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Platelet RNA modules point to coronary calcification in asymptomatic women with former preeclampsia

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HIGHLIGHTS

- Network construction shows transcript modules in platelet RNA.
- Modules associate with calcification in asymptomatic women with former preeclampsia.
- Platelet activation and coagulation pathways are enriched in the calcification modules.
- Relevant modules are enriched for coronary artery disease susceptibility loci.
- Platelet RNA gene modules may be useful to detect abnormalities in the circulation.

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ABSTRACT

Background and aims: Women who develop preeclampsia during pregnancy are at a higher risk for developing cardiovascular disease. As platelets are affected by preeclampsia, we set out to identify whether platelets carry information in their transcriptome on cardiovascular risk in women with former preeclampsia.
Methods: Platelets were isolated from asymptomatic women with previous preeclampsia, who underwent screening with coronary computed tomography angiography. Platelet RNA was isolated and used to construct gene networks using an unbiased approach. Platelet gene modules assembled from the network were related to risk factors and clinical traits of these women, including coronary artery calcium scores (CACS).
Results: We found multiple gene modules which correlated with CACS (correlation coefficients: 0.44 to 0.59, $p = 0.05$ to 0.007). The genes from two clinically relevant modules were expressed at a higher level in the group with calcifications ($p = 3.9 \times 10^{-10}$ and 0.02) and enriched for platelet-related gene-sets such as platelet activation. The first of these modules was also enriched ($p_{permutation} = 0.0546$) for genes mapped to known coronary artery disease susceptibility loci. Additional unbiased network analyses in platelet RNA of patients with overt cardiovascular disease underlined the importance of the identified modules for disease by high preservation. ($p = 1.6 \times 10^{-9}$ to 1.7×10^{-47}).
Conclusions: We found platelet RNA modules that correlated with CACS in asymptomatic women with previous preeclampsia. Whether or not platelets directly contribute to this disease trajectory, or reflect the underlying plaque substrate remains to be determined, but enrichment for coronary artery disease susceptibility genes emphasizes the importance for the disease.

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1. Introduction

Women who develop preeclampsia (PE) during pregnancy are at a higher risk for developing cardiovascular disease (CVD) later in life as compared to women with uncomplicated pregnancies [1,2]. PE is characterized by sudden onset hypertension, proteinuria and an abnormal platelet function. We have recently shown that arterial calcifications commonly occur in asymptomatic women after one or more pregnancies complicated with PE at age 45–55 years, a relatively young population [3]. The coronary artery calcium score (CACS), used to measure coronary artery calcifications, is an independent predictor of CVD events [4].

Women with former PE show problems in their microcirculation as much as 15–25 years after the complicated pregnancy [5], and other tissues might be affected as well. Platelets show a distorted function in PE [6], which may persist over time. As platelet transcriptomes have been previously studied for their potential to discern subtle differences, for example in cancer [7], we set out to determine whether platelets also carry information in their transcriptome regarding cardiovascular risk associated to PE. We therefore studied platelet transcriptomic profiles of asymptomatic women with previous PE, who were recruited for coronary computed tomography angiography. Using an unbiased approach, we correlated gene modules with risk factors and clinical traits of these women, among which CACS. We demonstrate a correlation between CACS and the presence of gene modules in platelet RNA, which are enriched for genes partaking in platelet activation, and coagulation. We further annotated clinically relevant modules with data from genome-wide association studies (GWAS) for coronary artery disease (CAD). The module with the highest gene significance for CACS, which is enriched for platelet activation, is also enriched for genes mapped to CAD susceptibility loci. Finally, we show that these modules are also present in platelet RNA of an established cardiovascular disease cohort.

2. Materials and methods

2.1. Study design CREw-IMAGO

We selected study participants (n = 20) from the CREw-IMAGO study, of which design, rationale, and clinical results have been published before [3,8]. The selection was generated and based on the highest and lowest CACS scores in the population while keeping confounders such as age and diabetes similar. The study was in accordance with the principles of the declaration of Helsinki and written informed consent was provided by subjects. As CREw-IMAGO is a screening study, there was no clinical indication for the coronary computed tomography angiography. ESC HEART SCOREs were calculated by using the European Low Risk Chart from the European Society of Cardiology.

2.2. Library preparation and sequencing

Platelet RNA isolation is described in the Supplemental Material. The CEL-seq2 protocol was used for library preparation [9]. Sequencing was performed on an Illumina NextSeq500 using 75-basepair paired-end sequencing (Utrecht Sequencing Facility). Data was processed according to a previously published method [10].

