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ORIGINAL ARTICLE

Cancer-specific risk prediction with a serum microRNA signature

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Abstract

We recently derived and validated a serum-based microRNA risk score (miR-score) that predicted colorectal cancer (CRC) occurrence with very high accuracy within 14 years of follow-up in a population-based cohort study from Germany (ESTHER cohort). Here, we aimed to evaluate associations of the CRC-specific miR-score with the risk of developing other common cancers, including female breast cancer (BC), lung cancer (LC), and prostate cancer (PC), in the ESTHER cohort. MicroRNAs (miRNAs) were profiled by quantitative real-time PCR in serum samples collected at baseline from randomly selected incident cases of BC ($n = 90$), LC ($n = 88$), and PC ($n = 93$) and participants without diagnosis of CRC, LC, BC, or PC (controls, $n = 181$) until the end of the 17-year follow-up. Multivariate logistic regression models were used to evaluate the associations of the miR-score with BC, LC, and PC incidence. The miR-score showed strong inverse associations with BC and LC incidence [odds ratio per 1 standard deviation increase: 0.60 (95% confidence interval [CI] 0.43–0.82), $p = 0.0017$, and 0.64 (95% CI 0.48–0.84), $p = 0.0015$, respectively]. Associations with PC were not statistically significant but pointed in the positive direction. Our study highlights the potential of serum-based miRNA biomarkers for cancer-specific risk prediction. Further large cohort studies aiming to investigate, validate, and optimize the use of circulating miRNA signatures for cancer risk assessment are warranted.

KEYWORDS
breast cancer, circulating miRNA, colorectal cancer, lung cancer, prostate cancer, risk prediction, risk stratification

Abbreviations: BC, breast cancer; CI, confidence interval; CRC, colorectal cancer; FC, fold change; LC, lung cancer; miRNA, microRNA; miR-score, microRNA risk score; *n*, number; OR, odds ratio; PC, prostate cancer; SD, standard deviation.

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1 | INTRODUCTION

Cancer incidence and mortality continue to increase worldwide. In 2020, there were an estimated 19.3 million new cases of cancer and almost 10.0 million deaths from cancer globally.[1,2] Population-based screening has the potential to lower cancer incidence and mortality through detection of asymptomatic precancerous lesions or early detection of invasive tumors. Tailoring such screening to those at highest risk has the potential to improve the effectiveness of screening programs[3,4] while minimizing potential harms such as overdiagnosis and false-positive tests.[5–7] Multiple risk prediction models incorporating cancer-specific lifestyle, clinical, genetic, and non-genetic risk factors have been proposed for identifying high-risk populations for risk-adapted screening for a variety of common cancers, including breast cancer (BC),[8–11] lung cancer (LC),[12–14] and prostate cancer (PC).[15–17]

Blood-based biomarkers such as circulating microRNAs (miRNAs) have emerged as potential candidates to improve cancer risk prediction.[13,18–23] MicroRNAs are small endogenous non-coding RNAs that regulate gene expression and control various cellular mechanisms.[24] MicroRNAs released into blood during tumorigenesis are detectable in serum or plasma in a highly stable form.[25] Using blood-based miRNA profiles for cancer risk assessment offers the advantages of a minimally invasive, low cost and quick PCR-based test. We recently derived and validated a microRNA risk score (miR-score) based on serum levels of seven miRNAs, which predicted the risk of developing colorectal cancer (CRC) with very high accuracy within 14 years of follow-up in the large population-based ESTHER (Epidemiologische Studie zu Chancen der Verhütung, Früherkennung und optimierter Therapie chronischer Erkrankungen in der älteren Bevölkerung) study.[23] The miR-score was derived using samples collected at baseline from 198 study participants who developed CRC during 14 years of follow-up and 178 randomly selected controls. Its performance was compared to that of established CRC risk models including an environmental risk score (ERS) and a polygenic risk score (PRS). The miR-score predicted CRC risk with very high accuracy (i.e. an optimism-corrected area under the receiver operating characteristic curve [AUC] of 0.794). In participants with all scores available, the optimism-corrected AUC was 0.802 for the miR-score, while the AUC (95% confidence interval [CI]) was 0.557 (0.498–0.616) for the ERS and 0.622 (0.564–0.681) for the PRS. Thus, the miR-score outperformed established approaches for CRC risk prediction. While the miR-score was highly predictive of CRC risk, to what extent the observed miRNA dysregulations are CRC-specific needs to be explored. In this study, we aimed to evaluate associations of the CRC-specific miR-score with the risk of developing BC, LC, and PC.

