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Original research

Small-RNA sequencing reveals potential serum biomarkers for gallbladder cancer: Results from a three-stage collaborative study of large European prospective cohorts

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ABSTRACT

Gallbladder cancer (GBC) is an aggressive disease with limited treatment options but high prevention potential. GBC tumours take 10–20 years to develop, a timeframe that holds potential for early detection. MicroRNAs (miRNAs) play a central role in abnormal cell processes, and circulating miRNAs may constitute valuable biomarkers of early disease. We used microarray data to pre-select differentially expressed miRNAs in formalin-fixed paraffin-embedded (FFPE) gallbladder tissue samples (GBC n = 40, normal n = 8). We then applied small-RNA sequencing to screen for miRNA expression differences in serum samples from three European prospective cohorts (n = 37 GBC case-control pairs), and validated the most promising candidates in three independent cohorts (n = 36 GBC case- control pairs). Statistical analyses included robust linear regression, pathway and meta-analysis, and examination of expression correlation between miRNAs and target genes. MiR-4533 and miR-671–5p were overexpressed in GBC tissue and serum samples, and meta-analysis confirmed the overexpression of miR-4533 in GBC serum samples from the prospective cohorts (p-value = 4.1×10^{-4}), especially in individuals of female sex, under 63.5 years, or with a BMI below 26.2 kg/m^2. Pathway and correlation analyses revealed that miR-4533 targets *SIPA1L2* in the Rap1 signalling pathway, and *SIPA1L2* was downregulated in GBC serum samples. Our study highlights the advantage of integrating small-RNA sequencing results from different types of samples and independent datasets, and the need for international research collaborations to identify and validate biomarkers for secondary prevention of rare tumours such as GBC. The function of miR-4533 and its interaction with *SIPA1L2* in GBC development need to be further investigated.

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1. Introduction

Gallbladder cancer (GBC; International Classification of Diseases, 10th Revision, diagnosis code C23) is an aggressive malignancy that causes approximately 89,000 deaths worldwide each year, a number that is expected to increase by 74 % by 2045 [1]. GBC primarily affects low- and middle-income countries, with 83 % of cases occurring in Asia and Latin America, while only 11 % of cases are diagnosed in Europe and North America, where GBC is relatively rare [1].

Gallstones are an important risk factor for the development of GBC, particularly in Europe and Latin America, where around 90 % of GBC cases are attributable to gallstone disease [2,3]. Other GBC risk factors include female sex, overweight and obesity as reflected by an elevated body-mass index (BMI), a family history of GBC, and recently identified susceptibility variants [4–8].

GBC generally develops within 10–20 years through the sequence "gallstones and inflammation → dysplasia → GBC" [9]. However, 5-year survival rates after GBC diagnosis vary between 5 % and 30 %, depending on the country [10–12]. Complete tumour resection is currently the only curative treatment option for GBC, but due to delayed and non-specific symptoms, most patients are diagnosed too late to undergo curative surgery [13,14]. Understanding the molecular changes responsible for GBC development, and identifying novel biomarkers for early diagnosis are therefore crucial.

Micro-RNAs (miRNAs) are a class of small, single-stranded RNAs of approximately 22–25 nucleotides [15]. Abnormal miRNA expression levels have been linked to a wide range of diseases, including cancer [16,17]. Through hybridization to the 3'-untranslated regions of mRNAs, miRNAs are important trans-regulators of gene expression [18]. Several studies have demonstrated that miRNAs can be released from cancer cells, leading to altered expression levels in human tissues, including blood and blood derivatives [19]. MiRNAs hold therefore a large potential as cancer biomarkers for risk prediction, early diagnosis and therapeutic intervention [20–23]. However, GBC research has been largely neglected, and the function of miRNAs in GBC development, and their potential for early detection, have not been sufficiently explored. Most miRNA studies on GBC conducted to date have investigated Latin American and Asian patients, as the disease is more common in these regions. However, these patients are exposed to different risk factors, have access to different health systems, and have a different genetic background than European patients [4,5,24].

The present study attempts to identify miRNAs as serum biomarkers for GBC risk prediction and early detection, and to elucidate the role of the identified miRNAs in GBC pathogenesis. After pre-selection of miRNAs using microarray data from formalin-fixed, paraffin-embedded (FFPE) gallbladder tissue samples from German GBC and gallstone disease patients, we sequenced small RNAs in serum samples from the large European prospective cohorts (screening: Norwegian and German cohorts, validation: Norwegian, Swedish and Finnish cohorts), combined the results from individual cohorts in a meta-analysis, and investigated the interaction between identified miRNAs and target genes using pathway analysis.

2. Materials and methods

2.1. Study design and investigated datasets

Figure 1 shows the study design. We followed a three-stage approach to (1) pre-select (2) screen, and (3) validate differentially expressed miRNAs in GBC and control samples from European patients. After pre-selection and screening, and before sequencing the small RNAs for validation, we registered the study and the miRNAs to be validated at the German Clinical Trials Register (DRKS, German Clinical Trials Register (drks.de), March, 5th 2021) and the International Clinical Trials Registry Platform of the World Health Organization (WHO, https://trialsearch.who.int/Trial2.aspx?TrialID=DRKS00024573).

The pre-selection dataset was used to identify miRNAs differentially expressed in FFPE tissue samples from normal, non-neoplastic gallbladders (n = 8) and GBC (n = 40). This patient collective has already been described in detail [24]. Briefly, tissue samples from patients who underwent surgical removal of the gallbladder (cholecystectomy) were obtained by the tissue bank of the National Centre for Tumour Diseases

