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Circulating miRNAs and inflammatory markers – Associations between miRNAs and cytokine levels point to miRNA-mediated sCD40L release from platelets

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ABSTRACT

MicroRNAs (miRNAs) are gaining increasing attention, particularly because of their involvement in immune-related signaling pathways. We investigated the association between 179 plasma-circulating miRNAs (Plasma Focus microRNA PCR Panel) and 47 cytokines ("MILLIPLEX® panel) in 692 participants of the population-based SHIP-TREND cohort (age range 21–79) and two additional cohorts to present a comprehensive map of miRNA-cytokine relations. Multivariate linear regression models identified Bonferroni-corrected significant associations between miRNAs and cytokines for EGF (pro-epidermal growth factor), PDGF-AA, PDGF-AB/BB (platelet-derived growth factor subunit A and B), VEGF-A (vascular endothelia growth factor A), and sCD40L (soluble CD40 ligand) with sCD40L showing the most robust pattern. These models were adjusted for age, sex, platelet count, BMI, smoking, and technical parameters. In the follow-up sample ($N = 191$, 7 years after initial sampling), we confirmed that the observed associations were stable over time and replicated our findings in an independent clinical cohort ($N = 74$). Furthermore, the causal mediation results provide evidence for the involvement of platelet activity in the regulation of sCD40L mediated by five miRNAs in the range of 25 %–69 % of the effect being mediated (strongest mediation for *hsa*-miR-223-3p). Our study highlights a strong and stable miRNA-mediated modulation of sCD40L, at the stage of platelet activation with potential subsequent effects on the interaction of immune cells and haemostasis pointing to a complex regulatory mechanism. Future research is needed to determine the clinical relevance of our observations in the context of vascular thrombosis, immunological disorders, and neurodegeneration.

1. Introduction

Cytokines represent a heterogeneous group of small, soluble proteins with profound regulatory effects on immune system and inflammatory processes, as well as on various physiological functions including cell proliferation, differentiation, and apoptosis [1]. Their complex network of signaling pathways forms a cornerstone of immune regulation, serving as mediators in the communication between immune cells and between the immune system and other tissues. Cytokines are produced by a myriad of cell types, including immune cells such as T-cells, B-cells, macrophages, and dendritic cells, as well as non-immune cells like epithelial and endothelial cells [2]. The fundamental importance of

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cytokines in immune regulation is underscored by their involvement in nearly all aspects of the immune response, from the initiation of inflammatory cascades to the resolution of immune reactions [1]. Their pleiotropic and redundant nature allows for fine-tuning of immune responses and adaptation to various environmental challenges. However, dysregulation in cytokine release or signaling pathways can lead to pathological states characterized by dysregulated immune response, inflammation, or autoimmunity and affect a wide spectrum of diseases in diverse medical areas [3,4].

On the regulatory level, genetic, environmental, and epigenetic mechanisms intricately modulate cytokine expression, shaping immune responses and influencing disease susceptibility. Biological regulation involves transcriptional, translational, and post-translational processes that control cytokine production, secretion, and signaling, as well as cell-cell communication [5,6]. Such epigenetic regulation modulates gene expression patterns without altering the underlying DNA sequence, thereby exerting short- and long-term effects on cytokine expression profiles [7].

In this work we focus on the post-transcriptional regulation via non-coding circulating microRNAs (miRNAs) which play a critical role in the regulation of mRNA stability and translation and thus have the ability to fine-tune cytokine expression patterns and immune cell differentiation and mediating rapid responses to external factors. A number of circulating miRNAs have been associated with inflammatory states and diseases affecting the immune system [8–10]. Understanding this intricate regulatory network between miRNAs and cytokine release is essential for deciphering the molecular basis of immune responses and identifying and understanding dysregulated patterns in immunological reactions and diseases. We used data from the general population based Study of Health in Pomerania (SHIP-TREND cohort) and a psychiatric patient cohort of the Greifswald Approach to Individualized Medicine (GANI_MED). The objective of this study was to examine the association between plasma levels of 47 cytokines, chemokines and growth factors (hereafter referred to as cytokines or analytes) and 179 circulating miRNAs in these cohorts, to create a map of cytokine/miRNA association patterns in plasma and thereby identify biologically relevant correlations.

2. Materials and methods

2.1. SHIP-TREND

The assessment in the *Study of Health in Pomerania* (SHIP) was carried out in accordance with the Declaration of Helsinki, including written informed consent from all participants. The survey and study methods were approved by the institutional review boards of the University Medicine Greifswald.

SHIP is a population-based cohort project conducted in the northeast of Germany [11]. The main aim was to assess the prevalence and incidence of common diseases and lifestyle-associated risk factors and their interactions in the general adult population. From 2008 to 2012 the SHIP-TREND-0 baseline sample (TREND-0) was recruited including 4420 participants that underwent a standardized computer-assisted personal interview, during which they provided information on disease status, sociodemographic and lifestyle factors. From 2016 to 2019, a follow-up of TREND-0 with similar assessments was conducted, called SHIP-TREND-1 (TREND-1), where 2507 subjects from the baseline TREND-0 sample participated. In both examinations basic blood counts were analyzed as part of the clinical routine and biological samples were collected to assess various blood-based biomarkers [11]. The blood samples were taken during morning and late morning between 7 and 12 am.

2.2. Plasma-circulating miRNA data in TREND

Blood samples in TREND were taken from the cubital vein [12] and EDTA plasma aliquots were frozen at −80 °C in the Integrated Research Biobank of the University Medicine Greifswald and used in accordance with its regulations. MiRNA levels were profiled in a subsample of TREND-0 participants ($N = 708$) as described previously [13] and in a subsample of TREND-1 participants ($N = 198$). Circulating miRNAs were prepared from 200 µl EDTA plasma using the miRCURY™ RNA Isolation Kit –Biofluids (Qiagen, Hilden, Germany). TREND-0 samples were measured in two batches (TREND-0 batch 1: $N = 371$; TREND-0 batch 2: $N = 337$), TREND-1 samples in one batch. For RT-qPCR based miRNA analysis the Serum/Plasma Focus microRNA PCR Panel (Qiagen, Hilden, Germany) V3.M and V4.M were used, covering 179 miRNAs. Further methodological details can be found in the supplement and in [15].

For each batch, a miRNA was selected for further analysis if Ct ≤ 37 was detected in at least 40 % of the samples. In order to consider the influence of technical parameters, the Ct values of synthetic spiked-in miRNAs, the interplate calibrator (UniSp3), as well as the storage time of the biosamples (dt_biobank) were regressed out of the data. The resulting residuals in TREND-0 and TREND-1 were used as independent variables in later models to detect associations between miRNAs and cytokines of interest. A batch was included in the analysis for a specific miRNA if at least 100 subjects contained a valid measurement. To account for hemolysis while preparing the plasma probes, we calculated the ΔCt(miR-23a – miR-451) as hemolysis indicator that was used as covariate in the statistical regression [14].