2 | MATERIALS AND METHODS

2.1 | Study design and population

We selected participants from the ESTHER study, an ongoing population-based longitudinal cohort study conducted in Saarland, Germany. Details of the ESTHER study design have been previously described.[26] Briefly, 9940 participants aged 50–75 years were recruited between July 2000 and December 2002 by their general practitioners in the context of a general health screening examination. The distribution of sociodemographic baseline characteristics and the prevalence of common diseases in the ESTHER study population closely resembled that of a representative German National Health Survey of 1998 within the corresponding age range.[27] These participants were regularly followed up thereafter. Information regarding participants' socio-demographic characteristics, lifestyle factors, and health status at baseline was obtained using standardized self-administered questionnaires. In addition, biological samples (blood, stool, and urine) were collected, processed, and stored at −80°C until analysis. Prevalent and incident cancers were determined by record linkage with the Saarland Cancer Registry. Saarland is a federal state in South-Western Germany with a total population of approximately 990,000 people in 2015–2020.[28] In Saarland, cancer reporting is mandatory by law. The Saarland Cancer Registry receives cancer notifications from hospitals, radiotherapy departments, outpatient clinics, screening programs, and doctors in private practice as well as reports from pathology laboratories and death certificates from local health authorities. The registry's case ascertainment was estimated to be almost complete.[28,29] The ESTHER study was approved by the ethics committees of the Medical Faculty of the University of Heidelberg and of the state medical board of Saarland, Germany. All participants provided written informed consent.

For the current study, miRNA profiling was performed in serum samples collected at baseline from randomly selected incident cases of BC ($n=90$), LC ($n=88$), and PC ($n=93$) and participants without diagnosis of CRC, LC, BC, or PC (the four most frequent cancers) until the end of the 17-year follow-up (further referred to as "controls," $n=181$). Levels of seven miRNAs (let-7g-5p, miR-19a-3p, miR-23a-3p, miR-92a-3p, miR-144-5p, miR-21-5p, and miR-27a-3p), identified in preliminary work[23] to be predictive of CRC risk, were quantified in selected serum samples using quantitative real-time PCR (qPCR) technology (Figure 1).

2.2 | miRNA quantification by quantitative PCR

The samples were thawed on ice and centrifuged at 3000×*g* for 5 min at 4°C. Total RNA was extracted from the samples using an miRNeasy Serum/Plasma Advanced Kit (QIAGEN, Germany) following the manufacturer's instructions. Next, 2 μL RNA was reverse transcribed in 10 μL reactions using the miRCURY LNA RT Kit (QIAGEN). Complementary DNA (cDNA) was diluted 50× and assayed in 10 μL PCR reactions according to the protocol for miRCURY LNA miRNA PCR. Spike-in controls UniSp2, UniSp4, and UniSp6 were added to the control for RNA extraction efficiency and possible cDNA synthesis inhibitors. Hemolysis was assessed by determining the levels of miR-451 and miR-23a via qPCR. miR-451 is expressed in red blood cells, and miR-23a is relatively stable in serum and not affected by hemolysis.[30–32] A quantification cycle (Cq) ratio between miR-23a and miR-451 higher than 7.0 was

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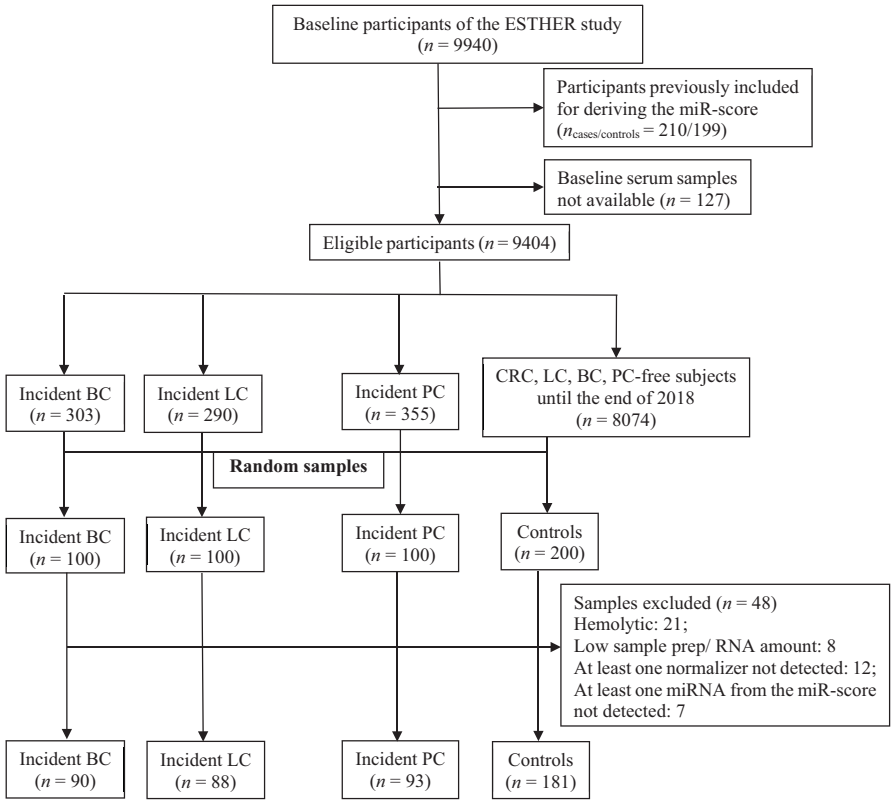