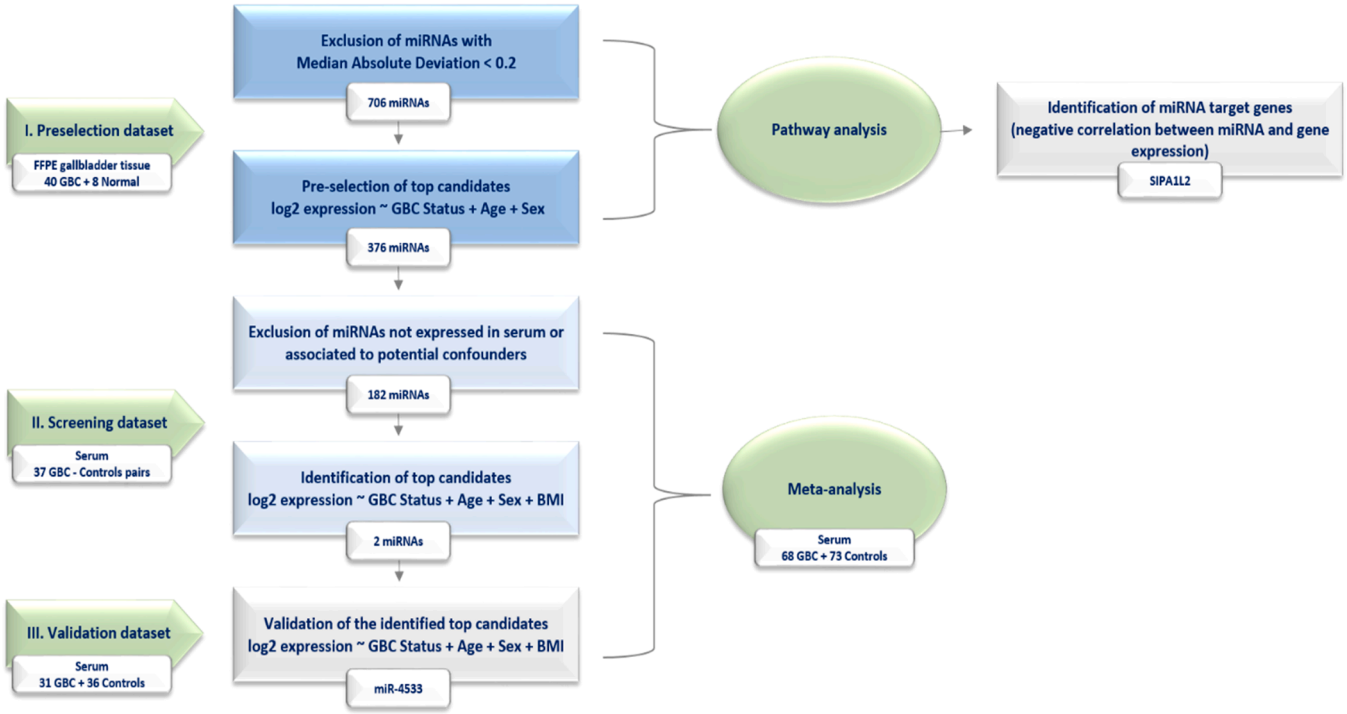

Fig. 1. Flowchart describing the overall study design. miRNAs: microRNAs; FFPE: formalin-fixed paraffin-embedded; GBC: gallbladder cancer; BMI: body-mass index.

(NCT Heidelberg, Germany). GBC patients underwent cholecystectomy at the time of diagnosis and received no treatment prior to sampling. GBC cases were histologically confirmed by at least two specialized pathologists at the Institute of Pathology at Heidelberg University Hospital. Non-neoplastic gallbladder tissue samples were obtained from patients who underwent cholecystectomy due to gallstone disease and served as the reference group for normal tissue in our study.

After miRNA pre-selection based on FFPE gallbladder tissue, we analysed 74 serum samples from three European prospective cohorts (n = 37 GBC case-control pairs, screening dataset) to determine which pre-selected miRNAs exhibited consistent expression differences in serum samples from GBC cases and matched controls. Data and samples were provided by the Norwegian Janus Serum Bank (n = 27 GBC case-control pairs); the German Early Detection and Optimised Therapy of Chronic Diseases in the Elderly Population (ESTHER) study (n = 9 GBC case-control pairs), and the German Heinz Nixdorf Recall (HNR) study (n = 1 GBC case-controls pair). As only one case-control pair was available from the HNR study, this cohort was merged with the ESTHER study, both of which consist of German individuals. Controls were matched by age and sex with GBC cases, and the main characteristics of the three cohorts are briefly summarized in the supplementary material.

The most promising miRNAs identified in the screening dataset were subsequently investigated in the validation dataset, which included data and samples (n = 36 GBC case-control pairs) from three large European prospective cohorts - the Finnish FINRISK cohort (n = 9 GBC case-control pairs), the Norwegian Helseundersøkelsen i Nord-Trøndelag (HUNT) study (n = 15 GBC case-control pairs), and the Swedish Twin Registry (n = 12 GBC case-control pairs). Controls were matched by age and sex with GBC cases. The main features of these cohorts are also briefly summarised in the supplementary material.

We also examined the mRNA expression levels of 10 GBC cell lines (G-415, GB-d1, Mz-Cha-1, NOZ, OCUG-1, OZ, SNU308, TGBC1 (also known as TGBC1TKB), TGBC2 (also known as TGBC2TKB) and YoMi). All cell lines were regularly tested for mycoplasma contamination using MycoAlert (Lonza, Basel, Switzerland) and authenticated by short tandem repeat analysis. Detailed information on the characterisation of the cell lines can be found in Reference [25].

2.2. RNA extraction, small RNA sequencing and genotyping

The protocol followed for RNA extraction, isolation, and profiling from FFPE gallbladder tissue has been described previously [24]. Briefly, miRNA samples were purified for microarray hybridization from microdissected FFPE material using the miRNeasy FFPE Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Agilent SurePrint Human miRNA microarrays (G4872A, miRBase Release 19.0, Agilent Technologies, Santa Clara, CA), which include 2006 human miRNAs, were used for miRNA profiling of normal gallbladder and GBC tumour samples. Labelling, hybridization and data processing were performed following the manufacturer's recommendations.

The protocol applied for small RNA extraction and sequencing from serum samples has also been described [26,27]. Briefly, RNA was extracted from 2 × 200 µl (screening) and 1 x 200 µl of serum (validation) using phenolchloroform separation and the miRNeasy Serum/Plasma kit (Cat. no 1071073, Qiagen) on a QIAcube (Qiagen). Glycogen (Cat. no AM9510, Invitrogen) was used as carrier during the RNA extraction step. The eluate was concentrated using Ampure beads XP (Agencourt). The NEBNext Small RNA kit was used to produce RNA sequencing libraries, which were sequenced on the HiSeq 2500 and 4000 (screening), and Novaseq 6000 (validation) platforms (Illumina, San Diego, CA, USA) for average depths of 18 M (screening) and 22 M reads per sample (validation), enabling us to capture mapped miRNA fragments of up to 47 base pairs. RNA counts were calculated using the sncRNA pipeline (https://github.com/sinanugur/sncRNA-workflow/) [28]. Reads were adapter-trimmed (AdapterRemoval v2.1.7), mapped to the human genome (hg38) by Bowtie2 v2.2.9 aligner in end-to-end mode, and HTSeq was used to count reads mapped to miRbase regions (v22.1).

Genomic DNA was extracted from peripheral blood using standard commercial kits and following standard laboratory procedures. Intraplate and interplate replicates, and blinded duplicates were included (at 5 %) as quality control measures. Study participants were genotyped with Illumina's OmniExpress and Global Screening arrays. Both arrays included more than 700,000 genome-wide single nucleotide polymorphisms. Missing genotypes were imputed with the minimac4 imputation software and the TOPMed reference sample via the TOPMed imputation server, accessible at https://imputation.biodatacatalyst.nhlbi.nih.gov/.

