Genetic variation in the *ADIPOQ* gene, adiponectin concentrations and risk of colorectal cancer – a Mendelian Randomization analysis using data from three large cohort studies

Katharina Nimptsch[1,2], Mingyang Song[2,3,4,5], Krasimira Aleksandrova[6], Michalis Katsoulis[7], Heinz Freisling[8], Mazda Jenab[8], Marc J. Gunter[8], Konstantinos K. Tsilidis[9,10], Elisabete Weiderpass[11,12,13,14], H Bas Bueno-De-Mesquita[9,15,16], Dawn Q Chong[4,5,17], Majken K. Jensen[2,18], Chunsen Wu[19], Kim Overvad[19] , Tilman Kühn[20], Myrto Barrdahl[20], Olle Melander[21], Karin Jirström[21], Petra H. Peeters[22,23], Sabina Sieri[24] , Salvatore Panico[25], Amanda J. Cross[9], Elio Riboli[9] , Bethany Van Guelpen[26], Robin Myte[26] , José María Huerta[27, 28], Miguel Rodriguez-Barranco[28,29], José Ramón Quirós[30], Miren Dorronsoro[31], Anne Tjønneland[32], Anja Olsen[32], Ruth Travis[33], Marie-Christine Boutron-Ruault [34,35], Franck Carbonnel[34,35,36], Gianluca Severi[34,35,36,37], Catalina Bonet[38], Domenico Palli[39], Jürgen Janke[1], Young-Ae Lee[40], Heiner Boeing[6], Edward L. Giovannucci[2,3,18], Shuji Ogino[3,18,41,42], Charles S. Fuchs[18,41], Eric Rimm[2,3,18], Kana Wu[2], Andrew T. Chan[4,5,18,43], Tobias Pischon[1,44]

[1] Molecular Epidemiology Research Group, Max Delbrück Center for Molecular Medicine (MDC), Berlin, Germany

[2] Department of Nutrition, Harvard T.H. Chan School of Public Health, Boston, Massachusetts, USA

[3]Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, Massachusetts, USA

[4] Division of Gastroenterology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts, USA

[5] Clinical and Translational Epidemiology Unit, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts, USA

[6] Department of Epidemiology, German Institute of Human Nutrition (DIfE), Potsdam-Rehbruecke, Nuthetal, Germany

[7] Hellenic Health Foundation, Athens, Greece

[8] International Agency for Research on Cancer (IARC-WHO), Lyon, France

[9] Department of Epidemiology and Biostatistics, The School of Public Health, Imperial College London, London, United Kingdom

[10] Department of Hygiene and Epidemiology, University of Ioannina School of Medicine, Ioannina, Greece

[11] Department of Community Medicine, Faculty of Health Sciences, University of Tromsø, The Arctic University of Norway, Tromsø, Norway

[12] Department of Research, Cancer Registry of Norway, Oslo, Norway

[13] Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, Stockholm, Sweden

[14] Samfundet Folkhälsan, Helsinki, Finland

[15] Department for Determinants of Chronic Diseases (DCD), National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands

[16] Department of Social & Preventive Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

[17] Division of Medical Oncology, National Cancer Centre Singapore, Singapore

[18] Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital, and Harvard Medical School, Boston, Massachusetts, USA

[19] Section for Epidemiology, Department of Public Health, Aarhus University, Aarhus, Denmark

[20] Division of Cancer Epidemiology, German Cancer Research Center, Heidelberg, Germany

[21] Department of Clinical Sciences Lund, Lund University, Malmö, Sweden

[22] Dept of Epidemiology, Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht, the Netherlands

[23] MRC-PHE Centre for Environment and Health, Department of Epidemiology and Biostatistics, School of Public Health, Imperial College, London, UK

[24] Epidemiology and Prevention Unit Fondazione IRCCS Istituto Nazionale dei Tumori, Milano, Italy

[25] Dipartimento di Medicina Clinica e Chirurgia, Federico II University, Naples, Italy

[26] Department of Radiation Sciences, Oncology, Umeå University, Umeå, Sweden

[27] Department of Epidemiology, Murcia Regional Health Council, IMIB-Arrixaca, Murcia, Spain

[28] CIBER Epidemiología y Salud Pública (CIBERESP), Spain

[29] Escuela Andaluza de Salud Pública. Instituto de Investigación Biosanitaria ibs.GRANADA. Hospitales Universitarios de Granada/Universidad de Granada, Granada, Spain

[30] Public Health Directorate, Asturias, Spain

[31] Public Health Direction and Biodonostia Research Institute- Ciberesp, Basque Regional Health Department, San Sebastian Spain

[32] Danish Cancer Society Research Center, Copenhagen, Denmark

[33] Cancer Epidemiology Unit, Nuffield Department of Population Health, University of Oxford, Oxford, UK

[34] Université Paris-Saclay, Université Paris-Sud, UVSQ, CESP, INSERM, Villejuif, France

[35] Gustave Roussy, F-94805, Villejuif, France

[36] Human Genetics Foundation (HuGeF), Torino, Italy

[37] Cancer Council Victoria and University of Melbourne, Australia

[38] Unit of Nutrition and Cancer, Cancer Epidemiology Research Programme, Catalan Institute of Oncology (ICO), Barcelona, Spain.

[39] Cancer Research and Prevention Institute (ISPO) Florence, Italy

[40] Genetics of Allergic Disease Research Group, Max Delbrück Center for Molecular Medicine (MDC), Berlin, Germany

[41] Department of Medical Oncology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts, USA

[42] Division of MPE Molecular Pathological Epidemiology, Department of Pathology, Brigham and Women's Hospital, and Harvard Medical School, Boston, Massachusetts, USA

[43] Broad Institute, Cambridge, Massachusetts, USA

[44] Charité Universitätsmedizin, Berlin, Germany

Corresponding author:

Dr. Katharina Nimptsch

Molecular Epidemiology Research Group

Max Delbrück Center for Molecular Medicine (MDC)