FIGURE 1 Flow diagram for selection of participants. BC, breast cancer; CRC, colorectal cancer; LC, lung cancer; miR-score, microRNA risk score; n, number of participants; PC, prostate cancer.

considered indicative of sample hemolysis.[33] Twenty-one samples displaying significant hemolysis and eight samples with low sample preparation or low RNA quantity were excluded from downstream analyses.

For samples meeting the quality control criteria, RNA extraction efficiency, monitored using UniSp2 and UniSp4, was acceptable with raw Cq values being consistent across the dataset (UniSp2: Cq 18.69 ± 1.12, UniSp4: Cq 25.63 ± 1.33). The cDNA synthesis reactions, monitored using UniSp6, indicated constant efficiency of the reverse transcription step with no signs of inhibition (Cq 16.64 ± 0.25). These samples were used to quantify levels of seven target miRNAs (let-7 g-5p, miR-19a-3p, miR-23a-3p, miR-92a-3p, miR-144-5p, miR-21-5p, and miR-27a-3p) and three normalizer miRNAs (miR-93-5p, miR-1246, and miR-223-3p). Each miRNA was assayed once on a custom panel using miRCURY LNA SYBR Green Master Mix. Negative controls excluding template from the reverse transcription reaction were performed and profiled like the samples. The amplification was performed in a LightCycler 480 Real-Time PCR System (Roche) in 384 well plates. The amplification curves were analyzed using the Roche LC software, both for the determination of Cq (Cq was calculated as the 2nd derivative) and for melting curve analysis. The amplification efficiency was calculated using algorithms similar to the LinReg software.[34] All assays were inspected for distinct melting curves and the melting temperature was confirmed to be within known specifications for the assay. Furthermore, assays within 5 Cq of the negative control or Cq > 37 were excluded from further analyses. Detectable miRNAs were those with a Cq < 40.

All laboratory analyses were performed blinded with respect to disease status.

2.3 | Statistical analysis

A combination of three miRNAs (miR-93-5p, miR-1246, and miR-223-3p) was identified and used to normalize qPCR data in our previous study.[23] In the current study, these normalizers exhibited good stability across the selected samples (stability value = 1.02×10^{-3}) using the NormFinder method[35] and were identified as suitable to normalize the qPCR data. Relative expression levels of miRNAs were calculated using $2^{-\Delta Ct}$ method. Samples with missing (raw Cq value ≥40) values for any of the normalizers ($n = 12$) or miRNAs from the miR-score ($n = 7$) were excluded from further analyses. The data was normalized to the average Cq value of the normalizers. For all further analyses involving BC and PC, we restricted controls to the respective sexes.

For each cancer type, we compared the expression levels of individual miRNAs between cases and controls using the Mann–Whitney test. For each cancer type, these analyses were repeated with follow-up time restricted to ≤4, ≥4–8, and ≥8 years after recruitment.

Next, an miR score was calculated for each participant using the formerly derived[23] linear predictor:

miR-score = 0.1899 + let-7 g-5p*0.2351 + miR-19a-3p*-0.2024 + miR-23a-3p*1.6595 + miR-92a-3p*0.4794 + miR-144-5p*0.2002 + miR-21-5p*-1.6772 + miR-27a-3p*0.1014.