2.3. Statistical analyses

MiRNA read counts were log2-transformed and miRNAs with low expression variability (median absolute deviation - MAD) were excluded from subsequent analyses. In the pre-selection dataset, quantile normalization was first applied separately to GBC and normal samples, and then simultaneously to all samples. In the screening and validation datasets, quantile normalization was first applied to each cohort separately, then to GBC cases and controls, and finally to the complete dataset. Principal component analysis (PCA) was performed for unsupervised examination of global expression profiles and identification of potential outlying samples, which were subsequently excluded based on Mahalanobis statistical depth (5 % of samples with the lowest statistical depth were removed). The R package "stats" was used for PCA and statistical depth calculation [29].

Pre-selection, screening and validation of differentially expressed miRNAs were based on robust linear regression. The pre-selection regression models included GBC status, age categorised into quartiles, and sex. The screening and validation regression models additionally included BMI categorised into quartiles (BMI information was not available in the pre-selection dataset). In the pre-selection stage, p-values from robust linear regression were adjusted for multiplicity using the Bonferroni method (for subsequent screening) and the false discovery rate (FDR, for pathway analysis), taking into account the number of miRNAs with MAD greater than zero. In the screening stage, Bonferroni and FDR adjustments for multiplicity considered the number of pre-selected miRNAs that were expressed in the serum samples, and miRNAs with expression levels associated with potential confounders (age, sex, smoking, BMI and physical activity) were excluded [27]. In the validation stage, Bonferroni adjustment for multiplicity considered the number of differentially expressed miRNAs identified in the screening stage. Robust linear regression models were fitted using the function "rlm" in the R package "MASS" [30], and the corresponding p-values were calculated using the function "rob.pvals" in the R package "repmod" [31].

After validation, we performed a meta-analysis to combine the results from all prospective cohorts using the "rma" function in the "Metafor" package [32]. The input values for the function were beta estimates with their corresponding standard errors from each cohort, and the cohort sample sizes as weights. We performed both fixed-effects and random-effects meta-analysis, used the function "forest", also in the "Metafor" package, to plot the results of the meta-analysis, and created the remaining plots using the R package "ggplot2" [33].

Survival data were only available for GBC patients in the pre-selection dataset, and we performed Kaplan-Meier survival analyses to investigate the association between miRNA expression and overall survival based on these patients. miRNA expression levels were dichotomized at the median value for this purpose. All analyses were conducted using R version 4.2.2, and the source code to reproduce all calculations is available at www.biometrie.uni-heidelberg.de/StatisticalGenetics/Software_and_Data.

2.4. Polygenic risk scores for gallstone disease, pathway and miRNA-gene expression correlation analysis

Genotype information was available for some participants in the ESTHER (n = 18), HUNT (n = 29), FINRISK (n = 16), and TwinGene (n = 17) studies, and we examined difference in miRNA expression as a function of individual polygenic risk scores (PRS) for gallstone disease. We used summary statistics on the association between genetic variants and gallstone disease from the UK Biobank (18,417 gallstone disease cases and 390,150 controls) for variants that were robustly (p-value $< 5 \times 10^{-8}$) associated with gallstone disease in the study by Ferkingstad et al. to calculate the PRS [34,35]. After excluding variants and samples with missing call rates of more than 5 %, variants with a minor allele frequency of less than 1 %, linkage disequilibrium pruning ($r^2 > 0.1$), and harmonisation of reference and alternative alleles in the UK Biobank and in the investigated prospective cohorts, PRS were calculated by multiplying the estimated additive genetic effects by the individual allele counts.

Based on the list of pre-selected miRNAs based on FFPE gallbladder tissue, we used the web-based software DIANA-miRPath v3.0 (http://diana.imis.athena-innovation.gr) for miRNA-based pathway analysis, and sorted the overrepresented pathways by FDR-corrected p-values. In addition to miRNA expression, mRNA expression values based on small RNA sequencing were also available for the analysed serum samples, and we used this information to investigate the relationship between miRNA and mRNA expression for the validated miRNAs. The total number of genes in the five pathways with the smallest FDR-corrected p-values was considered for Bonferroni adjustment of p-values from one-sided Spearman tests (negative miRNA-mRNA correlation), and possible differences in mRNA expression between GBC cases and controls were assessed by robust linear regression models adjusted for age, sex, and BMI. Finally, we visually inspected the miRNA-mRNA relationship, differences between GBC cases and controls in mRNA expression, and mRNA expression in GBC cell lines using scatter and dox-and-box plots.

3. Results

Table 1 shows the main characteristics of the datasets investigated. The pre-selection dataset (FFPE gallbladder tissue samples) contained more women (63 %) than men (37 %), 44 % of patients were older than 71 years, and information on BMI and smoking was not available. Most GBC patients were affected by T2 (43 %) and T3 (38 %) tumours (Table S1). Lymph node status was unknown in 48 % of patients, and 85 % of patients showed no evidence of metastasis. The majority of tumours (65 %) were classified as intermediate grade (G2). Apart from cholecystolithiasis, which occurred in 43 % of patients, most GBC patients exhibited no other pre-existing biliary condition.

Women were also overrepresented in the screening dataset (Janus: 74 %, ESTHER+HNR: 90 %), with 50 % of Janus participants under 54 years, while 55 % of participants in the ESTHER and HNR studies were aged 64–71 years. We also noticed differences in BMI between the Norwegian and German cohorts: the proportion of individuals with a BMI over 26.2 kg/m^2 was 38 % in the Janus study, compared to 65 % in the ESTHER and HNR cohorts. In terms of number of years between blood collection and GBC diagnosis, 63 % of Janus participants were diagnosed 9 years after blood sampling, while all ESTHER and HNR participants were diagnosed within 9 years.

In the validation dataset, women were overrepresented in the HUNT cohort (85 %), but not in FINRISK (44 %) or TwinGene (50 %). The proportion of individuals older than 71 years was 23 % in HUNT, 41 % in FINRISK and 45 % in TwinGene. Percentages of participants with a BMI over 29.4 kg/m^2 were 28 % in HUNT, 53 % in FINRISK and 19 % in TwinGene. Regarding the time between blood sampling and GBC diagnosis, the proportion of participants diagnosed at least 9 years after blood draw was 73 % in HUNT, 26 % in FINRISK and 0 % in TwinGene. Summing up, the datasets investigated were heterogeneous in terms of age, sex, BMI and time from blood collection to GBC diagnosis.

Among the 2006 miRNAs detected in FFPE gallbladder tissue, 1300 showed low expression variability (MAD less than 0.2) and were excluded from further analysis (Fig. 1). A PCA plot based on the remaining 706 miRNAs revealed different global expression profiles in GBC and normal gallbladder tissue samples (Fig. 2A), with the first principal component explaining 19 % of the overall variance in miRNA expression. P-values from robust linear regression adjusted for multiplicity using the Bonferroni method identified 376 miRNAs differentially expressed in GBC compared to normal gallbladder tissue (Fig. 2C, Table S2). In particular, we found that 215 miRNAs were overexpressed, and 161 miRNAs were underexpressed in GBC tissue.