2.3. Circulating inflammatory markers in TREND

In total, 25 µl neat EDTA plasma of TREND-0 ($N = 1175$) and TREND-1 ($N = 876$) participants – without prior freeze-thaw cycles of specific samples - was used for profiling of 47 cytokines using the "MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A – Immunology Multiplex Assay PC48" (HCYTA-60 K-PX48, Merck Millipore, Boston, MA, USA) by a bead-based multiplex assay. The following analytes were simultaneously measured: CD40 ligand (sCD40L), pro-epidermal growth factor (EGF), eotaxin (CCL11), fibroblast growth factor 2 (FGF-2), Fms-related tyrosine kinase 3-ligand (FLT-3 L), fractalkine (CX3CL1), granulocyte colony stimulating factor (CSF3, alias: G-CSF), granulocyte-macrophage colony stimulating factor (CSF2, alias: GM-CSF), C-X-C motif chemokine ligand 1 (CXCL1; alias: GRO-alpha), interferon-alpha 2 (IFNα2), interferon gamma (IFNγ), interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β), interleukin-1 receptor antagonist protein (IL1RA), interleukin (IL) IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, interleukin-12 subunit beta p40 (IL-12p40), interleukin-12 p70 (IL-12p70), IL-13, IL-15, IL-17A, IL-17E/interleukin-25 (IL-17E/IL-25), IL-17F, IL-18, IL-22, interleukin-17D (IL-27), C-X-C motif chemokine 10 (CXCL10, alias: IP-10), C—C motif chemokine 2 (CCL2, alias: MCP-1), C—C motif chemokine 7 (CCL7, alias: MCP-3), macrophage-colony stimulating factor 1 (CSF1, alias: M-CSF), C—C motif chemokine 22 (CCL22, alias: MDC), C-X-C motif chemokine 9 (CXCL9, alias: MIG), C—C motif chemokine 3 (CCL3, alias: MIP-1α), C—C motif chemokine 4 (CCL4, alias: MIP-1β), platelet-derived growth factor subunit A (PDGF-AA), platelet derived growth factor subunit B (PDGF-AB/BB), pro-transforming growth factor alpha (TGFα), tumor necrosis factor (TNFα), lymphotoxin-alpha (TNFβ), vascular endothelial growth factor (VEGF-A), hereafter referred to as analytes or cytokines (complete list in supplementary Table S3/S4). Of note, TREND-1 samples ($N = 867$) were profiled in parallel to corresponding TREND-0 samples to ensure that all timepoints per individual were measured under the same conditions. All analytes were log-transformed prior to analysis ($\log_{10}(1 + \text{analyte})$) to reduce skewness and ensure positivity. Details on measurement, data pre-processing, and biological/technical confounding factors have been published, recently [15]. The concentration for each analyte is provided in pg/ml. Both miRNA and cytokine profiles were available for TREND-0 participants ($N = 692$) and TREND-1 participants ($N = 191$), respectively. In TREND-0 nearly the whole sample (96 %) was fasting for at

least eight hours, in TREND-1 around 75 %.

2.4. GANI_MED

The GANI_MED study has been conducted in accordance to the Declaration of Helsinki. The study protocol was approved by the medical ethics committee of the University of Greifswald. Written informed consent was obtained from each of the study participants. The psychiatric patient sample GANI_MED (Greifswald Approach to Individualized Medicine) [16] is described in more detail in the supplemental material. In brief, GANI_MED is a multidisciplinary clinical cohort from different medical areas including patients from the Department of Psychiatry of the University Medicine Greifswald. Basic blood count was available as part of the clinical routine based on non-fasting blood samples. EDTA-plasma samples of 154 patients (38 males, 116 females) were used to measure 179 circulating miRNAs using Serum/Plasma Focus microRNA PCR Panel V4M (Qiagen, Hilden, Germany) [17].

2.5. Circulating inflammatory markers in GANI_MED

Blood samples of GANI_MED participants were collected from peripheral blood using ante-cubital venipuncture and EDTA plasma aliquots were frozen at −80 °C. In total, 25 µl neat EDTA plasma of each GANI_MED participant ($N = 150$) was used for profiling of 47 cytokines using the "MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A – Immunology Multiplex Assay PC48" (HCYTA-60K-PX48, Merck Millipore, Boston, MA, USA), according to the protocol used for the processing of TREND samples. The overlap of miRNA and cytokine data obtained in the GANI_MED cohort revealed $N = 74$ patients and were used as an independent replication sample for TREND-0. Further methodological details can be found in the supplement and in [13].

2.6. Covariates

Differential blood count was available as part of the clinical routine in TREND, BMI was calculated using anthropometric information on height and weight, and smoking status was separated into never, former, and current smokers in self-assessment. High-sensitivity C-reactive protein (hs-CRP) concentrations were determined in serum by nephelometry on the Dimension VISTA (Siemens Healthcare Diagnostics, Eschborn, Germany).

2.7. Statistical analyses

Subject characteristics of the final study sample (TREND-0 $N = 692$, TREND-1 $N = 191$, GANI_MED $N = 74$) were assessed by mean and standard deviation for metric variables and by numbers and percentages for categorical data. Differences between males and females or between samples were tested for significance via *t*-test for metric variables and Chi^2-test for categorical data. Analyses were performed in R 4.3.3 [18].

Discovery analysis: To assess the associations between 47 analytes and 179 circulating miRNAs in the general population of TREND-0, linear regression models with the log-transformed analytes as outcome and the miRNA residuals as predictors of interest were calculated adjusted for age, sex, BMI, platelet count (PLT), smoking status (never, former, current smoker), miRNA and cytokine batch, cytokine panel per day, storage time of the blood sample (dt_biobank), and miRNA-derived haemolysis indicator. Age was modelled non-linear as restricted cubic splines placing three knots (2 degrees of freedom) at percentiles 0.1, 0.5, and 0.9 reflecting the age of 31, 50, and 68 years.

$$\begin{aligned}\textbf{Cytokine} \sim \textbf{miRNA} + \text{age} + \text{sex} + \text{BMI} + \text{as.factor(smoking)} + \text{PLT} \\ + \text{as.factor(batch\_miRNA)} + \text{as.factor(batch\_cytokine)} \\ + \text{as.factor(panel)} + \text{dt\_biobank} + \text{hemolysis}\end{aligned}$$

Multiple testing correction was performed by applying Bonferroni correction ($p_{BO} = 0.05/\#\text{tests}$) per analyte. We decided to use this relatively conservative method because of the known correlation among the individual miRNAs [12] and cytokines [15] which impedes independency between the statistical tests which is a pre-requirement for multiple testing corrections such as FDR (false discovery rate).