Robert-Rössle-Straße 10

13125 Berlin

Tel.: ++49 30 / 9406 – 4573

Fax: ++49 30 / 9406 – 4576

e-mail: katharina.nimptsch@mdc-berlin.de

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Abstract

Higher levels of circulating adiponectin have been related to lower risk of colorectal cancer in several prospective cohort studies, but it remains unclear whether this association may be causal. We aimed to improve causal inference in a Mendelian Randomization meta-analysis using nested case-control studies of the European Prospective Investigation into Cancer and Nutrition (EPIC, 623 cases, 623 matched controls), the Health Professionals Follow-up Study (HPFS, 231 cases, 230 controls) and the Nurses' Health Study (NHS, 399 cases, 774 controls) with available data on pre-diagnostic adiponectin concentrations and selected single nucleotide polymorphisms (SNPs) in the *ADIPOQ* gene. By summing genotypes associated with higher adiponectin concentration using either study-specific weights (internal score) or weights from genome-wide association studies (external score), we created allele-scores that explained between 3% and 4% of the interindividual variation in adiponectin concentrations. Neither the internal (pooled OR per score-unit 0.99, 95% CI 0.95, 1.03) nor the external (pooled OR 0.97, 95% CI 0.90, 1.06) *ADIPOQ* allele-scores were associated with risk of colorectal cancer in logistic regression analyses. Genetically determined two-fold higher adiponectin was not significantly associated with risk of colorectal cancer using the internal (pooled OR 0.82, 95% CI 0.45, 1.51) or external score (pooled OR 0.83, 95% CI 0.44, 1.55) as instrumental variables as well as in a summary instrumental variable analysis using previously published data (OR 1.00, 95% CI 0.84, 1.19), that had higher statistical power. Thus, our study does not support a large causal effect of circulating adiponectin on colorectal cancer risk. Due to the limited genetic determination of adiponectin, larger Mendelian Randomization studies are necessary to clarify whether adiponectin is causally related to lower risk of colorectal cancer.

Keywords: adiponectin, *ADIPOQ*, colorectal cancer, Mendelian Randomization

Background

Obesity, in particular abdominal obesity is an established risk factor for the development of colorectal cancer [1]. Although the underlying biological mechanisms have not been fully elucidated, it is widely accepted that the adipose tissue, particularly visceral adipose tissue, is an active endocrine organ secreting various bioactive substances collectively named adipokines, which may provide an important link between body fatness and colorectal cancer risk [2]. In contrast to many other adipokines, adiponectin expression is suppressed in obesity and plasma concentrations are lower in obese than in lean individuals [3]. Adiponectin has been suggested to play a protective role in the development of cancer either directly through inhibition of cell growth (e.g. via RAS signaling [4]) and induction of apoptosis, or indirectly through improved insulin sensitivity and reduced inflammation [5]. The association between circulating adiponectin concentrations and risk of colorectal cancer has been investigated in several prospective cohort studies, with mixed findings: Higher plasma adiponectin concentrations were associated with lower risk of colorectal cancer (slightly stronger in women than men, but no statistically significant sex-differences) in the European Prospective Investigation into Cancer and Nutrition (EPIC) [6] and in the Health Professionals Follow-up study (HPFS), while no association was observed in the Nurses' Health Study (NHS) [7]. A meta-analysis of ten case-control or nested case-control studies (not including the data from EPIC, NHS or HPFS) reported a statistically significant two percent lower risk of colorectal cancer or adenoma for a 1 µg/mL increment in adiponectin in men whereas among women no association was observed [8].

To date, it remains unclear whether adiponectin plays a causal role in the development of colorectal cancer not least because it cannot be excluded that residual confounding and/or reverse causation bias might have introduced bias in observational associations (Figure 1). Mendelian Randomization is a statistical approach that can improve causal inference [9]. The principle is that under the assumption of the random assortment of alleles at conception, genetic variants that are associated with biomarker levels can be used as relatively unbiased proxies for biomarker concentrations due to two advantages. First, since the genotype of an individual is determined at gamete formation and cannot be altered later on (e.g. by disease onset), there is no possibility of reverse causation [10]. Second, the relationship between genetic variants and disease risk can be assumed to be not confounded by lifestyle and behavioral factors that can confound the observed association between circulating biomarkers and risk of disease. Therefore, using genetic variants associated with circulating biomarker concentrations in a Mendelian Randomization approach

may provide insight into the underlying causal relationships by circumventing reverse causation and residual confounding. In a pooled analysis from the Genetics and Epidemiology of Colorectal Cancer Consortium (GECCO), which includes data from NHS, HPFS and eight other studies comprising overall more than 7,000 colorectal cancer cases and approximately the same number of controls, genetic variants in the gene encoding adiponectin (*ADIPOQ*) were not associated with colorectal cancer risk [11]. However, a simultaneous analysis of adiponectin concentrations, *ADIPOQ* genetic variants and colorectal cancer was not conducted, because adiponectin plasma levels were only available in a subset of included studies, namely NHS and HPFS. With a dataset including individual participant data on genetic variants, biomarker concentration and disease outcome, a traditional Mendelian Randomization analysis taking into account the actual strength of the association between *ADIPOQ*-SNPs and adiponectin concentrations in the study population can be performed, which has the advantage that instrumental variable assumptions can be directly assessed [12, 13]. The aim of our investigation aims was therefore to improve causal inference in the association between circulating adiponectin and colorectal cancer risk using *ADIPOQ* genetic variants in a Mendelian Randomization meta-analysis with individual participant data from the EPIC, HPFS and NHS cohorts.

Methods

Study population

The three studies included in the present investigation were all nested case-control studies of large prospective cohorts with long follow-up. In all nested case-control studies, colorectal cancer was defined according to the International Statistical Classification of Diseases, Injury and Causes of Death (ICD-10) as cancers of the colon (C18.0-C18.7), cancers of the rectum (C19-C20) and tumors that were overlapping or unspecified (C18.8-C18.9). Blood samples were collected prior to diagnosis and matched control participants were selected using incidence density sampling, i.e. selection was performed among study participants who were alive and free of cancer (except non-melanoma skin cancer) at the time of diagnosis of the colorectal cancer case.

The EPIC study is a large multicenter prospective cohort including more than 520,000 study participants from 10 European countries who were aged between 35 and 70 years at recruitment which took place from 1992 to 2000 [14]. Baseline examinations included anthropometric

measurements, standardized ascertainment of lifestyle characteristics and medical history information as well as collection of blood samples. The EPIC study was approved by the ethical review board of the International Agency for Research on Cancer (IARC, Lyon, France) and the institutional review boards of each participating study center and informed consent was obtained from all participants. Incident cancer cases including colorectal cancer cases were determined through record linkage with local cancer registries in most countries (Denmark, Italy (except Naples), the Netherlands, Norway, Spain, Sweden, United Kingdom, complete up to 2003). In some countries (France, Germany, Naples (Italy), Greece, complete up to 2002) active follow-up was organized by contact of participants or next of kin through mailed questionnaires, followed by verification of self-reported cases by study physicians using health insurance data, data from cancer and clinical registries as well as medical records provided by the treating physicians. In the present analysis, colorectal cancer cases with available prediagnostic blood samples and DNA were included. As has been described previously [6], the nested case-control design matched each case to one control using incidence density sampling. Control participants were selected matched on age at blood collection (2 months to 4-year intervals), study center, fasting status (<3, 3-6, or>6 hours) as well as menopausal status and hormone use in women. The nested case-control study was designed to be applicable for several biomarker studies, which explains inclusion of the latter matching criteria which were not relevant for the present analysis. The number of cases and matched controls included in the present study is 1,246 (623 cases, 623 matched controls) which is 52% of the study size of the previous analysis on circulating adiponectin and risk of colorectal cancer in EPIC (1,206 cases, 1,206 matched controls) [6]. This difference is largely explained by unavailability of DNA samples from the Danish EPIC centers due to local technical and organizational issues.