Fig. 2B shows the global expression profiles based on MAD-positive

Table 1
Main patient characteristics in the investigated datasets. The preselection dataset consisted of FFPE gallbladder tissue samples from GBC and gallstone disease patients recruited in Germany. The screening dataset included serum samples from three European prospective cohorts (Janus in Norway, and ESTHER and HNR in Germany). The validation dataset comprised serum samples from three prospective cohorts (HUNT in Norway, FINRISK in Finland and TwinGene in Sweden).

		Preselection		Screening				Validation					
		FFPE		Janus		ESTHER+HNR		HUNT		FINRISK		TwinGene	
Variable	Level	n	%	n	%	n	%	n	%	n	%	n	%
Disease Status	GBC	40	0.83	27	0.50	10	0.50	15	0.50	8	0.47	8	0.40
	Normal/Control	8	0.17	27	0.50	10	0.50	15	0.50	9	0.53	12	0.60
Age (years)	Q1: 25 −54	8	0.17	27	0.50	1	0.05	5	0.17	3	0.18	0	0
	Q2: 54 −64	10	0.21	21	0.39	6	0.30	6	0.20	2	0.12	0	0
	Q3: 64 −71	9	0.19	1	0.02	11	0.55	12	0.40	5	0.29	11	0.55
	Q4: 71 −89	21	0.44	5	0.09	2	0.10	7	0.23	7	0.41	9	0.45
Sex	Female	30	0.63	40	0.74	18	0.90	24	0.85	7	0.44	10	0.50
	Male	18	0.37	14	0.26	2	0.10	4	0.14	9	0.56	10	0.50
BMI (kg/m^2)	Q1: 18.1 −23.3	-	-	18	0.35	4	0.20	8	0.28	1	0.06	3	0.19
	Q2: 23.3 −26.2	-	-	14	0.27	3	0.15	7	0.24	3	0.18	6	0.38
	Q3: 26.2 −29.4	-	-	12	0.23	7	0.35	6	0.21	4	0.24	4	0.25
	Q4: 29.4 −45.9	-	-	8	0.15	6	0.30	8	0.28	9	0.53	3	0.19
Smoking	Never	-	-	16	0.31	8	0.57	11	0.42	6	0.38	-	-
	Former	-	-	15	0.28	4	0.29	9	0.34	7	0.44	-	-
	Current	-	-	22	0.41	2	0.14	6	0.23	3	0.18	-	-
Follow-up time (years)	Q1: 0 −3.5	-	-	4	0.15	5	0.50	0	0	4	0.50	4	0.50
	Q2: 3.5 −9	-	-	6	0.22	5	0.50	3	0.20	2	0.25	4	0.50
	Q3: 9 −12.5	-	-	7	0.26	0	0	5	0.33	1	0.13	0	0
	Q4: 12.5 −18	-	-	10	0.37	0	0	6	0.40	1	0.13	0	0

Abbreviations: GBC: gallbladder cancer; FFPE: formalin-fixed paraffin-embedded; BMI: Body-mass index; Q1-Q4: first to fourth quartiles