To evaluate the associations of the miR-score with BC, LC, and PC incidence, the participants were stratified into four risk categories using quartile thresholds of the score in the controls. The associations were quantified for each cancer type separately by logistic regression models, taking the lowest quartile as the reference group. Models were calculated first without adjusting for any potential confounders (Model 1), then additionally adjusting for age and sex (Model 2),

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and odds ratios (ORs) with corresponding 95% CIs were calculated. Additionally, for each cancer type, ORs per standard deviation (SD) increase in the miR score were estimated using logistic regression models. To address possible reverse causality, sensitivity analyses were conducted, in which cancer cases that occurred in the first 2 years of follow-up were excluded. All statistical analyses were conducted using R software, version 4.1.2 (R Foundation, Vienna, Austria). Two-sided *p*-values less than 0.05 were considered statistically significant.

3 | RESULTS

3.1 | Characteristics of the study population

The study population included 90, 88, and 93 participants with incident BC, LC, and PC (cases), respectively, and 181 controls (81 males and 100 females) from the ESTHER study (Table 1). The median time (interquartile range) between sample collection and cancer diagnosis was 7.0 (4.4–10.6) years for BC, 8.6 (5.0–12.1) years for LC, and 7.6 (4.8–12.3) years for PC. The median age of the participants at sampling was between 60 and 64 years in all groups.

3.2 | Comparison of serum miRNA levels between incident cases of different cancers and controls

An evaluation of fold changes and corresponding *p*-values of individual miRNA levels when comparing participants with incident CRC with controls in our previous study and when comparing participants with incident BC, LC, and PC with controls in the current study is presented in Table 2. While our previous results were based on cohort data including 14 years follow-up, our current results are based on case–control comparisons, using incident cases and controls who remained free of the cancers during 17 years of follow-up. In the current study, when comparing BC cases with controls, miR-19a-3p was found to be upregulated before correction for multiple testing but failed to remain statistically significant after the correction. MiR-19a-3p was also observed to be significantly upregulated when comparing LC cases with controls. The observed upregulation of miR-19a-3p is contradictory to its observed downregulation when comparing CRC cases with controls in our previous study. Furthermore, in the comparison between LC cases and controls, miR-92a-3p was found to be downregulated before multiple testing correction but did not remain statistically significant after the correction. Conversely, our previous study results had revealed miR-92a-3p to be upregulated when comparing CRC cases with controls.

We then repeated these analyses comparing cases diagnosed in specific time windows (≤4, ≥4–8 and ≥8 years) after recruitment with controls (Table S1). For the cancers diagnosed ≤4 years after recruitment, no significant dysregulations were observed for any cancer type. For the cancers diagnosed between 4 and 8 years after recruitment, upregulation of miR-19a-3p was observed in BC and LC cases compared to controls. This direction of dysregulation remained consistent at all time windows and was also significant for all LC cases and controls (for the complete follow-up time of ≤18 years). For the cancers diagnosed ≥8 years after recruitment, no significant dysregulations were observed for any cancer type.

TABLE 1 Characteristics of the study populations.

	Previous study[23]		Current study			
Characteristics	CRC cases (*n* = 198)	Controls (*n* = 178)	BC cases (*n* = 90)	LC cases (*n* = 88)	PC cases (*n* = 93)	Controls (*n* = 181)
Age at sampling						
Mean (SD)	64.6 (5.9)	62.2 (6.6)	60.2 (6.0)	62.2 (6.3)	63.8 (6.0)	62.4 (6.8)
Median (range)	65 (50–75)	62 (50–75)	59.5 (50–73)	61.0 (50–75)	64.0 (50–75)	62.0 (50–75)
Gender counts (%)						
Male	122 (61.6)	89 (50.0)	0 (0.0)	53 (60.2)	93 (100.0)	81 (44.8)
Female	76 (38.4)	89 (50.0)	90 (100.0)	35 (39.8)	0 (0.0)	100 (55.2)
TNM stage at diagnosis counts (%)						
Stage I	8 (4.0)	–	0 (0.0)	14 (15.9)	15 (16.1)	–
Stage II	11 (5.6)	–	0 (0.0)	2 (2.3)	12 (12.9)	–
Stage III	4 (2.0)	–	24 (26.7)	16 (18.2)	5 (5.4)	–
Stage IV	27 (13.6)	–	2 (2.2)	20 (22.7)	3 (3.2)	–
Unknown	148 (74.7)	–	64 (71.1)	36 (40.9)	58 (62.4)	–
Time between sampling and diagnosis						
Range	0.0–14.3	–	0.0–17.7	0.1–17.6	0.3–18.0	–
Median (interquartile range)	6.8 (3.3–9.6)	–	7.0 (4.4–10.6)	8.6 (5.0–12.1)	7.6 (4.8–12.3)	–

Abbreviations: BC, breast cancer; CRC, colorectal cancer; LC, lung cancer; *n*, number of participants; PC, prostate cancer; SD, standard deviation.