2.3. Bioinformatics

All analyses after count table formation were performed in R (version 3.5.1).

Data was normalized for depth of sequencing by transforming count values into reads per million reads sequenced. This also removes any effect of the average age of the platelets in the different groups, as younger platelet contain more RNA than older platelets [11]. Genes with zero variance were removed. Network constructions [12], gene enrichments [13,14] and permutations are described in the Supplemental Material.

2.4. Second network construction in established disease cohort

A sample (n = 54) was selected from the Circulating Cells study, of which design and rationale have been published before [15]. Samples were selected to have a fair representation of controls and patients with different CAD symptoms. Platelet RNA was isolated and processed as published previously [7], leaving 51 samples after a cut-off of 100,000 counts expressed, of which 15 were healthy controls, 14 stable angina patients, 7 unstable angina patients, and 15 non-ST elevated myocardial infarction patients. A baseline table is provided in Supplemental Table 1. The network was constructed as described for the other

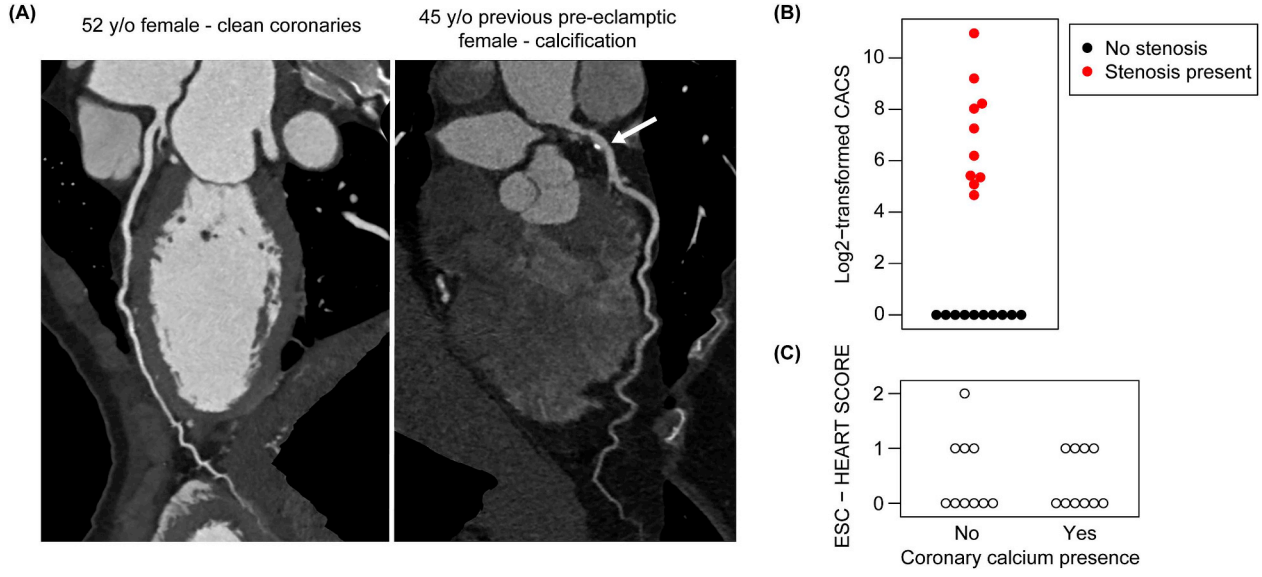

Fig. 1. Coronary computed tomography angiography and CACS.
(A) A typical coronary computed tomography angiography image of coronary arteries with (right image) and without calcification (left image). (B) The distribution of log2-transformed CACS is depicted. Every dot represents one individual. (C) The distribution of ESC HEART SCOREs for women with and without coronary calcification is shown. Every dot represents one individual.

network in the supplemental material. The same genes as for the first network were used to build the network, provided that they were present in the dataset.

3. Results

Baseline traits and clinical phenotypes of the asymptomatic women with previous PE selected for this study (n = 20) are depicted in Supplemental Table 1. Half of the women show calcification (CACS > 0) and stenosis in their coronary artery (Fig. 1A and B). ESC HEART SCOREs were calculated for the women included to address confounding and do not differ between women with and without a positive calcium score (Fig. 1C). Platelet RNA was isolated and sequenced to identify whether platelets carry information about CVD risk in these women.

To ascertain that the transcriptome was platelet-like, we checked expression of mitochondrial and platelet-abundant genes, as well as gene enrichment of the highest expressed genes, and compared them with previous studies [16–18]. After we noticed how similar these highly expressed genes were, we fed the top 25% detected transcripts (8061 genes) into WGCNA for unbiased signed network construction [12]. A complete workflow of the analyses performed within this study can be found in Suppl Fig. 1.