Sensitivity analyses: 1. In order to test the robustness of the results from the discovery analysis, additional confounders were included into the statistical model covering the following biological areas - blood coagulation, systemic inflammation, and seasonality of blood draw. Similar to age seasonality was modelled non-linearly. 2. In conditional analyses we aimed to identify the biological relevant cytokines in cases of overlapping results:

$$\textbf{Cytokine1} \sim \textbf{miRNA} + \textbf{Cytokine2} + \text{covariables}$$

Replication analysis: All associations surviving sensitivity analyses were included for replication in the follow-up TREND-1 sample ($N = 191$) and the independent clinical GANI_MED sample ($N = 74$). This includes all analytes that showed Bonferroni corrected significant associations with miRNAs in the discovery sample. In the replication analyses, similar statistical models were applied. In the GANI_MED sample, smoking status was not available.

Mediation analysis: As platelets are a main source of circulating miRNAs, we performed mediation analyses for the remaining significant cytokines testing the mediating role of the miRNAs on the path from platelets to analytes (PLT → miRNA → cytokine). Prior to analysis, linear relationships between the variables was tested as well as normal distribution of residuals. The mediating analyses were adjusted for the same covariates as the main analyses. Causal mediation analysis was performed using the *mediate()* function from the *mediation* package in R including percentile confidence intervals. All metric variables were scales prior to the analyses to achieve comparability of estimates.

In advance, we tested for the association between platelet count and miRNAs in our samples to identify miRNAs most likely released by platelets.

$$\textbf{PLT} \sim \textbf{miRNA} + \text{age} + \text{sex} + \text{BMI} + \text{as.factor(batch\_miRNA)} + \text{hemolysis}$$

3. Results

Association analyses in TREND-0 were performed in 692 subjects with a mean age of 50.1 years and nearly equal ratio between males and females (Table 1). Significant differences between sexes were only observed for smoking behavior and platelet count, there were no sex differences regarding miRNA or cytokine batches. For the final analyses 44 cytokines and 174 miRNAs were available with sufficient sample sizes. All miRNAs with a sample size of at least 100 were included into the analyses. For each batch a minimum sample size of 30 was required. By that, also miRNAs available in only one of the two batches were analyzed if the sample size of 100 was reached. A complete list of available miRNAs and cytokines for analysis per batch and sample is provided in the supplement (Fig. S1, TableS1–4). A general overview of the analysis workflow is provided in Fig. 1.

3.1. Discovery analyses: associations between cytokines and miRNAs in TREND-0

In sum, 7155 regression analyses were performed assessing the associations between miRNA abundance and the level of 44 cytokines in plasma. Requiring a minimum sample size of 100, between 114 and 174 miRNAs could be analyzed for each analyte with a sample size ranging between 101 and 692 for each individual analysis. The overall pattern of the results for each cytokine-miRNA pair is given in supplementary FigureS2.

Taking the 44 individual analytes, significantly associated miRNAs (Bonferroni correction per analyte) were observed for EGF (6), PDGF-AA (5), PDGF-AB/BB (6), sCD40L (52), and VEGF-A (3) (Fig. 2,

Table 1
Sample description of the analytic sample in TREND-0.

	Males ($N = 351$, 50.7 %)	Females ($N = 341$, 49.3 %)	Combined ($N = 692$)	Comparison
Age in years	49.6 (14.4), [22–79]	50.7 (13.1), [21–79]	50.1 (13.8), [21–79]	$T = -1.00$, $p = 0.32$
BMI in kg/m^2	27.6 (3.6), [17.7–39.0]	27.1 (4.9), [18.5–48.1]	27.4 (4.3), [17.7–48.1]	$T = 1.50$, $p = 0.13$
Smoking				Chi^2 = 29.3, $p = 4.4 \times 10^{-7}$
Never	111 (31.6 %)	174 (51.0 %)	285 (41.2 %)	
Former	162 (46.2 %)	100 (29.3 %)	262 (37.9 %)	
Current	78 (22.2 %)	67 (19.7 %)	145 (21.0 %)	
Cytokine batch				Chi^2 = 0.08, $p = 0.96$
1	149 (42.4 %)	142 (41.6 %)	291 (42.0 %)	
2	121 (34.5 %)	121 (35.5 %)	242 (35.0 %)	
3	81 (23.1 %)	78 (22.9 %)	159 (23.0 %)	
miRNA batch				Chi^2 = 0, $p = 1$
1	182 (51.9 %)	177 (51.9 %)	359 (51.9 %)	
2	169 (48.1 %)	164 (48.1 %)	333 (48.1 %)	
Platelet count (Gpt/l)	214 (46), [102–381]	241 (49), [93–392]	227 (49), [93–392]	$T = -7.45$ $p = 2.8 \times 10^{-13}$
ATC B01	31 (0.9 %)	17 (0.5 %)	48 (0.7 %)	Chi^2 = 3.35 $p = 0.07$

For metric variables mean, standard deviation and range are listed, for categorical variables numbers and percentage; BMI = Body Mass Index; ATC B01 = anticoagulant medication code; PLT: platelet count in giga per liter. T – test statistic for *t*-test; Chi^2 – test statistic for Chi^2-test. The sex definition (males/females) is based on biological sex.

supplementary TablesS5–9) with no miRNA showing significant associations with all these five analytes. Correcting for all 7155 performed tests ($p_{BO} = 6.9 \times 10^{-6}$), significant results were still observed for PDGF-AA (*hsa*-miR-10b-5p, β = 0.055 [0.032–0.079], $p = 3.8 \times 10^{-6}$), PDGF-AB/BB (*hsa*-miR-10b-5p, β = 0.047 [0.030–0.065], $p = 2.0 \times 10^{-7}$; *hsa*-miR-223-3p, β = −0.062 [−0.087 – −0.037], $p = 1.3 \times 10^{-6}$; *hsa*-miR-29a-3p, β = 0.066 [0.039–0.094], $p = 3.5 \times 10^{-6}$), and sCD40L (32, Supplementary Table S5) with *hsa*-miR-10b-5p being associated with all three analytes. For sCD40L, 30 % of the miRNAs exhibited a significant association after Bonferroni correction and 60 % of the miRNAs were at least nominal significant. All of the significant miRNAs from the different analytes were at least associated with sCD40L (Fig. 2B) with the same direction of effect. This is not surprising given the high correlation among these five analytes (supplementary Fig. S3) with the highest correlation observed between PDGF-AA and PDGF-AB/BB ($r = 0.87$) and the lowest between EGF and PDGF-AA ($r = 0.25$). The results for the cytokines that do not exhibited significant associations with miRNAs are outlined in Supplementary File 2.