The HPFS and NHS are two large US cohort studies, detailed descriptions of which are provided elsewhere [15, 16]. In brief, the HPFS started in 1986, including 51,529 men aged 40-75 years, and the NHS started in 1976 and included 121,701 women aged 30-55 years. In both cohorts, study participants provided information on medical history and lifestyle at recruitment. Since then, follow-up questionnaires were administered biennially to collect and update medical and lifestyle information and to elicit medical diagnoses. The follow-up rates in both cohorts exceeded 90% in each 2-year cycle and the cumulative follow-up rate (percentage of potentially collected person-years) was 94% in HPFS and 93% in NHS. Blood specimens were provided by

18,225 HPFS participants (35%) between 1993 and 1995 and by 32,826 NHS participants (27%) between 1989 and 1990 by overnight courier. Details on the procedures of blood collection as well as handling and storage of blood samples have been described previously [17, 18]. Among the participants for whom blood samples and DNA were available, 231 colorectal cancer cases were confirmed after blood collection in HPFS (up to January 1st 2008) and 399 in NHS (up to October 1st 2008). For each case up to two controls were randomly selected using incidence density sampling. The majority of individuals included in the nested case-control studies were of Caucasian ancestry in both HPFS (95.5%) and NHS (99.9%). All study participants provided informed consent and the study protocol was approved by the Institutional Review Board of the Brigham and Women's Hospital and the Harvard T.H. Chan School of Public Health.

The total number of participants in the present investigation is 2,880 (1,253 cases and 1,627 controls), including 1,246 in EPIC (623 cases and 623 controls), 461 in HPFS (231 cases, 230 controls) and 1,173 in NHS (399 cases, 774 controls).

Adiponectin measurement

Total circulating adiponectin concentration was measured using enzyme-linked immunosorbent assays from ALPCO Diagnostics (Salem, New Hampshire) in the three studies [6, 7]. Based on quality control samples, interbatch coefficients of variation were 8.3% in EPIC and 8.6% in HPFS and NHS. Adiponectin measurements in n=300 paired samples from HPFS showed high reliability, with intraclass correlation coefficient of 0.85 when measured within the same persons one year apart [19].

SNP selection and genotyping

In EPIC, a set of tagging SNPs covering variations in the *ADIPOQ* gene in populations of European ancestry was selected using HapMap 22/phase II CEPH population data (Utah residents with northern and western European descent) applying stringent criteria (minor allele frequency >5% and pairwise $r^2 \geq 0.8$). A total number of 15 SNPs were genotyped using TaqMan methodology (genotype call rates >99.2% for all the assays), of which one (rs7649121) was not in Hardy-Weinberg equilibrium in control participants ($p<0.0001$) and therefore was excluded from analysis. In HPFS and NHS SNPs in the *ADIPOQ* gene were selected based on previous evidence from genome-wide association studies (GWAS) on circulating adiponectin

concentrations [20-23]. Additional SNPs in adiponectin-related genes that have been associated with colorectal cancer risk were genotyped [24-26]. A total of 19 SNPs were genotyped using Illumina HumanOmniExpress as part of the GECCO project[7]. Missing SNPs were imputed to HapMap II release 24. All genotyped SNPs were in Hardy-Weinberg equilibrium in control participants. Eight *ADIPOQ* SNPs were available in all three included studies (rs1063539, rs16861194, rs822394, rs17300539, rs17366568, rs17366743, rs266729, rs1501299) and minor allele frequencies were comparable.

Statistical analysis

For the Mendelian Randomization analysis we first investigated the relation between individual *ADIPOQ*-SNPs and adiponectin concentrations. We used these results to construct *ADIPOQ* allele scores, which were then used to derive Mendelian Randomization estimates using two different approaches: Firstly, we analyzed the *ADIPOQ* allele scores in relation to colorectal cancer risk. Secondly, we applied an instrumental variable approach, simultaneously incorporating *ADIPOQ*-SNPs and plasma adiponectin concentrations, to model the association between genetically determined circulating adiponectin and colorectal cancer risk. While the first approach, which is considered as an equivalent to the intention-to-treat analysis in a randomized controlled trial [27], can only test for the existence of a causal association, the second approach aims at estimating the magnitude of a causal association (e.g. risk estimate per 2-fold higher genetically determined adiponectin) [28].

ADIPOQ allele scores

The associations between each SNP and adiponectin concentrations were examined using linear regression models with robust variance in control participants [29]. Adiponectin concentrations were naturally log-transformed (because of skewed distribution) and we calculated the estimated relative change in percent in adiponectin per minor allele (with genotypes coded 0, 1 or 2 according to the number of variant alleles). In addition, R^2 and F-values as measures of instrument strength are presented. In the previous publication by Song et al. [11] the association between ADIPOQ SNPs and plasma adiponectin concentrations was presented for HPFS and NHS, but the here included colorectal cancer controls were only a small subset of the individuals included in that analysis. We created study-specific ("internal") allele scores by summing alleles that were statistically significantly associated with higher adiponectin using the estimated

coefficients from the linear regression models as weights. In addition, we created an ("external") allele score that was valid across studies. For this purpose, only SNPs that were statistically significantly associated with circulating adiponectin in at least two of the included studies were incorporated in the score. The weights for this "external" score were derived from a meta-analysis of GWAS on adiponectin levels [20]. To examine whether the allele scores are independent of potentially confounding factors, we compared baseline characteristics in each study across score categories.

Association between allele scores and colorectal cancer

The association between the internal and external allele scores (per score-unit) in relation to risk of colorectal cancer was calculated in each study. In EPIC, we used conditional logistic regression conditioning on the matching variables and calculating odds ratios (ORs) and 95% confidence intervals that approximate incidence rate ratios and can be interpreted as relative risks. In HPFS and NHS, we used unconditional logistic regression adjusted for matching variables (age at blood draw and date of blood draw) to estimate relative risks. In sensitivity analyses, we restricted the logistic regression models to individuals with Caucasian ancestry (n=16 excluded in HPFS and n=1 excluded in NHS). Because multivariable adjustment is per definition not required in Mendelian Randomization studies, only minimally adjusted (conditional logistic regression conditioned on the matching variables or unconditional logistic regression adjusted for matching factors) estimates are presented. We pooled the study-specific results for the internal and external scores using a meta-analytic approach with random effects (39), thereby also assessing potential heterogeneity across studies. We also investigated whether ORs were different after additional adjustment for measured adiponectin (missing adiponectin values (2% in EPIC, 26% in HPFS, 32% in NHS) were imputed with sex-specific median values for this sensitivity analysis).