3.1. Gene modules correlate to coronary calcification

Using WGCNA, we found 17 separate modules (Supplemental Fig. 2) in the platelet RNAs, which were merged to form eight final modules, as some of the modules were noticeably similar. Eight genes out of the 8061 genes were not assigned to a specific module and therefore conveniently termed the “grey” module. Correlation of baseline traits and biologically interesting traits in CVD and PE with the modules' eigengenes (Fig. 2A) showed multiple modules (green, black, lightcyan, and lightyellow to a lesser extent, see Supplemental Data File for module gene content) to be positively correlated with CACS (correlation coefficients ranging from 0.44 to 0.59, with $p = 0.05$ to 0.007), and to a

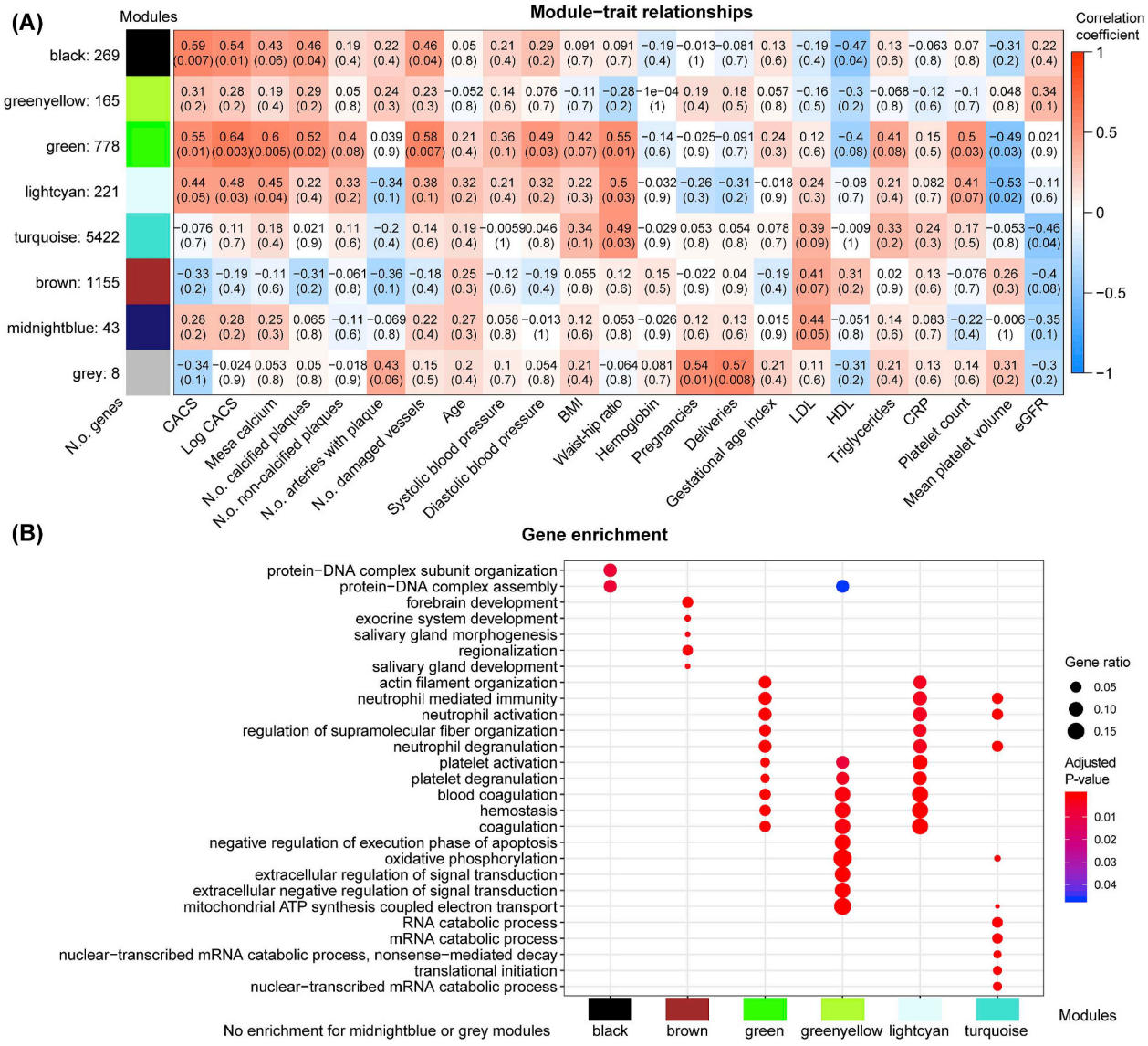

Fig. 2. Module-trait relationships and gene enrichments.
(A) A heatmap correlating gene modules (rows) and clinical variables (columns) is shown. The upper value in the box is the Pearson correlation coefficient and the bracketed value is the *p*-value of correlation. Modules are depicted with color and number of genes. (B) A gene enrichment dot-plot for the top 5 GO: Biological Process terms for every module, that has significant enrichment. Color indicates significance, with the size of the circle pointing to the ratio of genes of a gene set present in the module and the size of the module.

lesser extent with traits such as the number of calcified plaques and MESA calcium. These modules also correlate with a log-transformed CACS, showing that both low and high CACS contribute to the observed correlation. Other observed correlations of the modules' eigengenes to traits are not as strong (Fig. 2A). Two modules had significant different ME values between women with and without stenosis (green, $p = 0.0036$ and lightcyan, $p = 0.041$).

3.2. Gene enrichment analysis links to platelet biology

Gene enrichment analysis on the different modules pointed to terms involved in platelet biology, such as platelet activation, hemostasis and coagulation in the modules that correlated positively with CACS (green, lightcyan, and to a lesser extent greenyellow modules, Fig. 2B). The black module, also positively correlated to CACS, is enriched for "hemostasis" in a REACTOME pathway analysis ($p = 2.4*10^{-4}$). To make sure the platelet transcriptome is not always automatically enriched for platelet-related biology, we also looked at enrichment profiles in the other modules not strongly correlated to CACS. Indeed, the genes found in the other modules pointed to other pathways, like protein/RNA transport & metabolism (turquoise module) and development terms (brown module) (Fig. 2B).