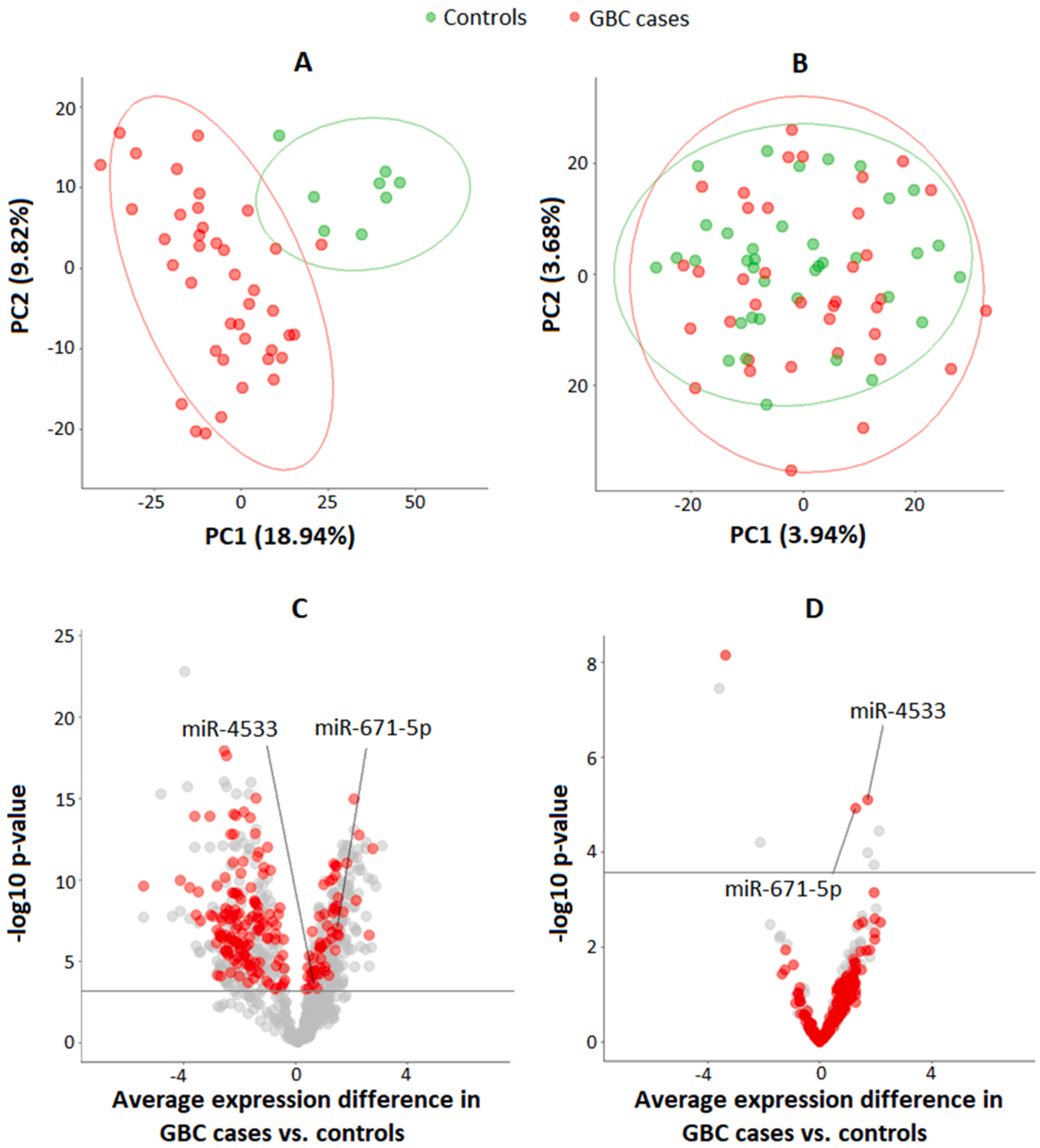

Fig. 2. Exploratory analysis of global miRNA expression profiles in the pre-selection (FFPE gallbladder tissue samples) and screening (serum samples) datasets. Panels A and B: Principal component analysis (PCA) plots of normalized log2 expression counts for miRNAs in the pre-selection (Panel A) and screening (Panel B) datasets. The x-axis shows the first principal component (PC1) and its explained variance in global miRNA expression; the y-axis shows the same information for the second principal component (PC2). Panels C and D: Volcano plots for miRNAs in the preselection (Panel C) and screening (Panel D) datasets. The x-axis shows the estimated average expression difference, and the y-axis shows the -log10 p-value from robust linear regression. Red dots represent miRNAs expressed in both FFPE gallbladder tissue and serum samples, and the grey horizontal lines show the statistical significance threshold (multiplicity-corrected Bonferroni p-value = 0.05). FFPE: formalin-fixed paraffin-embedded; GBC: gallbladder cancer; p-value: probability value.

miRNAs in the screening dataset. In contrast to the pre-selection dataset, which included gallbladder tissue samples, GBC cases and controls showed similar global miRNA expression patterns in serum. Among the 376 pre-selected candidates, 186 miRNAs were also detectable in serum (Fig. 2D). Four miRNAs associated with potential confounders in previous research were excluded from further analysis (miR-320d, miR-4466, miR-4516, miR-4755–3p). After robust linear regression analysis and multiplicity correction, three miRNAs were associated with GBC

risk. MiR-3925–5p showed a protective effect, while miR-4533 and miR-671–5p were associated with an increased risk of GBC. However, only miR-4533 and miR-671–5p showed consistent expression differences in gallbladder tissue and serum samples. MiR-3925–5p was underexpressed in GBC tissue but overexpressed in serum samples from GBC cases and was therefore excluded from further analyses. We also performed cohort-specific robust linear regression analyses stratified by age, sex, and BMI (Table S3).

Visual inspection of the global miRNA expression profiles in the validation dataset revealed the presence of five outlying samples, which were excluded from further analysis based on statistical depth (Fig. S1), resulting in 31 GBC cases and 36 controls ultimately used for validation. Robust linear regression detected no association between the two miRNAs identified in the screening dataset and GBC risk (Table S3), but stratified analyses confirmed overexpression of miR-4533 in prospective serum samples from GBC cases in HUNT cohort, especially in individuals with a BMI below 26.2 kg/m^2, and with an increased genetic susceptibility to gallstones (Table S3). miR-671–5p showed low overall expression in the validation dataset (Fig. 3C). Fig. S2 shows the global miRNA expression profiles of the screening and validation serum datasets.

Both fixed-effect and random-effect meta-analyses suggested that miR-4533 expression is associated with an increased risk of GBC (Fig. 3B), but no association emerged for miR-671–5p (Fig. 3D). Table 2 shows the overall and stratified results from robust linear regression models for the two candidates considering simultaneously all prospective cohorts investigated. Results adjusted for age, sex and BMI confirmed the increased expression of miR-4533 in prospective serum samples of GBC patients, particularly in individuals of female sex, younger than 63.5 years, or with a BMI below 26.2 kg/m^2. We found no correlation between sex and miR-4533 expression (p-value = 0.70), but the expression of miR-4533 decreased with age (p-value = 0.001) and BMI (p-value = 0.04). The results from survival analysis showed no association between overall survival and miR-4533 expression (Fig. S4, log-rank test p-value: 0.70).

Pathway analyses using the DIANA mirPath software indicated that miR-4533 is involved in the regulation of multiple cancer pathways. Sixty-five KEGG biological processes were significantly enriched (FDR-corrected p-value < 0.05). The top five pathways involving miR-4533 were related to proteoglycans in cancer, renal cell carcinoma, glioma, ErbB signalling, and Rap1 signalling (Table S4). These five pathways included 510 genes in total, but some of them belonged to several pathways and others were not expressed in our investigated serum samples, resulting in 308 genes examined in the miRNA-mRNA correlation analyses (Table S5).

Table 3 shows the results for the 10 genes most negatively and strongly correlated with miR-4533 expression. Among them, only *SIPA1L2* (Signal Induced Proliferation Associated 1 Like 2 gene) and *FAS* (Fas Cell Surface Death Receptor) were associated with GBC risk.