Instrumental variable analysis

For the joint analysis of adiponectin concentrations, genetic variants of the *ADIPOQ* gene and colorectal cancer risk, we performed an instrumental variable analysis using two-stage regression. In the first stage, adiponectin concentrations were predicted based on the genetic instruments (allele scores) by means of linear regression. In order to avoid potential bias [30], the first stage regression was performed only in control participants and genetically determined adiponectin was

predicted for the total study population including participants without measured adiponectin. In the second stage, a logistic regression of colorectal cancer on the predicted adiponectin concentrations was performed in each study. In EPIC, the second stage was a conditional logistic regression appropriate for the matched design, whereas in HPFS and NHS, the second stage was an unconditional logistic regression adjusted for matching factors. For HPFS and NHS, we restricted instrumental variable analyses to individuals of Caucasian ancestry in sensitivity analyses. The risk estimates resulting from the instrumental variable analysis display the association between 2-fold genetically determined higher adiponectin in relation to risk of colorectal cancer. Pooled associations were determined using random effects model and potential heterogeneity was assessed. Finally, to increase statistical power, we performed a summary instrumental variable analysis using published data [13]. Parameters for the association between the three SNPs included in the external score and circulating adiponectin were taken from GWAS data [20] and parameters for the association between the SNPs and colorectal cancer were derived from the analysis in GECCO [11].

All statistical tests are two-sided with significance at the 5% level. Instrumental variable analyses were performed using the STATA SE 12 (StataCorp, College Station, Texas, USA). Summary instrumental variable analyses were performed with a publicly available R-Studio application (Foundation for Statistical Computing, Vienna, Austria). All other analyses were performed using SAS (for EPIC data: SAS Enterprise Guide 4.3; for HPFS and NHS data: SAS 9.3; SAS Institute Inc., Cary, North Carolina, USA).

Results

Baseline characteristics of study participants in EPIC, HPFS and NHS are displayed in table 1. In EPIC and HPFS, incident cases had a higher body mass index (BMI) and waist circumference at baseline, whereas in NHS, these anthropometric measures did not differ between case and control participants. In EPIC, colorectal cancer cases consumed more alcohol and red and processed meat than control participants, whereas in the US cohorts, no such differences were observed. Other potentially confounding factors including physical activity and fiber intake did not differ remarkably between cases and controls in any study. In EPIC and HPFS, but not in NHS,

adiponectin concentrations were lower in cases than in control participants in univariate comparisons.

Of the 14 *ADIPOQ* SNPs available for analysis in EPIC, five SNPs (rs17300539, rs17366568, rs17366743, rs1501299, rs3774261) were statistically significantly associated with circulating adiponectin and incorporated in the internal weighted *ADIPOQ*-score for EPIC (Table 2). Each score unit was associated with 7.0% (95% 4.0, 10.0) higher adiponectin. The internal score explained 3.6% of the interindividual variation in adiponectin concentrations (F-value 22.9). In HPFS, only three (rs6810075, rs266729, rs1501299) of the 19 genotyped SNPs were statistically significantly associated with adiponectin concentrations (Table 3). After creating an internal allele-score with these SNPs, each score-unit was associated with 7.0% (95% CI 1.9, 12.3) higher adiponectin (F-value 7.3). In NHS, six of the 19 available SNPs were statistically significantly associated with circulating adiponectin (rs17300539, rs17366568, rs6773957, rs6444175, rs1501299, rs1063538). The resulting internal score was associated with 3.4% (95% CI 1.6, 5.2) higher adiponectin concentrations (F-value 14.2). Of the SNPs genotyped, eight were available in all three studies (rs1063539, rs16861194, rs822394, rs17300539, rs17366568, rs17366743, rs266729, rs1501299). Three *ADIPOQ* SNPs were associated with adiponectin concentrations in at least two studies: rs1501299 (EPIC, NHS, HPFS), rs17300539 (EPIC and NHS), rs1736658 (EPIC and HPFS). Using external weights we created an external allele score to be used across all studies (0.07*no. of T-alleles of rs1501299 + 0.18*no. of A-alleles of rs17300539 + 0.15*no. of G-alleles of rs17366568, divided by the sum of weights). The external score was statistically significantly associated with higher circulating adiponectin in EPIC (per score unit 8.4% higher, 95% CI 4.6, 12.4), HPFS (per score unit 9.5% higher, 95% CI 0.7, 19.1) and NHS (per score unit 10.2% higher, 95% CI 5.5, 15.2), explaining between 2.6% and 3.5 % of interindividual variance (F-values 19.5 in EPIC, 4.5 in HPFS, 18.6 in NHS).

Potentially confounding factors assessed in the three cohorts did not differ remarkably across categories (approximate tertiles) of the external *ADIPOQ*-score (all P-values >0.005, P-value Bonferroni-corrected for 10 tests; supplemental table 1).

We investigated whether the internal or external *ADIPOQ* scores are associated with lower risk of colorectal cancer in logistic regression analysis. Neither the internal (pooled OR 0.99, 95% CI 0.95, 1.03) nor the external (pooled OR 0.97, 95% CI 0.90, 1.06) *ADIPOQ* scores were

significantly associated with risk of colorectal cancer (table 4). The non-significant inverse parameter estimates in EPIC were slightly attenuated after adjustment for circulating adiponectin concentrations, while they remained unchanged in NHS and HPFS (supplemental table 2). Results were not altered when logistic regression analyses were restricted to Caucasian individuals in HPFS and NHS (internal scores, pooled OR 0.99, 95% CI 0.94, 1.03; external score, pooled OR 0.97, 95% CI 0.90, 1.05). In the instrumental variable analysis taking measured adiponectin and *ADIPOQ* genetic variation in our study population simultaneously into account (table 5), using the internal *ADIPOQ* scores, genetically determined two-fold higher adiponectin was not significantly associated with lower risk of colorectal cancer (pooled OR 0.82, 95% CI 0.45, 1.51). Similarly, using the external adiponectin score, no significant association with risk of colorectal cancer was observed per two-fold higher genetically determined adiponectin (pooled OR 0.83, 95% CI 0.44, 1.55). These associations were not altered by restriction to Caucasians in HPFS and NHS (internal scores as IV, pooled OR 0.81, 95% CI 0.45, 1.48; external score, pooled OR 0.82, 95% CI 0.44, 1.53)

In the summary instrumental variable analysis using published data (GWAS on adiponectin [20]; associations of *ADIPOQ*-SNPs with colorectal cancer published by the GECCO consortium [11]), no association between genetically determined higher adiponectin and risk of colorectal cancer was observed (OR per 2-fold higher adiponectin 1.00, 95% CI 0.84, 1.19) using the three SNPs included in the external score as instruments.