As almost half of the women included are postmenopausal, we checked the module eigengene values of every module after stratification for the occurrence of menopause (Supplemental Fig. 3). No significant differences were found between the pre- and postmenopausal women regarding module eigengene values in any module in our study. We also checked confounding by lifestyle habits such as smoking and alcohol consumption and medication use. We did not notice any correlations with smoking and alcohol consumption. For analyses on medication use, the numbers of women using medication were low (Supplemental Table 1).

Coupling clinical data to the eight constructed modules highlighted three gene modules (green, lightcyan, greenyellow) that are potentially relevant to subclinical atherosclerosis. To explore the importance of the modules further, we looked at the median expression of all genes within a module stratified by coronary artery calcification (Fig. 3). Expression levels were significantly different in three out of eight modules when comparing the group with and without coronary calcifications. The green, lightcyan, and turquoise modules have significantly higher expression ($p = 3.88 \times 10^{-10}$, 0.02, and 2.65×10^{-45} respectively) in platelets from women with coronary calcifications. The difference in means of the medians is largest in the green module, followed by the lightcyan module, both clinically relevant modules, indicating that platelet RNA modules that correlate with calcifications, enriched for coagulation terms, are more abundant in a population with subclinical atherosclerosis. This indicates that presence of coronary calcium associates with the platelet RNA transcriptome as well as severity of calcification.

3.3. Coronary calcification associated modules enriched for CAD loci

To gather more evidence for the implicated modules we tested them for enrichment of CAD susceptibility genes in known loci for CAD [19] using functional annotation and mapping [20]. We performed 10,000 permutations of CAD susceptibility gene status and mimicked a null-