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TABLE 2 Comparison of serum miRNA levels between participants with different incident cancers (cases) and controls in the ESTHER cohort.

	Previous results[23]				Current results								
	CRC cases[a] vs. Controls ($n_{cases/controls}$ = 198/178)				BC cases[b] vs. Controls ($n_{cases/controls}$ = 90/100)			LC cases[b] vs. Controls ($n_{cases/controls}$ = 88/181)			PC cases[b] vs. Controls ($n_{cases/controls}$ = 93/81)		
miRNA	FC		*p*-value[c]	*p*-adj[d]	FC	*p*-value[c]	*p*-adj[d]	FC		*p*-value[c]	*p*-adj[d]	FC	*p*-value[c]	*p*-adj[d]
let-7g-5p	1.26	↑	**<0.0001**	**<0.0001**	1.08	0.158	0.632	0.99		0.975	1.000	1.07	0.460	1.000
miR-19a-3p	0.61	↓	**<0.0001**	**<0.0001**	1.15	**0.015**	0.108	1.21	↑	**0.000**	**0.002**	0.90	0.348	1.000
miR-23a-3p	1.21	↑	**<0.0001**	**<0.0001**	1.00	0.883	0.883	0.96		0.158	0.635	0.95	0.308	1.000
miR-92a-3p	1.11	↑	**0.027**	**0.027**	0.89	0.056	0.336	0.88		**0.013**	0.080	0.94	0.564	1.000
miR-144-5p	1.53	↑	**<0.0001**	**<0.0001**	1.18	0.179	0.632	0.87		0.269	0.807	1.12	0.422	1.000
miR-21-5p	0.69	↓	**<0.0001**	**<0.0001**	1.14	0.068	0.338	1.00		0.933	1.000	0.96	0.675	1.000
miR-27a-3p	0.82	↓	**<0.0001**	**<0.0001**	1.06	0.415	0.830	0.94		0.127	0.635	0.92	0.196	1.000

Note: ↑ represents significant upregulation; ↓ represents significant downregulation.

Bold values indicate statistically significant results (p-value <0.05).

Abbreviations: BC, breast cancer; CRC, colorectal cancer; ESTHER, Epidemiologische Studie zu Chancen der Verhütung, Früherkennung und optimierter Therapie chronischer Erkrankungen in der älteren Bevölkerung; FC, fold change; LC, lung cancer; miRNA, microRNA; *n*, number; PC, prostate cancer.

[a]Incident during 14 years of follow-up.

[b]Incident during 17 years of follow-up.

[c]Values were generated from two-sided Mann–Whitney test.

[d]Multiple testing correction by the method of Bonferroni-Holm.

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3.3 | Associations of the colorectal cancer-specific miR-score with risk of different cancers

Associations of the miR-score with CRC incidence in our previous study and BC, LC, and PC incidence in the current study are presented in Table 3. In contrast to its strong positive association with CRC risk, the miR-score showed a strong inverse association with both BC and LC risk [adjusted OR per 1 SD increase in the miR score: 0.60 (95% CI, 0.43–0.82), $p=0.0017$, and 0.64 (95% CI, 0.48–0.84), $p=0.0015$, respectively]. Associations with PC were not statistically significant but pointed in the positive direction. Furthermore, results of the sensitivity analyses excluding cancer cases that occurred in the first 2 years of follow-up were consistent with the main results (Table S2).

4 | DISCUSSION

In this study, we evaluated associations of our previously reported[23] serum-based CRC-specific miR-score with the risk of developing BC, LC, and PC within the large population-based ESTHER cohort of older adults. MiRNAs were profiled by qPCR in serum collected at baseline from randomly selected participants with incident BC, LC, and PC and cancer-free controls identified within 17 years of follow-up. In our investigation, we observed strong inverse associations of the miR-score with the risk of developing BC and LC. Associations with PC were not statistically significant but pointed in the positive direction. Our results demonstrate that miRNA profiles associated with tumor progression differ across cancer types and could be useful for developing personalized cancer prevention strategies.

Quantification of serum miRNA levels in our study was done using the miRCURY LNA miRNA PCR custom panel, which has been reported to show high sensitivity and linearity for analyzing samples with low miRNA concentration.[36] Data obtained using this platform was normalized using a combination of three miRNAs (miR-93-5p, miR-1246, and miR-223-3p) that was identified in our previous study.[23] This normalizer combination was also found to be suitable for normalization in our current study, as it exhibited good stability across the selected samples using the NormFinder method.[35] Nevertheless, further validations are still needed to establish the utility of these miRNAs as optimal normalizers for qPCR data analysis.