3.2. Sensitivity analyses for significant targets

To assess the biological relevance of these findings in TREND-0, we performed a number of sensitivity analyses for the significant cytokines in Figure2B; (1) Adjusting for additional confounders addressing different biological aspects: *systemic inflammation* with white blood cells (WBC, lymphocytes, neutrophils), hs-CRP (high sensitive C-reactive protein) and fibrinogen [10]; *coagulation* with mean platelet volume (MPV) and anticoagulant medication (ATC B01); as well as effects of *seasonality* which was modelled similar to age but using 4 knots (3 degrees of freedom) instead of 3 to capture the more complex seasonal pattern. The knots were placed at quantiles 0.05, 0.35, 0.65, and 0.95

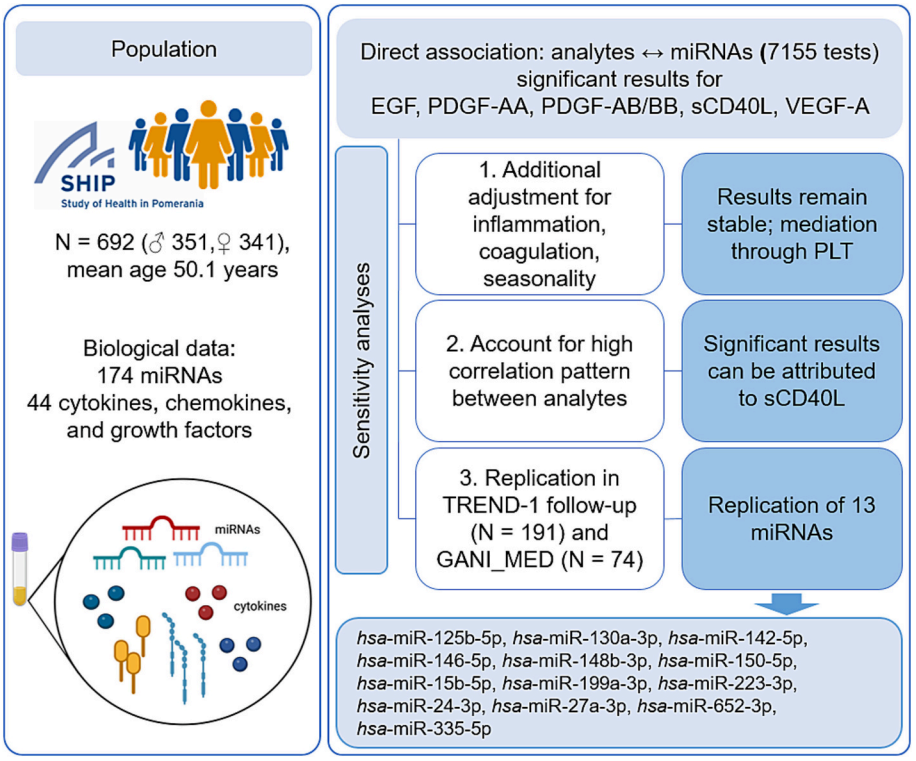

Fig. 1. Overview of the analysis workflow including discovery and subsequent analyses. SHIP is a general population-based study with measurements for cytokines and miRNAs in the TREND cohort. After the direct associations between all cytokine ~ miRNA pairs in TREND-0 (7155 tests performed, sample size $N = 692$) several sensitivity analyses were conducted. 1. Additional adjustment for relevant confounders covering different biological aspects, 2. Analysing correlation pattern among cytokines and miRNAs to identify the biological relevant associations, 3. Cross-sectional replication in the TREND-1 follow-up sample ($N = 191$) and a GANI_MED cohort ($N = 74$) with finally 13 miRNAs that could be replicated. Significant miRNAs were detected for sCD40L, EGF, PDGF-AA, PDGF-AB/BB, and VEGF-A with no miRNA significant for all these analytes.

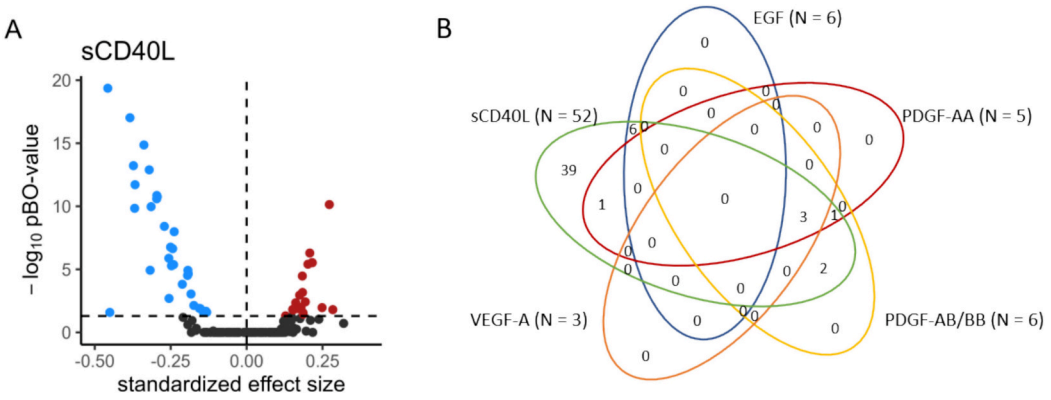

Fig. 2. Association results between cytokines and miRNAs. (A) The cytokine sCD40L was significantly associated with 52 miRNAs after Bonferroni correction ($p_{BO} < 0.05$). Left side of the volcano plot highlights the 32 miRNAs (blue) with significant negative estimates ($\beta < 0$) indicating higher miRNA levels with increasing sCD40L concentration (since smaller ΔCt values correspond to higher miRNA abundance). Positive ($\beta > 0$) associated miRNAs ($N = 20$) are highlighted in red, indicating lower miRNA levels with increasing sCD40L concentration. (B) The overlap of significant miRNAs associated with EGF, PDGF-AA, PDGF-AB/BB, sCD40L, and/or VEGF-A is based on the Bonferroni corrected results for each analyte. No miRNA was associated with all five cytokines. Created with *jvenn* [19]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reflecting the days 29, 131, 226, and 323 of the year; (2) conditional analyses for the significant cytokines addressing the high inter-correlation among the cytokines to identify the biological relevant associations.