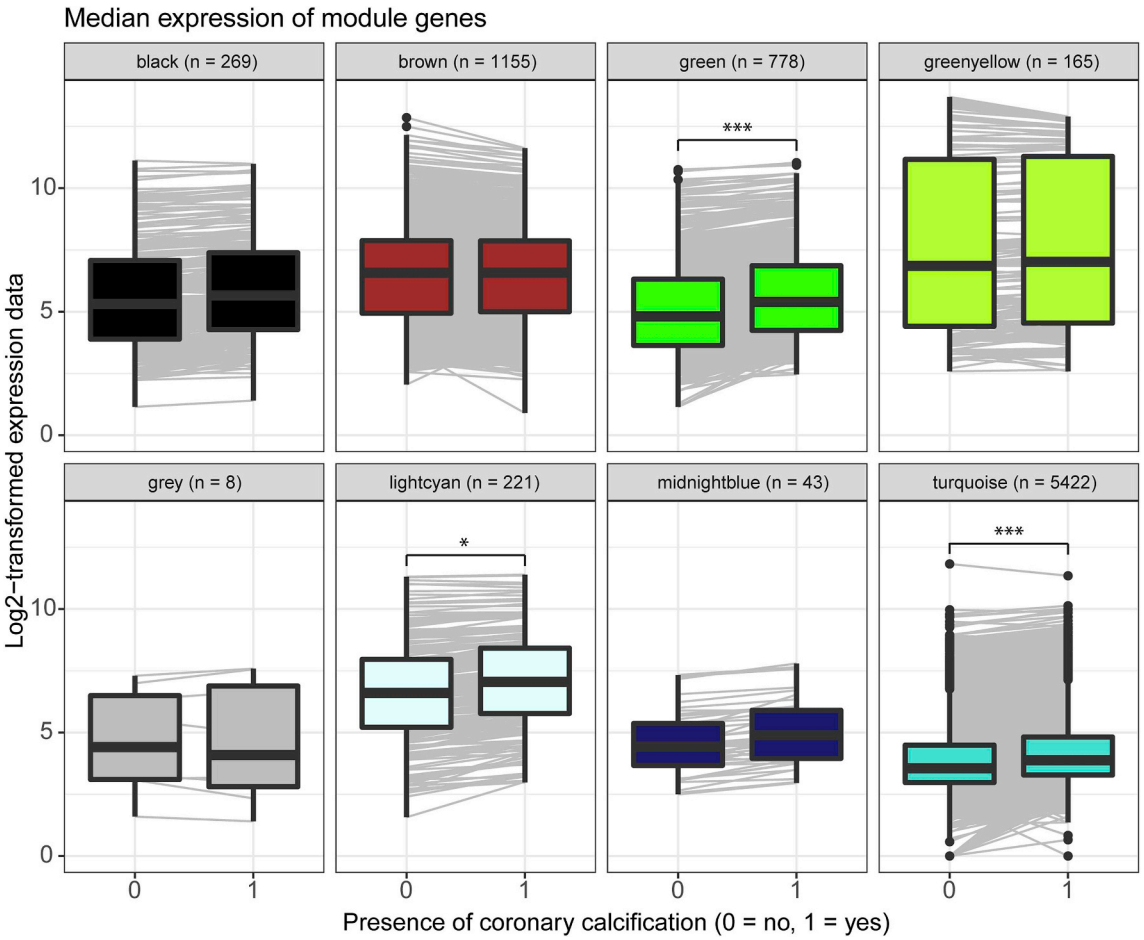

Fig. 3. Calcification and gene expression by modules.
Boxplots are shown for median expression of every gene per module stratified for the presence of coronary calcium. Lines are drawn to connect individual genes from every module. Asterisks indicate statistical significance (* = $p < 0.05$, *** = $p < 1 \times 10^{9}$).

distribution of CAD susceptibility gene proportions divided over the eight different modules from the first network (Fig. 4, Supplemental Data File). The green module, which is higher expressed in the calcification group, correlating with CACS, and enriched for coagulation terms, has a higher proportion of CAD susceptibility genes than expected (proportion: 3.8%, $p_{permutation} = 0.0546$, Fig. 4). A non-random enrichment for CAD susceptibility genes emphasizes the relevance of this module for cardiovascular disease. The grey and midnight blue modules show non-proper distributions, because these modules are small and can contain only a few CAD susceptibility genes. A supplemental data file containing the genes per module and whether or not it is a CAD susceptibility hit is provided in the Data Supplement.

3.4. Validation in a cardiovascular disease population

To assess whether the modules are important and maintained in cardiovascular disease in general, and not just specific to PE, we validated our results in the Circulating Cells study comprising individuals with and without diagnosed CAD [15] (n = 51). Baseline characteristics of the healthy controls and patients within this sample can be found in Supplemental Table 1. A new unbiased and signed network was constructed with the same genes from the first network, generating 24 modules, leading to 19 final modules after merging, with 2167 genes not assigned to a module (the “grey” module). Subsequently, the overlap of genes from the modules of the PE-network with these modules was determined. Fisher's exact tests showed that the clinically relevant calcification modules (green, lightcyan, and black) from our discovery are significantly overlapping with modules (dark red, black, and green) unbiasedly generated in the established cardiovascular disease network with $p = 1.6 \times 10^{-9}$ to 1.7×10^{-47} (Fig. 5A). This indicates that platelet RNA modules correlating with calcium scores in a population with subclinical atherosclerosis (CREw-IMAGO) are also present in platelets from patients with overt cardiovascular disease (Circulating Cells). As such, these three modules in the validation dataset are also enriched for platelet activation and coagulation terms (Fig. 5B). Interestingly, a very strong overlap between the largest modules created in both networks exists, consisting of genes implied in mRNA processing. These modules point towards a strong co-expression and preservation in platelets of mRNAs involved in RNA processing, as well as immune system-related processes.