Discussion

In this Mendelian Randomization analysis using data from three nested case-control studies of large prospective cohorts, we did not find evidence for a causal contribution of high adiponectin levels to lower risk of colorectal cancer. However, adiponectin concentrations were genetically determined only to a limited extent, which limited statistical power for our Mendelian Randomization analysis.

In a genetic association meta-analysis, the minor alleles of three *ADIPOQ* SNPs (rs1501299, rs2241766, rs266729) were associated with colorectal cancer risk [31], but associations were only seen in Asians and not in Caucasians. Individual SNPs at the *ADIPOQ* loci, including those incorporated in the *ADIPOQ* scores in the present study (rs17300539, rs17366568, rs17366743,

rs1501299, rs3774261 (the proxy SNPs rs2241766 was used in GECCO), rs6810075, rs266729, rs6773957, rs6444175, rs1063538) were unrelated to risk of colorectal cancer in GECCO [11]. In the same study, the allele-sum of 16 SNPs that have been related to higher adiponectin concentrations in previous GWAS were combined in a genetic score, which was not related to colorectal cancer risk in women (OR per ten-allele increment 1.08, 95% CI 0.95, 1.22) or men (OR 1.01, 95% CI 0.90, 1.13). In contrast, in a two-sample Mendelian Randomization meta-analysis [32] using the *ADIPOQ* SNP rs2241766 as instrumental variable, 1 mg/L genetically determined higher adiponectin was associated with a 20-40% higher risk of colorectal cancer.

The strength of our study is the ability to jointly investigate adiponectin, genetic variation in the *ADIPOQ* gene and risk of colorectal cancer. In contrast to a two-sample Mendelian Randomization design, a full sample design, where genetic information and intermediate phenotype data (i.e. measured adiponectin concentration) are available in the same study participants, generally requires less assumptions and allows for systematic evaluation of instrumental variable assumptions [33]. Thus, we were able to estimate the strength of the association between the *ADIPOQ* SNPs and adiponectin concentrations in our sample, thereby showing that the first Mendelian Randomization assumption was fulfilled. Furthermore, we showed that potentially confounding lifestyle factors did not vary substantially across categories of the instrumental variable, i.e. the second Mendelian Randomization assumption was also satisfied [34]. It should be noted that only potentially confounding factors measured in the three studies could be investigated, thus, it cannot be entirely excluded that unmeasured confounders varied by allele scores. Assessment of the third Mendelian Randomization assumption (instrumental variable is associated with the outcome only through the intermediate exposure of interest, i.e. no pleiotropy) is not as straightforward, but the use of multiple SNPs as instrumental variables argue against unknown pleiotropy. Also the observed attenuation of inverse estimates after additional adjustment for measured adiponectin in logistic regression analyses on *ADIPOQ*-scores in EPIC argues against violation of the third assumption.

However, our study has also several limitations: Given the limited genetic determination of adiponectin concentrations, our sample sizes from three nested case-control studies of prospective cohorts was limited to derive robust causal estimates. The *ADIPOQ*-score applied here explained only a low proportion (2.6%-3.6%) of the interindividual variation in adiponectin concentrations. With this genetic instrument and our sample size, the minimal OR that could have

been detected with 80% statistical power was 0.61 per standard deviation in adiponectin, which is a stronger association than has been observed in most observational studies [8]. Even with the relatively large sample size in GECCO (7,020 cases, 7,631 controls) no association between a genetic score of variants associated with adiponectin and colorectal cancer was detected [11]. Furthermore, in our summary data instrumental variable analysis, genetically determined higher adiponectin was not associated with colorectal cancer risk (minimal detectable OR with 80% power: 0.76 per standard deviation in adiponectin). A much larger sample size (n=33,960 cases, 33,960 controls) would be necessary to detect a moderate effect (e.g. OR 0.89 per 1 SD as observed in EPIC [6]) of adiponectin and colorectal cancer. Therefore, with our study and the summary instrumental variable analysis based on GECCO, we cannot rule out causality in the association between circulating adiponectin and risk of colorectal cancer. Although it has been suggested that the 2-stage instrumental variable estimator may result in biased estimates under case-control sampling, it has been shown to be unbiased under the null hypothesis of no causal effect as in the present study [35].

In conclusion, this Mendelian Randomization meta-analysis using data from three nested case-control studies of prospective cohorts does not support a large causal effect of circulating adiponectin on colorectal cancer risk. This lack of association may be related to the limited genetic determination of adiponectin and the limited sample size. Therefore, larger Mendelian Randomization studies are necessary to clarify whether adiponectin is causally related to lower risk of colorectal cancer.

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Figure 1. Directed acyclic graph (DAG) for Mendelian Randomization Study on adiponectin and colorectal cancer risk.

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Supplemental Table 1 Baseline characteristics by tertiles of the weighted ADIPOQ-score in control participants in EPIC, HPFS, and NHS

Table 4. Association between internal and external ADIPOQ-scores in EPIC, HPFS, and NHS

Supplemental Table 2. Association between internal and external ADIPOQ-scores in EPIC, HPFS, and NHS after additional adjustment for measured adiponectin

Table 5. Instrumental variable estimation of the association between genetically determined adiponectin concentrations with risk of colorectal cancer in EPIC, HPFS, and NHS

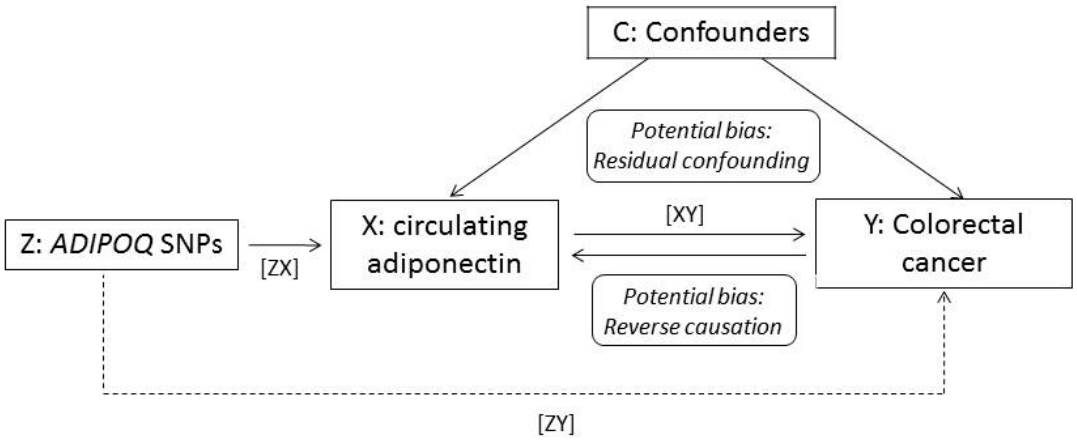

Figure 1. Directed acyclic graph (DAG) for Mendelian Randomization Study on adiponectin and colorectal cancer risk. X: modifiable exposure of interest; Y: outcome; C: confounder(s); Z: instrumental variable. NOTE: The effect of Z on Y should be mediated only through X (no pleiotropy), therefore this line is dashed. Associations [ZX] and [ZY] are used to estimate the causal effect of a biomarker on an outcome circumventing residual confounding and reverse causation.