(1) Additional covariates

Introducing additional covariates into the model did not change the overall significance of the findings. We tested this for all five significant analytes from the discovery analysis (data not shown). Thus, association results were highly stable and independent of systemic inflammatory markers, blood coagulation, and seasonal influences. Fasting time showed no association with either the significant cytokines or miRNAs and thus did not need to be included as covariate. As sex-differences are often reported in inflammatory pathways including cytokine signaling [20,21], we tested for sex differences in the association between miRNAs and the five significant analytes in the basic model. Sex-separated analyses as well as including a sex-interaction term indicated no sex-specific mechanisms and supported the former results (Supplementary Tables S10-S12).

(2) Conditional analysis for cytokines

There was a high inter-correlation among the cytokines especially for PDGF-AA, PDGF-AB/BB, and VEGF-A as well as for EGF and sCD40L, which are all produced in platelets (FigureS2). As all significant miRNAs for the five top analytes were also associated with sCD40L, we performed miRNA ~ analyte analyses additionally adjusted for sCD40L. None of the former results for EGF, VEGF-A, PDGF-AA, and PDGF-AB/BB remained significant after adjusting the model for sCD40L. However, adjusting the sCD40L analyses for EGF still revealed significant results (FigureS4). Thus, the main focus of the subsequent analyses was placed on sCD40L as biological important player affected by miRNA regulation. These associations were also not affected by the different cytokine or miRNA batches as batch-wise results for sCD40L showed a high consistency between the different batches (3 cytokine and 2 miRNA batches; FiguresS5/S6).

3.3. Replication analyses

We aimed for replication in the cross-sectional follow-up TREND-1 assessment ($N = 191$) as well as in an independent GANI_MED psychiatric patient sample ($N = 74$). A replication would indicate biological stability over time of the associations as well as provide strong biological evidence in an independent clinical cohort.

As SHIP-TREND is a longitudinal population-based study with regular follow-ups, we also analyzed the cross-sectional data of the follow-up sample TREND-1 that provided miRNA and cytokine measurements comparable to TREND-0. A comparison between both time-points is given in Table 2. As expected by design, subjects in TREND-1 were significantly older than in TREND-0 with a mean follow-up time of 7.6 years. Also smoking status changed as people switched from never to current to ever smokers. Interestingly, older age of the participants was not accompanied by higher mean BMI or platelet count suggesting stable health parameters in this subsample.

In TREND-1, similar regression models as in TREND-0 were calculated to replicate the findings for sCD40L and to validate the cross-sectional stability of miRNA-analyte associations. Analyses were only run for those miRNAs with at least 100 valid measures resulting in 160 miRNAs. For sCD40L 33 miRNAs reached nominal significance, and at least 6 were significant after Bonferroni correction. Of those 33, 23 also reached Bonferroni corrected significance in TREND-0 (FigureS7A, TableS13). For all these miRNAs directions of effect on sCD40L were identical in TREND-0 and TREND-1. The strongest effects were observed for miR-146a-5p, miR-151a-3p, miR-199a-3p, miR-24-3p, miR-27a-3p, and miR-27b-3p (all Bonferroni corrected significant in TREND-1, Fig. 3). Thus, the strong results for sCD40L were stable over time and could be replicated in TREND-1.

Table 2
Comparison between the sample of 191 subjects with data in TREND-0 and TREND-1 with a mean follow-up time of 7.6 years and the GANI_MED sample.

Variable	TREND-0 (N = 191)	TREND-1 (N = 191)	Comparison T0 vs. T1	GANI_MED (N = 74)
Biological			$Chi^2 = 0$	
Sex	90 (47.1 %)	90 (47.1 %)	$p = 1$	30 (40.5 %)
Males	101 (52.9 %)	101 (52.9 %)		44 (59.5 %)
Females				
Age in years	51.1 (12.4), [22–78]	58.6 (12.2), [31–85]	$T = -6.0$ $p < 0.001$	39.4 (12.8), [19–64]
BMI in kg/m²	27.5 (3.8), [18.8–38.1]	27.2 (4.1), [18.8–39.1]	$T = -0.44$ $p = 0.66$	27.4 (6.0), [16.4–42.9]
PLT in Gpt/l	227 (47), [122–366]	221 (48), [106–382]	$T = 1.22$ $p = 0.22$	264 (59), [174–526]
Smoking			$Chi^2 = 251$	NA
Never	81 (42.4 %)	71 (37.2 %)	$p < 0.001$	
Ever	79 (41.4 %)	94 (49.2 %)		
current	31 (16.2 %)	26 (13.6 %)		

PLT: platelet count in giga per liter; BMI: Body Mass Index; T – *t*-test statistic; Chi^2 – Chi^2 test statistic.