3.5. Sensitivity analysis

As one of the women in the discovery population had a CACS much higher than the others (a CACS of 1989), we performed the same analyses without this sample, to see whether the same modules would be found, and the same conclusions could be drawn (Supplemental Fig. 4). Modules that correlate with calcium scores are still formed by the analyses, and these are also the modules that heavily overlap with the network construction where the sample is included. Enrichment for platelet activation is present and a preservation with the established CVD cohort network holds true as well.

4. Discussion

Using an unbiased approach, we found platelet gene modules enriched with genes involved in platelet activation and coagulation that were most strongly correlated to CACS in a population of asymptomatic and formerly preeclamptic women. These particular gene modules were not strongly related to some of the other risk factors or clinical traits

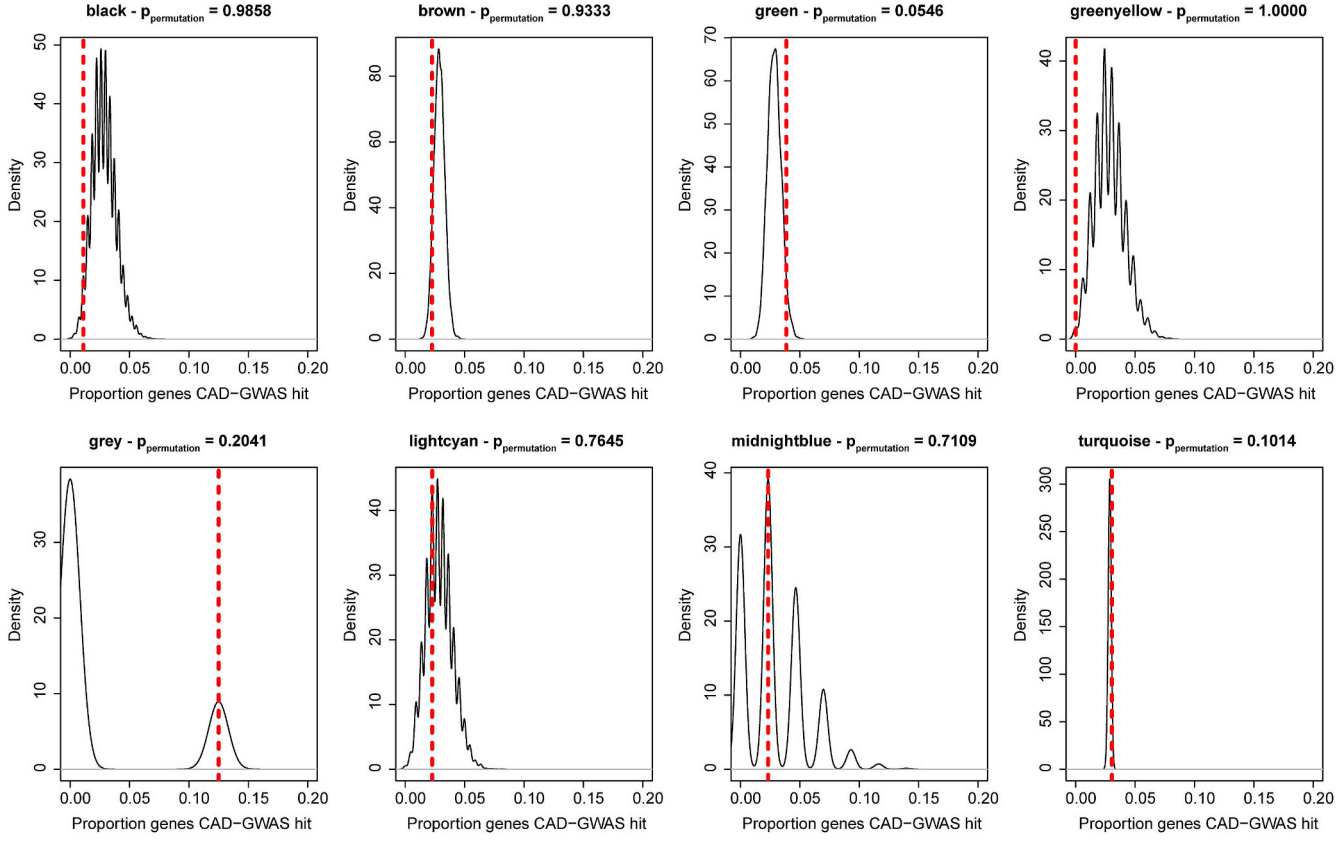

Fig. 4. CAD susceptibility gene proportions per module.
Density plots are shown from data derived from 10,000 permutations of CAD susceptibility gene status. The X-axis contains the proportion of genes of a module that is a CAD susceptibility gene. Actual observed CAD susceptibility gene proportions are drawn in red vertical lines. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