The miR-score was based on serum levels of seven miRNAs (let-7 g-5p, miR-19a-3p, miR-23a-3p, miR-92a-3p, miR-144-5p, miR-21-5p, and miR-27a-3p) that were found to be significantly dysregulated in incident CRC cases compared to controls identified during 14 years of follow-up in our previous study.[23] In the current study, when comparing incident BC, LC, or PC cases with controls identified during 17 years of follow-up, divergent patterns from those previously observed for CRC cases were observed. MiR-19a-3p was found to be upregulated in BC and LC cases compared to controls, in contrast to its previously observed downregulation in CRC cases compared to controls. Another miRNA, miR-92a-3p, also showed a divergent pattern between LC cases and controls (downregulated in LC cases before multiple testing correction but not significant after the correction) compared to that previously observed between CRC cases and controls (upregulated in CRC cases). Both miR-19a-3p and miR-92a-3p are members of the miR-17-92 cluster, a potent oncogenic cluster, also known as oncomir-1.[37,38] Numerous studies have reported dysregulation of the miR-17-92 and its role in promoting tumorigenesis in various human cancers. With respect to the role of miR-19a-3p in lung carcinogenesis, it is known to promote LC cell proliferation and migration.[39] Prior studies evaluating miR-19a-3p levels for LC detection have mostly used samples collected from patients after diagnosis. In one such study,[40] Lin et al. found elevated levels of miR-19a-3p in serum from established LC cases relative to controls, which is in agreement with our observations comparing incident LC cases to controls. Conversely, other previous studies also using samples collected post-diagnosis have reported lower levels of miR-19a-3p in serum[41] or plasma[42] of LC patients compared to healthy individuals. Another member of the miR-17-92 cluster, miR-92a-3p, has been shown to participate in tumorigenesis and tumor progression by regulating tumor cell proliferation, migration, and apoptosis.[43–46] Previous studies evaluating miR-92a-3p levels for LC detection have used samples from patients with a confirmed diagnosis, making it difficult to determine if they will be useful for risk prediction or have resulted from cancer progression. In one such study[41] by Fan et al., miR-92a-3p was reported to be downregulated in serum samples of non-small cell LC patients compared to healthy controls, which is in accordance with our findings in incident LC cases relative to controls (downregulated before multiple testing correction but not significant after the correction). However, in another study by Reis et al.[47] using plasma samples from post-diagnosis LC patients and healthy controls, miR-92a-3p levels were revealed to be higher in cases than controls.

It should be noted that while most previous studies evaluating miR-19a-3p or miR-92a-3p levels for LC or BC detection used samples from patients with an established cancer diagnosis, our findings are based on samples collected several years prior to a diagnosis from participants who later developed BC or LC during a 17-year follow-up in a prospective cohort study. Since an existing tumor is likely to affect the circulating miRNA profile, studies using post-diagnosis samples might not be useful for discovering biomarkers of cancer risk prior to disease onset. Concerns about changes in miRNA profiles after disease onset were addressed in our study by conducting sensitivity analyses excluding cancer cases that occurred in the first 2 years of follow-up. Results of these analyses were consistent with the main results. To further facilitate discovery of biomarkers of cancer risk, studies using prediagnostic samples from cancer patients in large prospective cohorts are warranted. Only a handful of such studies[13,18–22] have been performed to discover circulating miRNA biomarkers using samples obtained prior to BC or LC diagnosis. In line with findings from these studies, our study demonstrated circulating miRNA levels to be altered years before cancer occurrence.

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TABLE 3 Associations of the CRC-specific miR-score with the occurrence of different cancers in the ESTHER cohort.