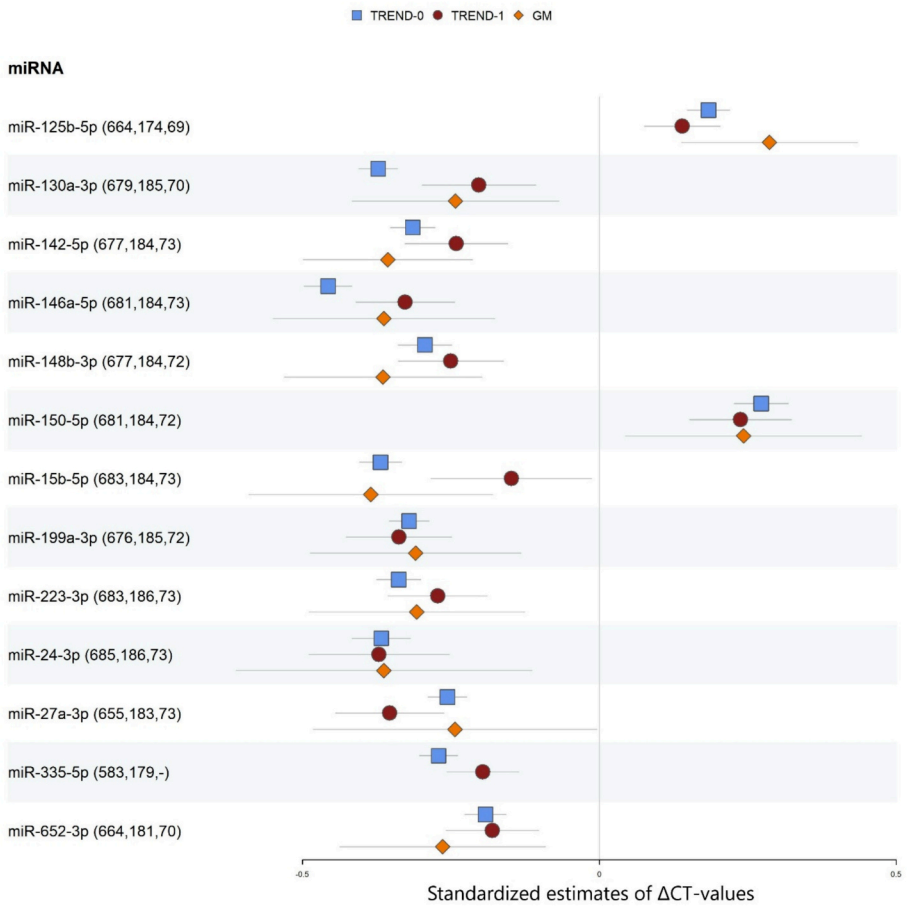

Fig. 3. Forest-plot of the significant miRNAs for sCD40L. MiRNAs with Bonferroni corrected significance in the discovery sample TREND-0 and at least nominal significance in both replication samples TREND-1 and GANI_MED (GM) and similar direction of effect. Standardized estimates and 95 % confidence intervals are displayed. MiR-125b-5p and miR-150-5p were down-regulated in association with sCD40L while all other miRNAs were up-regulated. The numbers in brackets give the sample size of TREND-0, TREND-1, and GANI_MED. MiR-335-5p was not available in GANI_MED and is thus missing here.

Compared to TREND, GANI_MED comprises a higher number of females and was considerably younger with a mean age of 39.4 and a mean BMI of 27.4 (Table 2). As smoking status was not available in the data and the analysis model this could be a source of bias and hamper the comparability of the results between the cohorts. In GANI_MED, the association between sCD40L and miRNAs could be tested for 124 miRNAs with a sample size above 50. Significant miRNAs identified in TREND-0 and replicated in TREND-1 could again be replicated on nominal significance level in the independent GANI_MED sample (Fig. 3, FigureS7B, TableS14). The 13 remaining significant miRNAs are listed in Fig. 3. A graphical representation of the significant miRNAs throughout the three samples is given in Fig. S8.

3.4. Mediating role of miRNAs

Thrombocytes, also known as platelets, are a main source of circulating miRNAs [22]. We found among significantly miRNA associated analytes the platelet-enriched PDGF-AA, PDGF-AB/BB, sCD40L, and VEGF-A which can be released by platelets in large amounts. Hence, we tested for the associations between miRNAs and platelet count in both TREND samples and in the independent GANI_MED sample. In TREND-0, four miRNAs exceeded Bonferroni corrected significance (19 Benjamini-Hochberg (BH)-corrected significance; supplementary Table S15). This strong association between miRNAs and platelets was also observed in TREND-1 and GANI_MED. However, the significance of this association was lower in both studies, which is probably due to the smaller sample size. In direct associations in the TREND-0 sample, sCD40L was also positively associated with platelet count ($p = 1.6 \times 10^{-4}$). Thus, we tested for possible mediation effects of the 13 significantly replicated miRNAs. The associations between the three factors (platelet count, miRNAs, and sCD40L) was linear with normal distribution of residuals (checked visually). Since the significance of the association results between miRNAs and sCD40L did not substantially change when excluding platelet count from the model (data not shown) we tested the causal mediation model with the miRNAs as mediator on the path from platelet count to sCD40L (PLT → miRNA → sCD40L; supplementary Fig. S9). For 5 out of the 13 replicated miRNAs a significant partial mediation of the effect of platelet count on sCD40L was observed and the proportion of effect mediated ranged from 25 % to 69 % (miR-125b-5p, miR-142-5p, miR-150-5p, miR-223-3p, miR-652-3p; Supplementary Table S16). The strongest mediation was found for miR-223-3p which mediated nearly 69 % of the effect of platelets on sCD40L.

Eight miRNAs exhibited effects on sCD40L that were statistically independent from platelet count (no significant mediation observed). Based on all analysis results, four theoretical mechanisms of sCD40L release can be separated: (I) direct release of sCD40L from activated platelets, (II) platelet-miRNA partially mediating the release of sCD40L, (III) activation via miRNAs independently of platelets, and (IV) release from various additional sources (e.g. other cell types) independent of platelets and the tested miRNAs (Fig. 4).

