918	0.99	0.91-1.07	0.77	
Obese	1284/1115	1.00	0.91-1.11	0.93	
Underweight	74/80	0.63	0.40-0.98	0.04	
Site					
Colon	4037/6285	0.98	0.94-1.03	0.53	0.61 ^c
Rectum	1184/6285	0.98	0.91-1.06	0.65	

Abbreviations: BMI, body mass index; CCFR, colon cancer family registry; CI, confidence interval; GRS, genetic risk score; OR, odds ratio per year.

^a all analyses adjusted for age, sex, study and principal components of genetic ancestry

^b P value calculated using likelihood ratio tests comparing the model with and without interaction term

^c P value for heterogeneity was obtained in case-only analysis of colon vs. rectal cancer