Table 1 Baseline characteristics of study participants in EPIC, HPFS and NHS

	EPIC (n=1,246)		HPFS (n=461)		NHS (n=1,173)	
	Controls (n=623)	Cases (n=623)	Controls (n=230)	Cases (n=231)	Controls (n=774)	Cases (n=399)
Female sex, n (%)	289 (46.4)	289 (46.4)	0 (0)	0 (0)	774 (100)	399 (100)
Age at blood collection, years, mean (SD)	58.3 (8.2)	58.3 (8.2)	65.6 (8.9)	66.1 (8.8)	59.1 (6.7)	59.2 (6.7)
Current smoking, n (%)	124 (19.9)	122 (19.6)	12 (5.4)	8 (3.6)	90 (11.7)	48 (12.1)
Physical activity (MET-hours/week), mean (SD)	89.1 (52.1)	90.4 (54.8)	34.5 (29.2)	35.8 (41.3)	16.4 (19.8)	16.5 (19.2)
Body mass index, kg/m^2, mean (SD)	26.4 (3.8)	27.1 (4.4)	25.2 (3.3)	26.0 (3.1)	25.4 (4.4)	25.3 (4.3)
Waist circumference, cm, mean (SD)	89.3 (12.3)	91.9 (13.1)	94.2 (9.4)	96.8 (8.4)	79.5 (10.7)	80.3 (10.9)
Alcohol intake, g/day, median (IQR)	6.4 (1.0-21.1)	7.8 (0.8-22.6)	7.0 (1.8-15.8)	6.9 (0.9-18.7)	1.1 (0.00-6.9)	1.8 (0.00-8.5)
Fiber, g/day, median (IQR)	21.8 (17.7-27.0)	21.5 (16.8-27.5)	22.7 (18.6-28.8)	22.2 (18.4-27.2)	18.1 (15.1-21.3)	17.7 (15.2-21.1)
Red and processed meat, g/day, median (IQR)	69.1 (45.4-101.5)	72.3 (49.4-108.8)	63.4 (33.5-98.1)	64.5 (37.5-105.5)	52.4 (33.3-81.1)	55.8 (33.4-91.7)
Total adiponectin (µg/mL), median (IQR)[a]	6.3 (4.8-8.7)	5.9 (4.3-8.2)	5.6 (3.9-8.2)	5.3 (3.5-7.2)	8.5 (6.0-10.9)	8.5 (6.0-11.5)

SD, standard deviation, IQR, inter-quartile range, MET, metabolic equivalent of task

[a] Adiponectin measurement was not available in n=16 controls and n=7 cases in EPIC, in n=63 controls and n=56 cases in HPFS, and in n=264 controls and n=112 cases in NHS; some study participants had missing values for the here displayed diet and lifestyle factors: in EPIC, there were missing values on physical activity (n=69 controls, n=67 cases) and waist circumference (n=65 controls, n=65 cases); in HPFS, there were missing values on smoking status (n=7 controls, n=6 cases), waist circumference (n=25 controls, n=46 cases), alcohol (n=4 controls, n=5 cases), fiber (n=1 case) or red and processed meat (n=4 controls, n=5 cases) intake; in NHS, there were missing values on alcohol (n=8 controls, n=1 cases), fiber (n=11 controls, n=2 cases) and red and processed meat (n=8 controls, n=1 case) intake.

Table 2. Association between all ADIPOQ SNPs genotyped in EPIC and plasma adiponectin levels in control participants

			Men and women (n=623)			
SNP		MAF	Relative change (95% CI), %[a]	p_{trend}	F-Value	R^2 (%)
rs1063539	G>C	13%	0.9 (-6.2; 8.6)	0.81	0.1	0.0
rs16861194	A>G	8%	-4.4 (-12.9; 4.8)	0.34	1.0	0.2
rs12495941	G>T	37%	0.1 (-5.0; 5.5)	0.96	0.0	0.0
rs822391	T>C	19%	5.2 (-1.7; 12.6)	0.14	2.2	0.4
rs822394	C>A	17%	4.1 (-2.9; 11.6)	0.26	1.3	0.2
rs17300539[b,c]	G>A	9%	**18.2 (8.5; 28.8)**	**<0.0001**	14.5	2.3
rs17366568[b,c]	G>A	11%	**-12.3 (-19.0; -5.0)**	**<0.0001**	10.3	1.7
rs17366743[b]	T>C	3%	**20.6 (3.0; 41.2)**	**0.02**	5.4	0.9
rs182052	G>A	35%	-2.8 (-7.8; 2.5)	0.29	1.1	0.2
rs266729	C>G	27%	-1.6 (-7.1; 4.2)	0.59	0.3	0.1
rs1501299[b,c]	G>T	28%	**6.3 (0.3; 12.5)**	**0.04**	4.3	0.7
rs2241766	T>G	13%	2.3 (-5.1; 10.3)	0.56	0.3	0.1
rs3774261[b]	G>A	41%	**5.3 (0.1; 10.9)**	**0.05**	3.9	0.6
rs3821799	C>T	47%	0.0 (-4.9; 5.2)	0.99	0.0	0.0
internal weighted ADIPOQ-score			**7.0 (4.0; 10.0)**	**<0.0001**	22.9	3.6
external weighted ADIPOQ-score			**8.4 (4.6; 12.4)**	**<0.0001**	19.5	3.2

MAF: Minor allele frequency; 95% CI, 95% confidence interval

[a] Percent change in adiponectin concentrations per copy of minor allele or score unit, estimated in univariable linear regression models.