Cancer type	Quartile[a]	Cases	Controls	Model 1[b] OR (95% CI)[d]	Model 1[b] *p*-value[d]	Model 2[c] OR (95% CI)[d]	Model 2[c] *p*-value[d]
Results from previous study[23]							
CRC[e]	Q1 (<−1.22]	15 (7.6)	45 (25.0)	Ref.		Ref.	
	Q2 (−1.22, −0.66]	12 (6.1)	44 (25.0)	0.82 (0.34–1.90)	0.6495	0.74 (0.30–1.78)	0.4974
	Q3 (−0.66, −0.08]	31 (15.7)	44 (25.0)	2.11 (1.00–4.40)	**0.0485**	2.07 (0.97–4.41)	0.0600
	Q4 (> −0.08)	140 (70.7)	45 (25.0)	9.33 (4.76–18.30)	**<0.0001**	8.81 (4.42–17.56)	**<0.0001**
	OR[f]			5.00 (3.49–7.17)	**<0.0001**	4.92 (3.41–7.11)	**<0.0001**
Results from current study							
BC[g]	Q1 (< −1.74]	31 (34.4)	25 (25.0)	Ref.		Ref.	
	Q2 (−1.74, −1.25]	30 (33.3)	23 (23.0)	1.05 (0.49–2.24)	0.8958	1.07 (0.50–2.31)	0.8631
	Q3 (−1.25, −0.763]	21 (23.3)	27 (27.0)	0.63 (0.29–1.36)	0.2390	0.64 (0.29–1.41)	0.2709
	Q4 (> −0.763)	8 (8.9)	25 (25.0)	0.26 (0.10–0.67)	**0.0054**	0.25 (0.09–0.65)	**0.0045**
	OR[f]			0.61 (0.45–0.84)	**0.0027**	0.60 (0.43–0.82)	**0.0017**
LC[g]	Q1 (< −1.64)	29 (33.0)	46 (25.0)	Ref.		Ref.	
	Q2 (−1.64, −1.11]	29 (33.0)	44 (24.0)	1.05 (0.54–2.02)	0.8950	0.98 (0.50–1.92)	0.9536
	Q3 (−1.11, −0.602]	21 (24.0)	45 (25.0)	0.74 (0.37–1.48)	0.3969	0.71 (0.35–1.44)	0.3391
	Q4 (> −0.602)	9 (10.0)	46 (25.0)	0.31 (0.13–0.73)	**0.0071**	0.28 (0.12–0.66)	**0.0039**
	OR[f]			0.66 (0.51–0.87)	**0.0029**	0.64 (0.48–0.84)	**0.0015**
PC[g]	Q1 (< −1.53]	21 (22.6)	20 (24.7)	Ref.		Ref.	
	Q2 (−1.53, −1.04]	28 (30.1)	21 (25.9)	1.27 (0.55–2.92)	0.5740	1.32 (0.57–3.07)	0.5120
	Q3 (−1.04, −0.47]	22 (23.7)	20 (24.7)	1.05 (0.44–2.48)	0.9160	1.12 (0.47–2.66)	0.8040
	Q4 (> −0.47)	22 (23.7)	20 (24.7)	1.05 (0.44 2.48)	0.9160	1.15 (0.48–2.76)	0.7540
	OR[f]			1.01 (0.75–1.36)	0.9610	1.03 (0.76–1.39)	0.8490

Note: Bold values indicate statistically significant results (p-value <0.05).

Abbreviations: BC, breast cancer; CI, confidence interval; CRC, colorectal cancer; LC, lung cancer; miR-score, microRNA risk score; OR, odds ratio; PC, prostate cancer; Q, quartile; Ref., reference category.

[a]Quartiles of the miR-score among controls.

[b]Model 1: without any adjustment for covariates.

[c]Model 2: adjusted for age (BC and PC) or for age and sex (CRC and LC).

[d]OR, 95% CI and two-sided p-values were generated from logistic regression model.

[e]Incident during 14 years of follow-up.

[f]OR per SD increase in the miR-score.

[g]Incident during 17 years of follow-up.

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In a previous study[11] from our research group, we assessed associations of a PRS, ERS, and methylation risk scores (MRSs), with BC incidence in the ESTHER cohort with up to 14 years of follow-up. While the PRS (based on 269 single-nucleotide polymorphisms (SNPs) derived from a GWAS[48]) and ERS (Dierssen–Sotos score[49]) showed positive individual associations with BC incidence, the MRS (based on 423 CpGs, derived from genetically predicted cytosine-phosphate-guanine (CpG) methylation levels[50]) was not significantly associated with BC incidence. Adjusted ORs per SD increase in the score were 1.48 (95% CI, 1.16–1.90), 1.40 (95% CI, 1.09–1.80), and 1.07 (95% CI, 0.84–1.36) for the PRS, ERS, and MRS, respectively. In the current study, the CRC-specific miR-score[23] integrating serum levels of seven miRNAs showed an even stronger inverse association with BC incidence within 17 years of follow-up in the same cohort [adjusted OR per SD increase in the miR-score, 0.54 (95% CI, 0.40–0.72)] compared to the PRS and ERS. Future research should explore further enhanced BC risk prediction by combining the miRNA-based risk score with other biomarker or risk factor-based scores such as PRS and ERS.