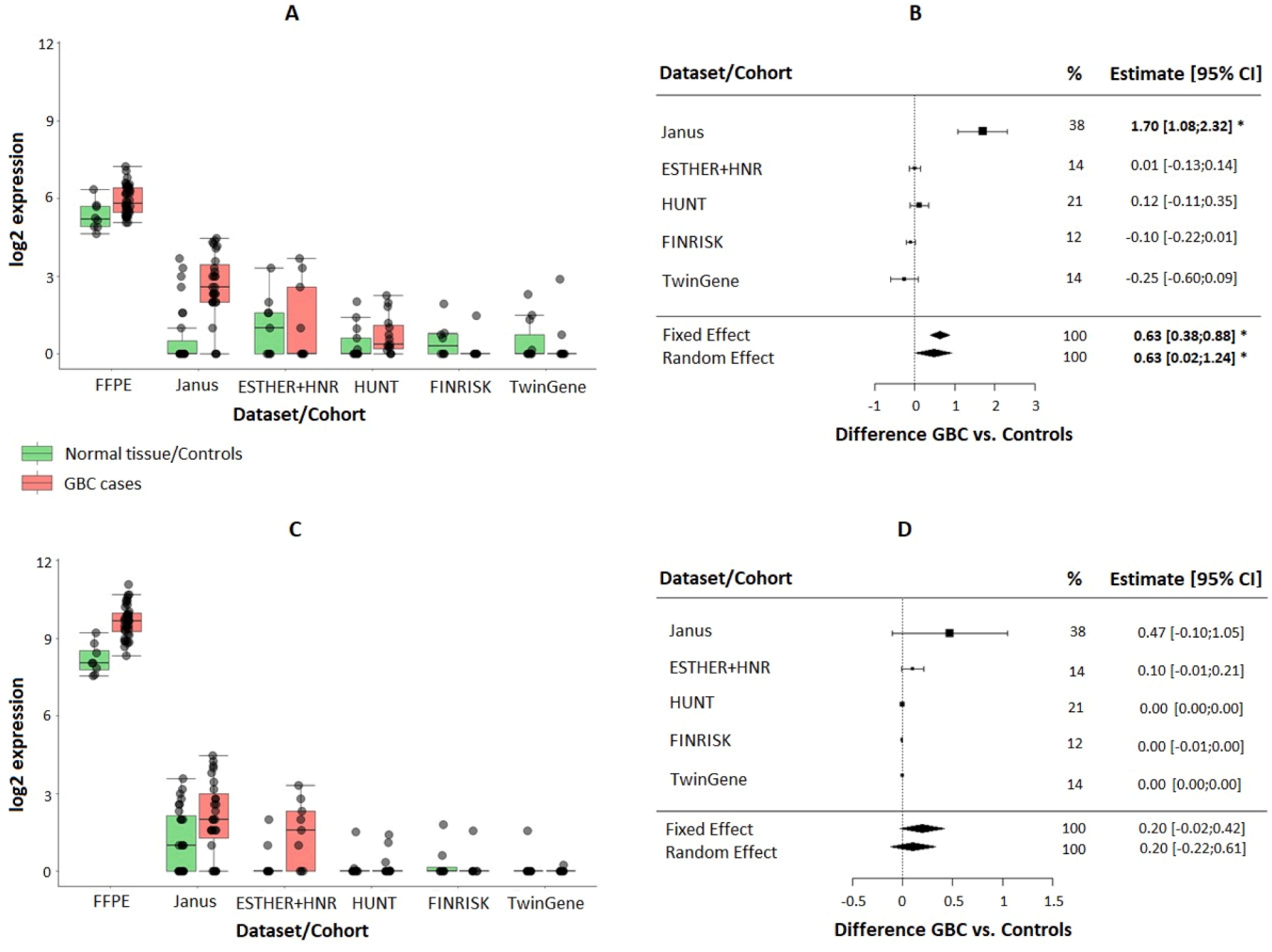

Fig. 3. Expression of miR-4533 and miR-671–5p in FFPE gallbladder tissue and serum samples, and average differences in serum expression between GBC cases and controls. Panels A and C: Dot-and-box plots of log2 expression in the preselection dataset and in the five investigated prospective cohorts (Panel A: miR-4533; Panel C: miR-671–5p). Panels B and D: Forest plots and combined average differences in serum expression between GBC cases and controls from fixed and random effects meta-analysis (Panel B: miR-4533; Panel D: miR-671–5p). FFPE: formalin-fixed paraffin-embedded; CI: confidence interval; GBC: gallbladder cancer.

Table 2
Overall and stratified differences in miRNA expression by age, sex, body-mass index and genetic susceptibility to gallstone disease between prospective GBC cases and controls.

Variable	Level	miR-4533		miR-671-5p	
		log2 expression in controls Median [5th; 95th]	GBC Case-ControlDifference [95 % CI]	log2 expression in controlsMedian [5th;95th]	GBC Case-Control Difference [95 % CI]
All	-	0.00 [0.00; 2.21]	**0.43** [0.17; 0.69]	0.00 [0.00; 2.85]	0.06 [−0.09; 0.20]
Age	< 63.5 years	0.00 [0.00; 2.42]	**1.17** [0.63; 1.71]	0.00 [0.00; 2.99]	0.36 [−0.08; 0.80]
	≥ 63.5 years	0.00 [0.00; 1.76]	0.01 [−0.07; 0.09]	0.00 [0.00; 1.21]	−0.00 [−0.001; 0.001]
Sex	Female	0.00 [0.00; 1.99]	**0.42** [0.14; 0.70]	0.00 [0.00; 2.69]	0.09 [−0.13; 0.30]
	Male	0.02 [0.00; 2.14]	0.32 [−0.21; 0.85]	0.00 [0.00; 2.91]	0.07 [−0.23; 0.38]
BMI	< 26.2 kg/m^2	0.00 [0.00; 1.89]	**0.83** [0.42; 1.24]	0.00 [0.00; 2.68]	0.26 [−0.01; 0.54]
	≥ 26.2 kg/m^2	0.00 [0.00; 1.97]	0.14 [−0.06; 0.34]	0.00 [0.00; 2.86]	−0.01 [−0.04; 0.02]
GSD-PRS*	< 2.88	0.00 [0.00; 1.36]	0.07 [−0.17; 0.31]	0.00 [0.00; 1.21]	−0.00 [−0.003; 0.001]
	≥ 2.88	0.01 [0.00; 1.15]	−0.15 [−0.37; 0.05]	0.00 [0.00; 1.15]	−0.04 [−0.10; 0.02]

Abbreviations: GBC: Gallbladder cancer; 5th; 95th: 5th and 95th percentiles; CI: Confidence interval; BMI: Body-mass index; GSD-PRS: Polygenic risk score for gallstone disease (Calculated for 80 out of 146 individuals with available genotype information). Bold type indicates the 95 % CI does not include zero.

Table 3
List of the top 10 genes with expression values most negatively correlated with miR-4533 expression in the 5 pathways with the smallest false-discovery-rate-corrected p-values.

Gene	Spearman rho correlation			Expression difference in GBC cases vs. controls		
	Estimate	95 % CI	p-value	Estimate	95 % CI	p-value
FLT4	−0.268	[−0.48; −0.05]	0.01	0.23	[−0.10; 0.56]	0.17
RAP1A	−0.262	[−0.51; −0.01]	0.01	−0.15	[−0.47; 0.17]	0.35
FGF7	−0.248	[−0.45; −0.03]	0.02	−0.01	[−0.20; 0.18]	0.93
SIPA1L2	**−0.247**	[−0.48; −0.02]	0.02	**−0.60**	[−1.18; −0.01]	0.04
ARNT2	−0.245	[−0.45; −0.02]	0.02	0.07	[−0.11; 0.27]	0.53
ITGAM	−0.189	[−0.39; 0.03]	0.05	0.00	[−0.16; 0.33]	0.97
MAPK9	−0.189	[−0.39; 0.02]	0.06	0.18	[−0.03; 0.67]	0.27
RAPGEF1	−0.187	[−0.41; 0.06]	0.06	−0.28	[−0.50; 0.19]	0.09
RAPGEF5	−0.187	[−0.42; 0.06]	0.06	0.12	[−0.10; 0.39]	0.41
FAS	−0.179	[−0.39; 0.06]	0.07	0.44	[0.16; 0.76]	0.01

Abbreviations: GBC: gallbladder cancer; CI: confidence interval; p-value: probability value; *FLT4*: Fms Related Receptor Tyrosine Kinase 4; *RAP1A*: Ras-related protein Rap-1A; *FGF7*: Fibroblast Growth Factor 7; *SIPA1L2*: Signal Induced Proliferation Associated 1 Like 2; *ARNT2*: Aryl Hydrocarbon Receptor Nuclear Translocator 2; *ITGAM*: Integrin Subunit Alpha M; *MAPK9*: Mitogen-Activated Protein Kinase 9; *RAPGEF1*: Rap Guanine Nucleotide Exchange Factor 1; *RAPGEF5*: Rap Guanine Nucleotide Exchange Factor 5; *FAS*: Fas Cell Surface Death Receptor. Bold type indicates the 95 % CI for Spearman rho correlation does not include zero, the gene expression difference is negative, and the 95 % CI for the expression difference does not include zero.