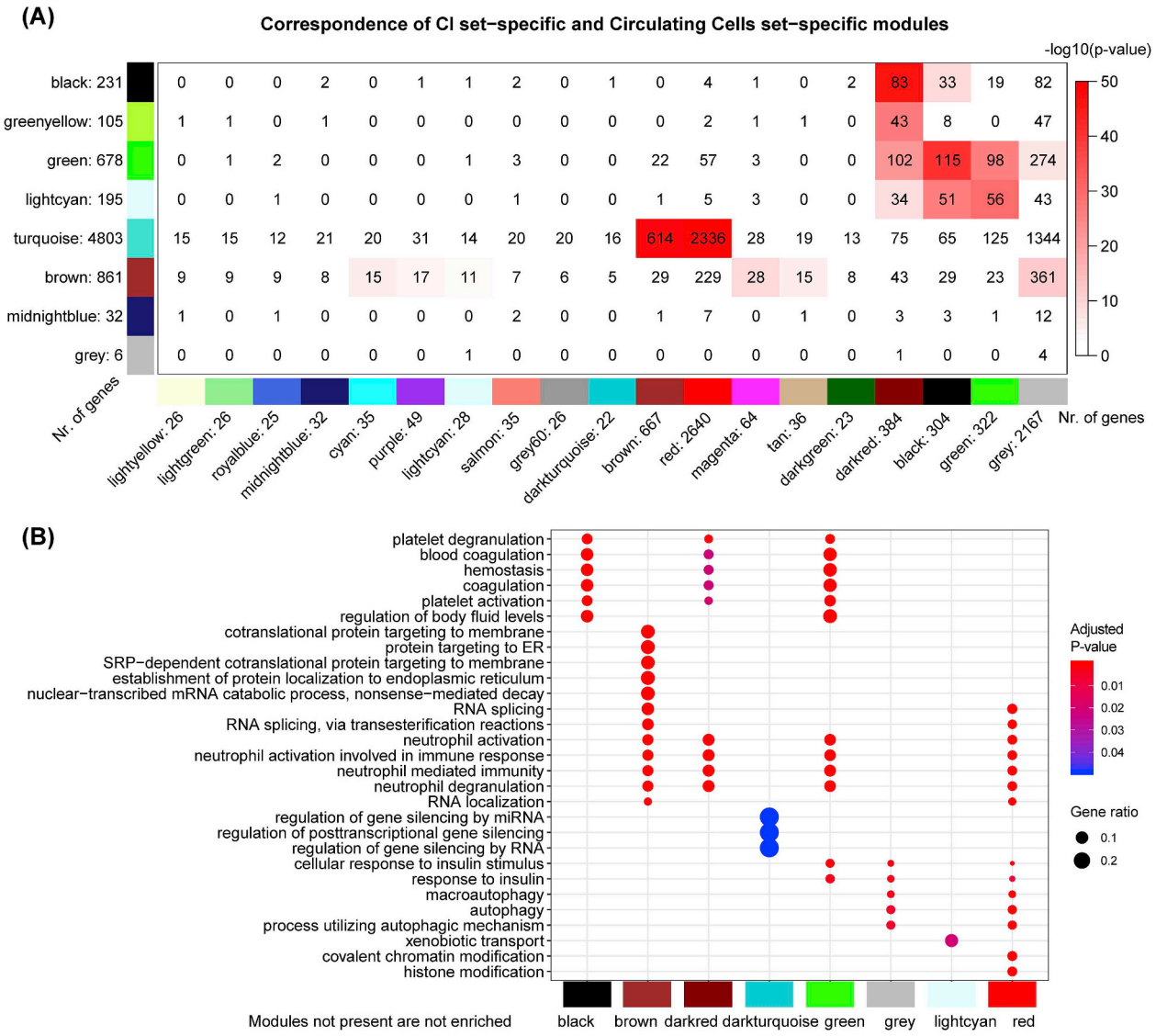

Fig. 5. Overlap between PE-network and the Circulating Cells-network.
(A) Genes in modules from the CREw-IMAGO (CI) set (PE-specific) were overlapped with genes in modules from Circulating Cells (an established CVD cohort [15]). Fisher's exact tests were performed to test for significance. Heatmap colors show the *p*-values from the Fisher's exact tests, with the number in the box showing how many genes are overlapping between two modules. (B) A gene enrichment dot-plot for the top 5 GO: Biological Process terms for every Circulating Cells-module, that has significant enrichment. Color indicates significance, with the size of the circle pointing to the ratio of genes of a gene set present in the module and the size of the module.