3.5. Correlation patterns between miRNAs

To rule out that the observed replication of TREND-0 results in the

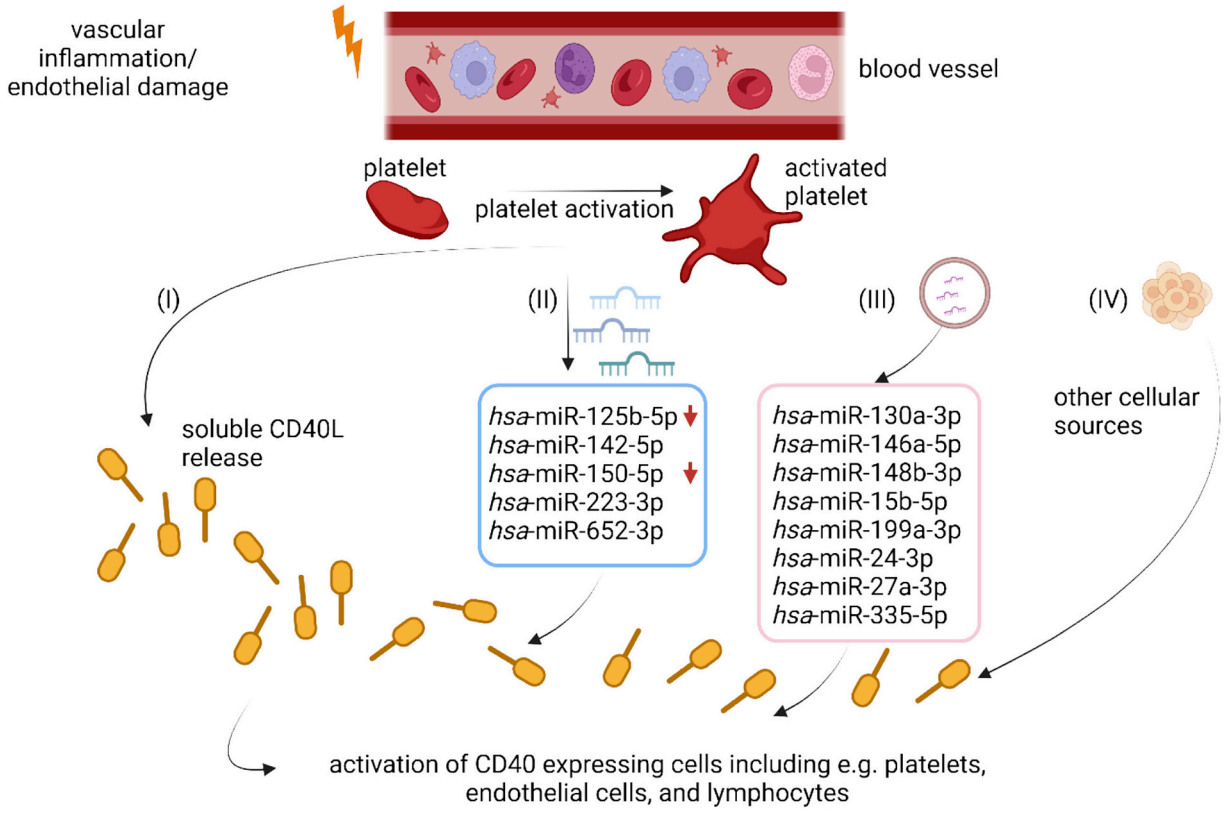

Fig. 4. (A) Hypothetic model of sCD40L release (I) direct release of sCD40L from activated platelets, (II) miRNAs (partial) mediating the effect of platelets on sCD40L, (III) activation via miRNAs independently of platelets, and (IV) release of sCD40L from various additional sources. miRNAs 125b-5p and 150-5p revealed decreased levels in association with sCD40L while all other significant miRNAs showed increasing levels. Created with Biorender.com.

TREND-1 follow-up sample was only due to the strong temporal stability of the miRNA data, we examined the correlation of miRNA abundance between the two time points (TREND-0 and TREND-1). The ΔCT-values of the miRNAs in TREND-0 and TREND-1 were correlated for each miRNA respectively for the 191 subjects with available data at both time points. These data were previously corrected for biobank storage time, calibrator miRNAs, batch and hemolysis (see Methods section). Overall, correlations between the 166 miRNAs with at least 50 subjects with available data at both time points were evaluated. The correlation coefficients ranged between $r = -0.25$ and 0.42 with a median of 0.08 (mean 0.084). Although the correlation between the miRNAs at the two different time points was rather small, the correlation patterns among the 13 significant miRNAs at each time point separately were highly stable (Fig. S10A/B). This stable correlation pattern was also observed in the independent psychiatric patient cohort GANI_MED (Fig. S10C). On the level of cytokines, values for sCD40L in TREND-0 and TREND-1 (after adjusting for technical covariates) showed a correlation of $r = 0.39$ between both time points but with no clear trend visible when comparing males and females (Fig. S11). Thus, although the individual correlations of miRNAs between the different time points were small, the in-between miRNA correlation patterns at each individual time point were stable as well as their associations with sCD40L over time. This points to a strong time independent and stable biological mechanism.

3.6. Biological relevance of miRNA enrichment

In a final step we wanted to determine the biological relevance of the 13 miRNAs on the level of diseases. Applying the miEAA tool (miRNA Enrichment and Annotation tool, https://ccb-compute2.cs.uni-saarland.de/mieaa/ [23]) with these miRNAs as input revealed a strong enrichment for immune system associated disorders and mechanisms of cytokine activation (Supplementary Table S17). The most significant enrichment was found for rheumatic disorders such as rheumatoid arthritis or fibromyalgia but also vascular disorders (cardiovascular, cerebrovascular), various cancer tapes, as well as neuropsychiatric disorders were enriched. With regard to biological processes in gene ontology, immune system processes such as leukocyte activation, immune system development, or cytokine activity were over-represented. The top miRNA from the TREND-0 analyses, *hsa*-miR-146a-5p, showed strong enrichment in immune system associated human tissues (https://www.ccb.uni-saarland.de/tissueatlas2) as well as in mouse brain microglia and immune cells (https://mirna.wustl.edu/) (Fig. S12).

4. Discussion

In the present study, we investigated the association patterns between circulating miRNAs and a panel of 44 plasma cytokines, chemokines and growth factors in the population-based SHIP-TREND cohort. Although previous research, including TREND and other population-based studies, has reported strong associations between miRNAs and various blood-based markers of systemic inflammation [10,24], only a few analytes from this inflammation panel showed significant associations with miRNAs in our analyses. These analytes include the soluble form of CD40 ligand (sCD40L) and mainly growth factors such as PDGF-AA (platelet-derived growth factor subunit A), PDGF-AB/BB (platelet-derived growth factor subunit B), VEGF-A (vascular endothelial growth factor A), and EGF (pro-epidermal growth factor). Post hoc analyses attributed these associations primarily to sCD40L, which exhibited significant and consistent miRNA associations that were replicable in the follow-up TREND-1 sample and the independent GANI_MED patient sample. For 13 miRNAs, Bonferroni-corrected significant associations in TREND-0 could be validated in both additional cohorts.

Although there was no consistent and significant correlation between the individual miRNAs measured at the two timepoints, TREND-0 and

TREND-1, the correlation pattern among the 13 miRNAs associated with sCD40L were highly comparable across all three samples, suggesting a strong biological determination. To identify the primary source of this plasma-based sCD40L, we examined associations with blood cell components. Consistent with both the literature and our findings, significant associations were found with platelets - the primary source of circulating sCD40L [25], and to a lesser extent, with fibrinogen, which plays a role in platelet activation [26]. Several miRNAs also showed strong association with platelet count. This platelet-associated mechanism was further investigated through causal mediation analyses where the significant miRNAs could be divided into a subsample partially mediating the effect of platelet count on sCD40L and a subsample revealing platelet independent effects on sCD40L.

In more detail, blood platelets are anucleate cells produced by the fragmentation of megakaryocytes. They play major roles in haemostasis and wound healing, as well as in immune response, by interacting with immune cells [27]. Upon activation, platelets secrete various inflammatory mediators, either releasing them into the plasma or presenting them on the cell surface to facilitate interaction with endothelial or T-cells. One such surface bound mediator is CD40 ligand (CD40L or CD154), which is secreted by platelets in alpha granules. CD40L is a transmembrane protein of the TNF superfamily essential for cellular interaction by binding to the CD40 receptor on expressing cells [27]. The CD40L/CD40 axis is crucial in many inflammatory processes. In blood plasma, platelets are the main source of soluble CD40L (sCD40L), which is cleaved from the platelet membrane upon activation [27]. Despite lacking a nucleus, platelets contain precursor miRNA inherited from megakaryocytes, which can be processed into mature miRNA and released into the cytoplasm [25]. The plasma concentrations observed in our samples might therefore directly reflect miRNA mediated CD40L synthesis in megakaryocytes and/or platelet activation.