However, *FAS* was overexpressed in serum samples from prospective GBC cases, and we decided to focus on *SIPA1L2* (Spearman rho correlation −0.247, average GBC case-control expression difference −0.60, Table 3). Fig. 4A depicts the negative relationship between miR-4533 and *SIPA1L2* expression in the investigated prospective serum samples from control participants. Fig. 4B shows that while miR-4533 is overexpressed, *SIPA1L2* is downregulated in serum samples from GBC cases compared to control. The correlation between miR-4533 and *SIPA1L2* serum expression was negative in all investigated prospective European cohorts and also in the serum samples from 138 Chilean patients affected by gallstones, but the magnitude of the negative association showed a wide variability, ranging from −0.03 (95 % CI −1.00 to 0.07) in the Chilean samples to −0.73 (95 % CI −0.97 to −0.26) in the FINRISK cohort (Fig. S5). *SIPA1L2* was expressed in 10 GBC cell-lines (Fig. 4**C**), showing its highest expression in YoMi.

Without attempting an exhaustive review of the literature, we also examined the expression in our serum samples of 34 miRNAs previously associated with GBC [24,36–39]. Most studies (74 %) were conducted in India, followed by China (24 %), and all but one study investigated gallbladder tissue samples (Table S6). Of the 34 miRNAs, eight showed an association between their serum expression levels and GBC risk (miR-145–5p, miR-144–5p, miR-196a-5p, miR-196b-5p, miR-32–5p, miR-3613–5p, miR-374a-5p, miR-378c). The expression of three miRNAs in serum (miR-144–5p, miR-145–5p in the Indian study (but not in the single European study) and miR-378c) was consistent with previous reports, where miR-144–5p and miR-145–5p were overexpressed in serum and gallbladder tissue of GBC patients, and miR-378c was downregulated in both types of samples.

4. Discussion

GBC is a very aggressive tumour with limited treatment options and a poor prognosis. As tumour development takes 10 to 20 years and cholecystectomy is a rather uncomplicated procedure, the implementation of primary and secondary prevention strategies would significantly improve the prognosis of GBC patients. Previous studies have shown the potential of miRNAs as risk and early detection biomarkers, as well as their regulatory role in many important biological processes [16,40]. The link between miRNA expression and GBC development has been partially investigated, but most previous studies have been conducted in individuals of Asian genetic background [24,36–38]. Given the aggressiveness and preventive potential of GBC, there is an urgent need to identify novel biomarkers for individuals of European ancestry as well.

In the present study, we aimed to identify circulating miRNAs as potential biomarkers for GBC prevention using data and serum samples from six large European prospective cohorts. We applied a three-step approach to (1) pre-select miRNAs using FFPE tissue samples, (2) screen miRNAs using serum samples from three cohorts, and (3) validate the identified miRNA-GBC risk associations in serum samples from three different cohorts. We combined the cohort-specific results by meta-analysis, and performed a pathway analysis to explore the role of validated miRNAs.

Two miRNAs, miR-4533 and miR-671–5p, were overexpressed in both GBC tissue and serum samples from GBC cases. Subsequent validation and meta-analysis confirmed that serum expression of miR-4533 was associated with an increased risk of GBC. Pathway analysis indicated that miR-4533 is involved in several cancer pathways, such as

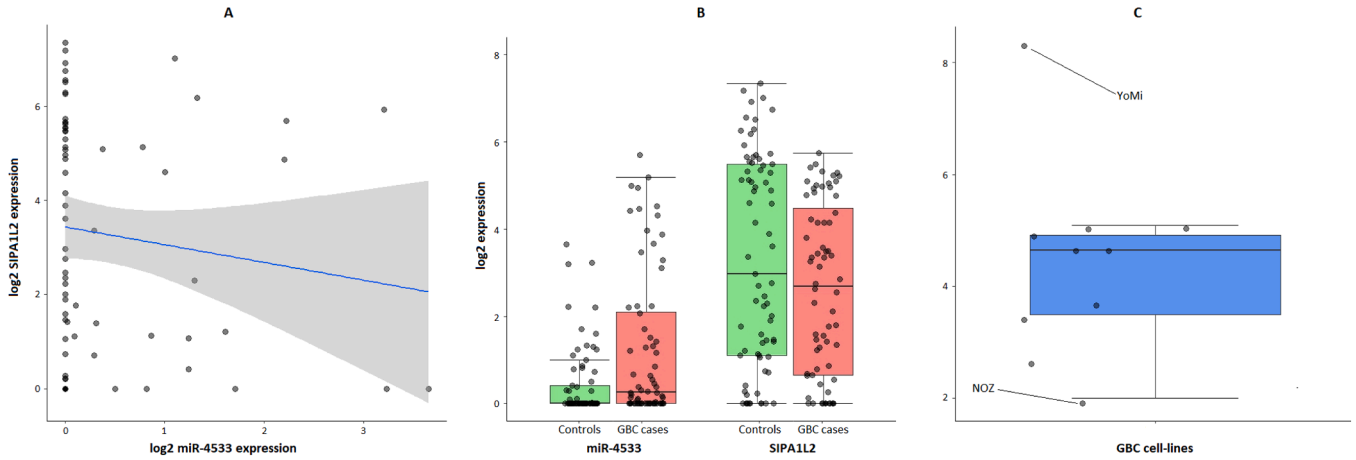

Fig. 4. miR-4533 and *SIPA1L2* expression in serum samples and GBC cell lines. Panel A: Scatterplot of log2 miR-4533 vs *SIPA1L2* expression in serum samples from control subjects. Panel B: log2 miR-4533 and *SIPA1L2* expression in serum samples from control subjects (green) and GBC cases (red) in the five prospective cohorts investigated. Panel C: log2 *SIPA1L2* expression in 10 GBC cell-lines. NOZ and YoMi were the two cell-lines with the lowest and highest log2 *SIPA1L2* expression, respectively. *SIPA1L2*: Signal Induced Proliferation Associated 1 Like 2 gene; GBC: gallbladder cancer.

proteoglycans in cancer, renal cell carcinoma, glioma, ErbB and Rap1 signalling. The negative correlation between the expression of *SIPA1L2* and miR-4533 suggested that *SIPA1L2* is a target gene of miR-4533, as indicated in the miRDB, miRWalk, and TargetScan databases [41–43], and *SIPA1L2* was underexpressed in serum samples from GBC cases. We used the DIANA software for pathway analysis, but the web tool "miEAA" indicated that miR-4533 is also involved in the pancreatic cancer pathway [44]. This pathway includes the gene *CASP8*, which was negatively correlated with miR-4533 expression, and downregulated in GBC cases. We also examined the expression of 34 miRNAs previously associated with GBC in our own prospective serum samples, and found that miR-144–5p and miR-145–5p (in an Indian but not a European study) were overexpressed in serum and gallbladder tissue of GBC patients, and miR-378c was downregulated in both types of samples.