In the same cohort, we previously also evaluated associations of a PRS (based on 31 previously identified SNPs) and MRS (based on 151 previously identified CpG loci) with LC incidence at up to 14 years of follow-up.[14] Both the PRS and MRS showed positive individual associations with LC incidence; in models comprehensively adjusted for smoking history, adjusted ORs comparing the highest with lowest quartile were 1.93 (95% CI, 1.05–3.71) and 5.64 (95% CI, 2.13–17.03) for the PRS and MRS, respectively. In another recent study,[13] we derived and validated an LC-specific miR-score that enhanced LC risk prediction when combined with pack-years or established risk factor-based LC risk models within 14 years of follow-up in the ESTHER cohort. This 3-miRNA-based score was strongly associated with LC risk after comprehensively adjusting for smoking history [adjusted OR per SD increase in the miR-score was 2.23 (95% CI, 1.71–2.90)]. In the current study, the CRC-specific 7-miRNA-based score[23] was inversely associated with LC incidence within 17 years of follow-up in the ESTHER cohort [age and sex-adjusted OR per SD increase in the miR-score 0.64 (95% CI, 0.48–0.84)]. Interestingly, the LC-specific and CRC-specific miR-scores were composed of distinct miRNAs indicating that miRNA profiles associated with tumor progression differ across cancer types and could be useful for developing personalized screening strategies.

The strengths of our study include evaluation of circulating miRNAs as biomarkers of risk of different cancers diagnosed within a large prospective cohort with a 17-year follow-up. Complete ascertainment of incident cancers in the study population was ensured by linking the ESTHER cohort data with data from the Saarland Cancer Registry. Even though measurement of miRNAs well before diagnosis is a major asset for risk assessment compared to the commonly employed approach with blood sampling after cancer diagnosis, a limitation of our study is that it is based on samples collected only once at baseline (i.e. at a single time point), which prohibited evaluation of longitudinal changes in miRNA profiles in relation to cancer development. The study design did not allow assessment of changing miRNA patterns and cancer risk over time. As miRNA levels have been reported to change during tumor development and proliferation,[51,52] further studies measuring miRNAs repeatedly over time in representative population cohorts are needed to more accurately define their potential use for cancer risk stratification. Furthermore, our study focused only on a specific miRNA panel and its association with the risk of developing different cancers. Other miRNAs not included in our panel might also be informative for risk assessment of BC, LC, and PC. Finally, we would like to note that our results based on the ESTHER cohort cannot be generalized to non-Caucasian populations and younger adults.

To conclude, we observed strong inverse associations of our previously reported[23] CRC-specific miR-score with the risk of BC and LC. We observed no statistically significant association of the miR-score with PC risk. Our study illustrates that serum miRNA levels are altered years before BC and LC occurrence, advancing the concept that circulating miRNAs might be potentially useful biomarkers for risk stratification in cancer screening. Compared to individuals remaining free of the four most frequent cancers, serum miRNA profiles in patients with subsequent cancer development varied across different cancer types. Thus, our findings highlight the potential of serum-based miRNA biomarkers for cancer risk stratification and their potential use for personalized, risk-adapted cancer screening. Further large cohort studies with longitudinal repeat measurement of serum miRNA profiles aiming to investigate, validate, and optimize the use of circulating miRNA signatures for cancer risk assessment are warranted.

AUTHOR CONTRIBUTIONS

Janhavi R. Raut: Formal analysis; methodology; project administration; writing – original draft. **Megha Bhardwaj:** Writing – review and editing. **Ben Schöttker:** Resources; writing – review and editing. **Bernd Holleczek:** Resources; writing – review and editing. **Petra Schrotz-King:** Methodology; project administration; writing – review and editing. **Hermann Brenner:** Conceptualization; methodology; resources; supervision; writing – review and editing.

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Germany); and the Bundesministerium für Bildung und Forschung (Federal Ministry of Education and Research, Berlin, Germany). The sponsors had no role in the study design, in the collection, analysis and interpretation of data, and the preparation, review, or approval of the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

ETHICS STATEMENT

- Approval of the research protocol by an Institutional Reviewer Board: Yes.
- Informed Consent: All participants provided written informed consent.
- Registry and the Registration No. of the study/trial: The study was approved by the ethics committees of the Medical Faculty of the University of Heidelberg and of the state medical board of Saarland, Germany.
- Animal Studies: N/A.

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