[b] incorporated in study specific *ADIPOQ*-score for EPIC

[c] incorporated in external *ADIPOQ*-score

in bold: statistically significant associations ($p<0.05$)

Table 3. Association between all ADIPOQ SNPs genotyped in HPFS and NHS and plasma adiponectin levels in control participants

		Men (n=167), HPFS					Women (n=510), NHS					
SNP		MAF	Rel. change (95% CI), %[a]	p_{trend}	F-Value	R^2 (%)	SNP		MAF	Rel. change (95% CI), %[a]	p_{trend}	F-Value	R^2 (%)
rs1063539	G>C	15%	-5.6 (-20.0; 11.5)	0.50	0.5	0.3	rs1063539	G>C	12%	-1.4 (-9.4; 7.4)	0.75	0.1	0.0
rs16861194	A>G	9%	-1.5 (-19.0; 19.8)	0.88	0.0	0.0	rs16861194	A>G	5%	-9.1 (-19; 1.9)	0.10	2.7	0.5
rs7615090	T>G	5%	-7.4 (-29.0; 20.8)	0.57	0.3	0.2	rs7615090	T>G	6%	-7.3 (-18; 4.8)	0.23	1.5	0.3
rs822394	C>A	15%	-2.3 (-16.3; 13.9)	0.76	0.1	0.1	rs822394	C>A	17%	6.1 (-1.3; 14.0)	0.11	2.6	0.5
rs17300539[d]	G>A	9%	13.5 (-5.8; 36.6)	0.19	1.8	1.1	**rs17300539[cd]**	G>A	8%	**13.3 (2.8; 25.0)**	**0.01**	6.3	1.2
rs17366568[d]	G>A	8%	-9.7 (-26; 10.1)	0.32	1.0	0.6	**rs17366568[cd]**	G>A	7%	**-18.5 (-26.8; -9.2)**	**<0.0001**	13.8	2.7
rs17366743	T>C	3%	0.9 (-26.4; 38.3)	0.96	0.0	0.0	rs17366743[d]	T>C	3%	2.5 (-11.7; 18.9)	0.75	0.1	0.0
rs6810075[b]	T>C	33%	**-11.8 (-21.2; -1.2)**	**0.03**	4.6	2.7	rs6810075	T>C	31%	-5.0 (-10; 0.7)	0.09	3.0	0.6
rs6773957	G>A	41%	9.2 (-2.6; 22.3)	0.13	2.3	1.4	**rs6773957[c]**	G>A	38%	**6.5 (0.9; 12.5)**	**0.02**	5.2	1.0
rs822354	G>A	36%	7.6 (-4.1; 20.9)	0.22	1.5	0.9	rs822354	G>A	33%	1.4 (-4.2; 7.4)	0.62	0.2	0.1
rs6444175[b]	G>A	28%	11.4 (-0.7; 25.0)	0.07	3.3	2.0	**rs6444175[c]**	G>A	27%	**7.6 (1.2; 14.5)**	**0.02**	5.5	1.1
rs266717	T>C	49%	-3.2 (-13.3; 8.0)	0.56	0.3	0.2	rs266717	T>C	47%	-3.8 (-8.8; 1.5)	0.16	2.0	0.4
rs1426810	A>G	37%	0.7 (-9.8; 12.4)	0.91	0.0	0.0	rs1426810	A>G	40%	3.9 (-1.7; 9.9)	0.17	1.9	0.4
rs1342387	T>C	43%	2.9 (-7.9; 15.0)	0.62	0.3	0.2	rs1342387	T>C	45%	-4.4 (-9.5; 1.0)	0.11	2.5	0.5
rs12733285	C>T	30%	-2.7 (-13.7; 9.8)	0.66	0.2	0.1	rs12733285	C>T	30%	-4.1 (-9.6; 1.7)	0.16	2.0	0.4
rs266729[b]	C>G	24%	**-12.6 (-22.8; -1.2)**	**0.03**	4.6	2.7	rs266729	C>G	26%	-4.4 (-10; 1.8)	0.16	2.0	0.4
rs1501299[bd]	G>T	26%	**13.4 (1.0; 27.4)**	**0.04**	4.4	2.6	**rs1501299[cd]**	G>T	27%	**8.6 (2.2; 15.5)**	**0.01**	7.0	1.4
rs1063538	C>T	41%	9.2 (-2.6; 22.3)	0.13	2.3	1.4	**rs1063538[c]**	C>T	38%	**6.6 (0.9; 12.5)**	**0.02**	5.2	1.0
rs3774262[b]	G>A	15%	-6.0 (-20.0; 10.5)	0.46	0.6	0.3	rs3774262	G>A	11%	-0.7 (-9.0; 8.4)	0.88	0.0	0.0
internal weighted ADIPOQ-score			**7.0 (1.9; 12.3)**	**0.01**	7.3	4.2	internal weighted ADIPOQ-score			**3.4 (1.6; 5.2)**	**<0.0001**	14.2	2.7
external weighted ADIPOQ-score			**9.5 (0.7; 19.1)**	**0.04**	4.5	2.6	external weighted ADIPOQ-score			**10.2 (5.5; 15.2)**	**<0.0001**	18.6	3.5

MAF: Minor allele frequency; 95% CI, 95% confidence interval

[a] Percent change in adiponectin concentrations per copy of minor allele or score unit, estimated in univariable linear regression models.

[b] incorporated in study specific *ADIPOQ*-score for HPFS

[c] incorporated in study specific *ADIPOQ*-score for NHS

[d] incorporated in external *ADIPOQ*-score

in bold: statistically significant associations ($p<0.05$)

Supplemental Table 1. Baseline characteristics by tertiles of the external *ADIPOQ*-score in control participants in EPIC, HPFS, and NHS