To explore this further, we tested the causal mediating effect of the 13 significant miRNAs on the association between platelet count and sCD40L as a marker of platelet activation. This analysis revealed two distinct groups: five miRNAs with strong mediating effects and eight miRNAs with a platelet-independent effect on sCD40L. All mediating miRNAs had previously been reported to be enriched in platelets [22,28–30]. It can be hypothesized that these five miRNAs (miR-125b-5p, miR-142-5p, miR-150-5p, miR-223-3p, miR-652-3p) exert their effects partially within platelets prior activation or during the megakaryocyte stage. In such cases, these miRNAs may influence sCD40L formation or platelet activation, which finally leads to the release of sCD40L into plasma. Platelet activation is connected with pro- or anti-inflammatory processes via the release of cytokines and signaling molecules, monocyte recruitment, polarization of macrophages, or interaction with endothelial and immune cells [25]. MiRNAs have the ability to modify platelet physiology and activation by targeting protein expression, as observed for *hsa*-miR-223 [22].

Another potential mechanism involves the release of miRNAs through passive leakage or from activated platelets via platelet-derived extracellular vesicles (microvesicles) or RNA-binding protein complexes. Platelets, as well as megakaryocytes, serve as source for miRNA-containing microvesicles, allowing for horizontal transfer of miRNAs to other cell types [31]. These plasma-based miRNAs could induce sCD40L release from platelets or other cells by binding receptors at the cell surface and even regulate gene expression in neighboring cells (e. g. epithelial cells or macrophages) [22,30,32,33]. In fact, platelet-derived microvesicles are a major source (around 45 %) of circulating miRNAs [30], and these miRNAs have been associated with various diseases, including cardiovascular diseases and cancer [30].

Interestingly, eight miRNAs showed a significant association with sCD40L independently of platelet count (Fig. 4), suggesting a platelet independent molecular mechanism after their release into plasma. These non-mediating miRNAs, which may not be inherited from platelets, could act as a signaling molecule in platelet activation leading to sCD40L release from platelets into plasma. The two miRNAs miR-199a-3p and miR-148b-3p, showing platelet-independent effects on sCD40L in our analyses, have previously been identified as markers of platelet activation [29]. These miRNAs may also originate from other blood cells or from various organs and tissues secreting miRNAs into the bloodstream [34,35]. These miRNAs could subsequently promote sCD40L release from platelets or other cells.

Regarding their biological roles, the 13 replicated miRNAs demonstrated significant enrichment in immune-system associated diseases and biological processes, as expected. Platelet count serves as a general marker in immunological diseases [36,37]. Since sCD40L is primarily expressed by platelets and is involved in immune cell activation and initiation of inflammatory response [27], it plays a major role in rheumatic disorders, facilitating sCD40L as a valuable biomarker for disease activity and treatment response [38–40]. In vascular diseases, including cardio- and cerebrovascular conditions, platelets contribute to arteriosclerosis, thrombosis, and vascular inflammation [41,42]. These processes may be driven by sCD40L and the CD40L/CD40 axis, with sCD40L being a widely discussed biomarker for cardiovascular events [43–45]. In neurodegenerative diseases, sCD40L has been investigated as a potential biomarker for inflammation-driven pathology. Chronic inflammation can promote endothelial dysfunction, damaging the blood-brain-barrier and increasing sCD40L levels [44,46]. Elevated sCD40L levels in blood have also been linked to increased risk of Alzheimer's Disease (AD) [47–49], stroke [50], or multiple sclerosis [51–53]. Cancer is another significant area of association, as the CD40L/CD40 axis plays a crucial role in tumor neoangiogenesis [44]. Furthermore, CD40L facilitates interactions with immune cells, promoting cell proliferation, and has been strongly associated with leukaemia and lymphoma [54,55].

5. Conclusions

In conclusion, our analyses provide a further step towards understanding the complex miRNA driven regulation of the human immune response. The study was primarily limited by the number of miRNAs available in the PCR panels, and the lack of experimental evidence as our hypothesis were based on association analyses. Additional limitations include limited ethical diversity, potential batch effects, and differences in adjustment sets across TREND and GANI_MED, all of which may reduce the generalizability and comparability of the findings. Furthermore, our cross-sectional design hampers the identification of causal relationships in association and mediation analyses, and we were only able to measure platelet count not platelet activation Thus, we cannot exclude alternative mechanisms involving the activation of additional immune cells contributing to sCD40L and miRNAs release. Despite these limitations, the consistent findings across three cohorts suggest potentially robust biological effects. Our study highlights the possible role of miRNA mediated platelet activation and sCD40L release, emphasizing the importance of platelets as a source of both circulating miRNAs and sCD40L, as well as their central role in inflammatory response. We identified 13 replicated candidate miRNAs with significant associations with sCD40L. Furthermore, our analyses indicated platelet-independent associations between miRNAs and sCD40L that warrant further investigation. These 13 miRNAs could serve as valuable targets for further mechanistic research into immune-mediated inflammatory disorders, including in-vitro validation of their cellular effects.

Availability of data and materials

The data of the SHIP and GANI_MED study cannot be made publically available due to the informed consent of the study participants, but it can be accessed through a data application form available at https://fvcm.med.uni-greifswald.de/ for researchers who meet the criteria for access to condential data.

CRediT authorship contribution statement

Sandra Van der Auwera: Writing – original draft, Visualization, Formal analysis, Conceptualization. **Sabine Ameling:** Writing – review & editing, Visualization, Resources, Data curation. **Anja Wiechert:** Writing – review & editing, Data curation. **Nele Friedrich:** Writing – review & editing, Resources, Data curation. **Matthias Nauck:** Writing – review & editing, Resources, Project administration. **Henry Völzke:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Barbara M. Bröker:** Writing – review & editing, Resources. **Hans J. Grabe:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Uwe Völker:** Writing – review & editing, Resources, Project administration.

Ethics approval and consent to participate

The assessment in the *Study of Health in Pomerania* (SHIP) was carried out in accordance with the Declaration of Helsinki, including written informed consent from all participants. The survey and study methods were approved by the institutional review boards of the University Medicine Greifswald. The GANI_MED study has been conducted in accordance to the Declaration of Helsinki. The study protocol was approved by the medical ethics committee of the University of Greifswald. Written informed consent was obtained from each of the study participants.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: HJG has received travel grants and speakers honoraria from Neuraxpharm, Servier, Indorsia and Janssen Cilag. All other authors declare that they have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2025.157012.

Data availability

The authors do not have permission to share data.

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