Our study has strengths and limitations. One strength was the registration of the miRNA validation after pre-selection and screening on the German and WHO Clinical Trials Registry platforms. Even after combining data and samples from large European cohorts and conducting the largest prospective study to date, the sample size was relatively small (67 GBC case-control pairs), as is often the case with rare diseases. Follow-up studies that include a larger number of study participants will lead to a higher number of identified and validated miRNAs, and more accurate estimates of individual GBC risk. The preselection of miRNA candidates based on gallbladder tissue was probably a strength of this study: In principle, we would expect higher expression levels of GBC biomarkers, and greater expression differences between patients and unaffected controls, in gallbladder tissue than in serum samples – this was the case for the two miRNAs that we sought to validate (Fig. 3A and B). An advantage of miRNAs over other potential circulating biomarkers (metabolites, proteins, etc.) is that they are very stable in serum even at extreme temperatures and long-term storage [45]. The heterogeneity of the prospective cohorts studied (age, sex, BMI, time from blood retrieval to GBC diagnosis) probably translates into a good representativeness of our results, but on the other hand, we likely overlook miRNA expression differences that are population-specific. Gallstones are an important risk factor for GBC in Europe, and the lack of information on gallstones was another limitation of this study, but we attempted to account for this risk factor by calculating a polygenic risk score based on genetic variants robustly associated with gallstone disease.

There is some evidence in the literature for the role of miR-4533 as a potential cancer biomarker. A miRNA study of colorectal cancer with participants of Caucasian, Hispanic, Asian and African ancestry found miR-4533 overexpression in the colorectal mucosa [46]. Upregulation of miR-4533 has also been found in breast and prostate cancer [47,48]. Since miR-4533 does not have a hairpin loop and is probably dicer independent, it may not be a canonical miRNA. However, miR-4533 is listed in the miRbase database, and our results suggest that it is a potential serum biomarkers for GBC [49]. The second identified but not validated candidate, miR-671–5p, has been involved in the development of biliary-tract cancer. The expression of miR-671–5p was found to be related to the progression of hepatocellular carcinoma, the overexpression of miR-671–5p has also been linked to poor prognosis in colorectal cancer, and mediation of miR-671–5p by HMGA1 has been reported to promote metastasis in renal cell carcinoma through APC targeting [50–52].

The role of the *SIPA1L2* gene in the tumour environment has been widely studied. According to The Human Protein Atlas, *SIPA1L2* is a biomarker for renal cancer [53]. *SIPA1L2* expression has also been associated with an unfavourable prognosis in intestinal-type gastric cancer [54]. *SIPA1L2* is downregulated in colorectal cancer, and reduced *SIPA1L2* expression is associated with a poorer survival of colorectal cancer patients [55]. Among the key pathways involving the set of preselected miRNAs, the ErbB signaling pathway has emerged as frequently altered in GBC, affecting up to 40 % of GBC tumours [56–58]. The proteoglycans in cancer pathway, on the other hand, plays a crucial role in the progression of inflammatory gallbladder lesions to invasive cancer [59].

Overall, our study demonstrates that miR-4533 holds promising potential for GBC risk prediction and early detection. The robustness of our results lies primarily in the approach used for miRNA identification and validation. We combined independent datasets for two types of biosamples, gallbladder tissue and serum, and our results are based on diverse cohorts in terms of age, sex, BMI and time from blood sampling to diagnosis. GBC is relatively rare and under-researched in high-income countries, and through our collaborative study of the large European prospective cohorts, we aimed to gain more insight into the mechanisms of this disease in patients of European descent.

Virtually all reviews on miRNAs and GBC include only tissue studies, reflecting that new studies on circulating biomarkers are still needed. In the sparse literature available, we found that results were inconsistent depending on the study population and sample size. For example, while Goeppert et al. found miR-145–5p to be underexpressed in gallbladder tissue from European GBC patients, Saxena et al. reported overexpression of miR-145–5p in gallbladder tissue from Indian GBC patients [24,38]. The possible dependence of miRNA expression on regional environmental and genetic characteristics may pose an additional difficulty in the identification of cancer biomarkers.

In conclusion, we used prospective samples collected by six large European cohorts for small-RNA sequencing to identify and validate serum biomarkers that can be used for individual risk prediction and GBC prevention in individuals of European descent. We identified miR-4533 as a potential GBC biomarker, whose expression was elevated in serum samples from GBC cases, particularly in individuals younger than 63.5 years, or with a BMI below 26.2 kg/m^2. Pathway and correlation analyses revealed that miR-4533 targets *SIPA1L2*, and *SIPA1L2* was underexpressed in GBC serum samples. These promising results need to be validated and further refined in future studies, also with regard to their transferability to other sample types (e.g. whole blood and plasma), and to non-European populations.

Ethics approval

Gallbladder tissue samples were provided by the Tissue Bank of the National Center for Tumour Diseases (NCT, Heidelberg, Germany) with approval of the ethics committee of Heidelberg Medical Faculty (Project 206/05). The serum samples analysed were provided by the following large European prospective cohorts participating in the collaborative study "Identification of biomarkers for gallbladder cancer risk prediction - Towards personalized prevention of an orphan disease": The Janus Serum Bank (2016/1688 REK sør-øst B), the ESTHER Study (project 58/2000 Heidelberg Medical Faculty), the Heinz Nixdorf Recall Study (Project Gallbladder Cancer/2019 University Hospital Essen), the Helseundersøkelsen i Nord-Trøndelag (HUNT) Study (project 2016/1222), the Swedish Twin Registry (project 2016/2:11), and the National FINRISK Study (project BB2016_32). All participants provided written informed consent prior to enrolment. Ethics approvals and material/data transfer agreements are available on request.

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Felix Boekstegers: Writing – review & editing, Data curation. **Dominique Scherer:** Writing – review & editing, Writing – original draft, Project administration, Methodology. **Hilde Langseth:** Writing – review & editing. **Trine B. Rounge:** Writing – review & editing, Funding acquisition. **Kristian Hveem:** Writing – review & editing. **Stephanie Roessler:** Writing – review & editing. **Sonali Pechlivanis:** Writing – review & editing. **Hermann Brenner:** Writing – review & editing. **Justo Lorenzo Bermejo:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Melanie Waldenberger:** Writing – review & editing. **Alice Blandino:** Writing – review & editing, Writing – original draft, Visualization, Funding acquisition, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejca.2024.115138.

Data Availability

The necessary input files and source code to reproduce all the results are available at www.biometrie.uni-heidelberg.de/StatisticalGenetics/Software_and_Data.

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