	EPIC (n=623)			
	Tertile 1	Tertile 2	Tertile 3	p-value
N	126	221	276	
Female sex, n (%)	63 (50.0)	95 (43.0)	131 (47.5)	0.40
Age, years, mean (SD)	56.9 (7.6)	58.5 (8.6)	58.8 (8.0)	0.05
Current smoking, n (%)	24 (19.0)	38 (17.2)	62 (22.5)	0.33
Physical activity (MET-hours/week), mean (SD)	87.50 (47.59-127.3)	77.00 (46.04-109.1)	92.47 (48.10-131.6)	0.06
Body mass index, kg/m2, mean (SD)	26.4 (4.1)	26.4 (3.8)	26.3 (3.6)	0.78
Waist circumference, cm, mean (SD)	88.3 (12.0)	89.8 (12.1)	89.3 (12.6)	0.64
Alcohol intake, g/day, median (IQR)	5.33 (1.05-17.73)	6.37 (0.83-18.25)	6.50 (1.13-23.42)	0.71
Fiber, g/day, median (IQR)	22.20 (17.73-27.37)	22.38 (18.36-28.06)	21.37 (16.76-26.37)	0.12
Red and processed meat, g/day, median (IQR)	75.11 (49.56-98.45)	63.56 (41.81-97.40)	71.11 (48.23-106.7)	0.29
Total adiponectin (µg/mL), median (IQR)	5.48 (3.81-7.75)	5.87 (4.80-8.33)	6.90 (5.13-9.08)	<0.0001
	HPFS (n=230)			
	Tertile 1	Tertile 2	Tertile 3	p-value
N	28	102	100	
Female sex, n (%)	0 (0)	0 (0)	0 (0)	
Age, years, mean (SD)	67.9 (7.4)	64.6 (9.2)	66.1 (8.9)	0.81
Current smoking, n (%)	1 (3.8)	7 (7.1)	4 (4.1)	0.61
Physical activity (MET-hours/week), mean (SD)	31.8 (30.2)	30.9 (23.4)	39.0 (33.6)	0.08
Body mass index, kg/m2, mean (SD)	25.4 (4.8)	25.2 (3.1)	25.1 (2.9)	0.69
Waist circumference, cm, mean (SD)	96.0 (10.2)	94.2 (9.4)	93.7 (9.1)	0.33
Alcohol intake, g/day, median (IQR)	7.0 (0.9-20.7)	7.7 (1.9-14.8)	6.2 (1.7-18.5)	0.98
Fiber, g/day, median (IQR)	22.5 (18.5-28.5)	21.7 (18.1-26.9)	23.1 (18.8-30.1)	0.29
Red and processed meat, g/day, median (IQR)	68.6 (30.6-101.5)	63.8 (27.7-98.1)	62.1 (38.5-92.1)	0.95
Total adiponectin (µg/mL), median (IQR)	5.3 (3.9- 7.7)	5.5 (3.8- 7.9)	5.8 (4.0- 8.9)	0.32
	NHS (n=774)			
	Tertile 1	Tertile 2	Tertile 3	p-value
N	102	324	348	
Female sex, n (%)	102 (100)	324 (100)	348 (100)	
Age, years, mean (SD)	58.7 (7.5)	59.1 (6.7)	59.3 (6.6)	0.47
Current smoking, n (%)	16 (15.7)	32 (9.9)	42 (12.2)	0.26
Physical activity (MET-hours/week), mean (SD)	13.7 (14.3)	15.8 (19.1)	17.7 (21.7)	0.06
Body mass index, kg/m2, mean (SD)	25.2 (4.4)	25.5 (4.7)	25.3 (4.1)	0.96
Waist circumference, cm, mean (SD)	81.5 (12.4)	79.5 (10.7)	79.0 (9.9)	0.08
Alcohol intake, g/day, median (IQR)	0.0 (0.0- 3.7)	1.8 (0.0- 7.5)	1.8 (0.0- 7.6)	0.02
Fiber, g/day, median (IQR)	17.5 (14.3-22.0)	17.8 (14.9-21.2)	18.4 (15.5-21.3)	0.53
Red and processed meat, g/day, median (IQR)	49.4 (29.4-86.9)	52.7 (34.3-81.3)	52.5 (33.3-80.0)	0.98
Total adiponectin (µg/mL), median (IQR)	7.3 (4.6- 9.3)	8.4 (6.4-10.7)	9.0 (6.3-12.0)	<0.0001

SD, standard deviation, IQR, inter-quartile range, HMW, high-molecular weight

P-values for were determined by Chi-Square test for variables expressed as %, by analysis of variance for variables expressed as means, and by Kruskal–Wallis test for variables expressed as medians. After Bonferroni-correction for 10 tests, a p-value <0.005 is considered statistically significant.

Table 4. Association between internal and external *ADIPOQ*-scores and colorectal cancer risk in EPIC, HPFS, and NHS

		Internal Score					External Score				
	No. Cases/No. Controls	% Diff[a]	OR	(95% CI)	p_{trend}	$p_{het.}$	% Diff[a]	OR	(95% CI)	p_{trend}	$p_{het.}$
EPIC[b]	623/623	7.0	0.97	(0.89, 1.06)	0.56		8.4	0.95	(0.85, 1.06)	0.37	
HPFS[c]	231/230	7.0	0.99	(0.88, 1.12)	0.87		9.5	1.00	(0.81, 1.22)	0.97	
NHS[c]	399/774	3.4	0.99	(0.94, 1.05)	0.81		10.2	1.00	(0.87, 1.15)	0.99	
Pooled	1253/1627		0.99	(0.95, 1.03)	0.58	0.92		0.97	(0.90, 1.06)	0.52	0.83

[a] Per score unit, estimates based on univariate linear regression in controls
[b] Conditional logistic regression, controlling for matching factors (age at blood collection, study center, fasting status, menopausal status and hormone use in women)
[c] Unconditional logistic regression, adjusted for matching factors(age at blood draw, date of blood draw)
OR, odds ratio; 95% CI, 95% confidence interval; $p_{het.}$, P value for heterogeneity by study

Supplemental Table 2. Association between internal and external *ADIPOQ*-scores and colorectal cancer risk in EPIC, HPFS, and NHS after additional adjustment for measured adiponectin

		Internal Score					External Score				
	No. Cases/No. Controls	% Diff[a]	OR	(95% CI)	p_{trend}	$p_{het.}$	% Diff[a]	OR	(95% CI)	p_{trend}	$p_{het.}$
EPIC[b]	623/623	7.0	1.00	(0.91, 1.09)	0.93		8.4	0.98	(0.87, 1.10)	0.68	
HPFS[c]	231/230	7.0	1.00	(0.89, 1.13)	0.98		9.5	1.01	(0.82, 1.24)	0.91	
NHS[c]	399/774	3.4	0.99	(0.94, 1.05)	0.78		10.2	1.00	(0.87, 1.15)	0.98	
Pooled	1253/1627		0.99	(0.95, 1.04)	0.80	0.99		0.99	(0.91, 1.07)	0.79	0.94

[a] Per score unit, estimates based on univariate linear regression in controls
[b] Conditional logistic regression, controlling for matching factors (age at blood collection, study center, fasting status, menopausal status and hormone use in women)
[c] Unconditional logistic regression, adjusted for matching factors (age at blood draw, date of blood draw)
OR, odds ratio; 95% CI, 95% confidence interval; $p_{het.}$, P value for heterogeneity by study

Table 5. Instrumental variable estimation of the association between genetically determined adiponectin concentrations with risk of colorectal cancer in EPIC, HPFS, and NHS

		Instrumental variable (IV)							
		Internal Score				External Score			
	# Cases/# Controls	IV-OR	(95% CI)	p_{trend}	$p_{het.}$	IV-OR	(95% CI)	p_{trend}	$p_{het.}$
EPIC	623/623	0.76	(0.32, 1.84)	0.55		0.65	(0.25, 1.70)	0.38	
HPFS	231/230	0.90	(0.28, 2.71)	0.87		0.97	(0.21, 4.48)	0.97	
NHS	399/774	0.87	(0.27, 2.66)	0.81		1.00	(0.38, 2.68)	0.99	
Pooled	1253/1627	0.82	(0.45, 1.51)	0.53	0.97	0.83	(0.44, 1.55)	0.56	0.81

IV-OR, instrumental variable odds ratio; 95% CI, 95% confidence interval; $p_{het.}$, P value for heterogeneity by study

IV-OR derived from two-stage regression. First stage was a linear regression. In EPIC, the second stage was a conditional logistic regression controlling for matching factors (age at blood collection, study center, fasting status, menopausal status and hormone use in women); in HPFS and NHS, the second stage was an unconditional logistic regression adjusting for matching factors (age at blood draw, date of blood draw)

$p_{het.}$, P value for heterogeneity by study