including CRP levels, suggesting an independent correlation that could not be explained by e.g. inflammation. As mentioned before, CACS are highly predictive for future CVD events [4], also in women. However, high CACS are not expected in women that are 45–54 years of age [21]. Our data indicate that platelet activation might play a role in a population of women that develops CVD much earlier.

Platelet activation has been shown in PE before [6], but much is still unknown about why some women develop CVD earlier than others. As PE influences the microvasculature as much as 15–25 years after pregnancy [5], it is possible that platelets are affected as well. However, the half-life of platelets is too short for them to still have an effect a decade after a complicated pregnancy. Epigenetic changes in megakaryocytes could possibly explain the abnormal platelet profile 15 years after a complicated pregnancy, as changes in DNA methylation in leukocytes of women with and without PE have been noted before as well [22]. Unfortunately, research regarding epigenetics in megakaryocytes is lacking.

As hypertension is one of the main symptoms of preeclampsia, it might shape the platelet transcriptome. It has been shown previously that hypertension induces a prothrombotic megakaryocyte and platelet phenotype [23], and a slightly positive correlation with diastolic blood pressure is present in the modules correlating with CACS that are enriched for platelet activation (Fig. 2A and B). A negative correlation between HDL-cholesterol and two CACS-modules (green and black) could be appreciated as well, but not for LDL-cholesterol. It is known that HDL affects platelet activation [24], and correlations with modules enriched for platelet activation reinforces those findings.

Recently, it has been shown that activated platelets aggravate calcific aortic valve stenosis in mice [25]. On top of that, activated platelets were found by scanning electron microscopy on endothelium-denuded areas of the calcified stenosis. This suggests that activated platelets can potentially contribute to calcifications of coronary artery stenosis, especially since the (green) platelet activation module correlated to CACS was also enriched for CAD susceptibility genes.

Despite a small sample size, participants were very well characterized and phenotyped. WGCNA allowed us to detect subtleties harbored

in the platelet transcriptome, which may be used in the future for prognostic or diagnostic means. The gene modules found to be correlating with CACS need to be validated in larger cohorts of women at high risk for CVD. We addressed the issue of overfitting correlation estimates due to a limited sample size by validating network construction in an established disease cohort that showed clear preservation of the detected modules, especially of the coronary calcification associated and CAD susceptibility enriched (green) module. Clinical characteristics of the two cohorts do not differ remarkably, except that the PE-group is more hypertensive, contains less smokers and less diabetics (Supplemental Table 1). Removing the outlying sample with the high CACS did not materially change the modules formed, as indicated by strong overlap of modules from the networks constructed with and without this sample (Supplemental Fig. 4). A limitation of our study is that we did not have a control group of women without pregnancy and therefore could not look into platelet signatures from women with unaffected pregnancies, which would be interesting to determine the effect of PE on the platelet transcriptome. In addition, we do not have follow-up data, so we do not know whether high CACS is associated with major cardiovascular end-points in this particular population.

Finally, an established CVD cohort like the Circulating Cells study [15] is bound to encompass patients with calcifications, thereby underlining the significance of the co-expressed signature in the two CVD populations and the identified gene modules in CVD in general. However, this group of patients is not an ideal domain for reproducibility and validity studies of our platelet signature and warrants further studies.

In conclusion, our findings suggest that platelet RNA modules may be useful indicators of early-stage coronary artery disease in an asymptomatic population known to be at risk for accelerated atherosclerosis. Whether the coronary calcification associated platelet modules are indicative of causative biology, the state of the circulation, or an effect from the disease on the platelet still needs to be determined.

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Author contributions

R.H. and H.d.R formulated the research question and designed the study; R.H., S.K., S.d.J., J.M., N.L., G.Z., G.P., R.U., I.H. performed experiments and/or provided materials/samples; R.H., M.M., S.v.d.L. analyzed the data. R.H. and H.d.R. interpreted the results and drafted the manuscript. All authors have contributed to critically revising the manuscript.

Declaration of competing interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.10.009.

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