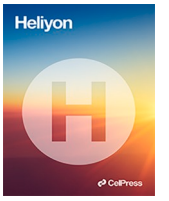
Heliyon 10 (2024) e34923

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

The disparity of platelet factor 4 and platelets in individuals of different ages

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A B S T R A C T

The aging process profoundly impacts the systemic milieu, with specific blood-borne factors playing critical roles in its regulation. Platelet Factor 4 (PF4), released by platelets, has emerged as a novel blood-borne factor that contributes to the rejuvenation of aging brains in rodents. However, the age-related disparity in PF4 levels in humans remains poorly understood. To explore the relationship between PF4 and the natural aging process in humans, we collected peripheral blood (PB) samples from young (23.40 ± 2.13 years, n = 15) and elderly (75.23 ± 4.19 years, n = 13) individuals, along with cord blood (CB) samples (n = 15). ELISA analysis revealed higher PF4 levels in platelet-rich plasma lysate from young PB compared with that from elderly PB. Consistent with this, qPCR results demonstrated the highest PF4 expression in young PB among the three groups. In addition, FACS analysis showed increased expression of CXCR3 in mononuclear cells of young PB, indicating a greater responsiveness to PF4. Finally, our RNA-sequencing analysis corroborated platelets as a sensitive element during the natural aging process, and indicated platelets play a pivotal role in antioxidant response during aging, as evidenced by significant enrichment of several age-related pathways. These findings reveal that, alongside PF4 levels, platelets undergo substantial alterations during aging. Taken together, our data identified age-related disparities in platelets and PF4-related elements during natural aging and underscored the potential of targeting platelet modulation as an intervention in the aging process.

1. Introduction

Systemic aging is a complex biological phenomenon within living organisms, involving cellular senescence and stem cell depletion among other factors [1]. Classic models such as heterochronic parabiosis and blood/plasma injections have been established to study the blood system's role in aging. These models highlight the significance of peripheral system in the natural aging process, profoundly impacting various organs and tissues including brain, pancreas, heart, kidney, and vasculature [2–11]. Mechanistic studies have identified specific blood-borne factors in peripheral system that play crucial roles in regulating the overall physiological environment, either promoting rejuvenation or aging [12,13]. These age-related blood-borne factors may offer potential avenues for rejuvenation interventions, although further investigation is warranted.

Platelets, generated from megakaryocytes within bone marrow and circulating throughout the body, are important components of the peripheral system [14]. During platelet maturation, various bioactive mediators are synthesized and stored within α-granules, dense granules, and lysosomes. Upon platelet activation, these factors are released into the peripheral environment, modulating the function of other cells [15,16]. Previous studies have also unveiled a potential link between platelet function alterations and the aging

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https://doi.org/10.1016/j.heliyon.2024.e34923
Received 6 February 2024; Received in revised form 10 July 2024; Accepted 18 July 2024
Available online 20 July 2024

process. Platelets exhibit increased ROS and mitochondrial dysfunction during aging, resulting in the activation of the mTORC1 pathway. Such alterations contribute to systemically increased platelet activation, driving age-related inflammatory and neurological syndromes [17]. However, the molecular changes in platelets and how platelets interact with systemic environment during aging are not fully understood.

Platelet Factor 4 (PF4) is a pivotal platelet-derived α-granule protein released upon platelet activation, playing a crucial role in maintaining the balance between coagulation and immunomodulation. PF4 constitutes approximately 25 % of the total protein content within platelet α-granules [18]. Recent studies have unveiled PF4 as a novel blood-borne factor responsible for transmitting the rejuvenating properties of youthful blood to aging brains, particularly in the hippocampus [19,20]. Systemic administration of exogenous PF4 has been shown to reduce hippocampal neuroinflammation, alter synaptic plasticity, and improve cognitive capacity in aged mice. Interestingly, the peripheral immune system responded actively to PF4 treatment and contributed to the central rejuvenation. Besides, exercise led to platelet activation and the release of PF4, which have been found essential for exercise-induced elevated proliferation ability of hippocampal precursor cells, improving neurogenesis to ameliorate regenerative and cognitive decline [21]. However, the reported rejuvenation effects of PF4 have mainly focused on rodents and the age-related discrepancy of PF4 expression and its correlation with natural aging process in humans remain unclear.

In this study, we demonstrate that PF4 content in platelets and PF4 expression level in mononuclear cells are higher in blood samples of young individuals compared to those of old individuals. The expression of CXC-chemokine receptor 3 (CXCR3), the primary receptor of PF4, is increased in the young group compared to the old group, indicating a greater responsiveness to PF4 in young peripheral blood samples. Additionally, RNA-seq identified significant changes in platelet transcriptomes and the enrichment of several age-related pathways during aging, suggesting that platelets are a sensitive element that plays a potential role in antioxidant response and other processes during natural aging.

2. Materials and methods

2.1. Blood sample preparation

Cord and peripheral blood samples were obtained from the Women's Hospital, Zhejiang University School of Medicine. This study was approved by the Ethics Committee of the Women's Hospital, Zhejiang University School of Medicine. All blood donors didn't take NSAID/aspirin/salicylate or any other antiplatelet medications before sample collection and didn't possess a history of cardiac or peripheral vascular disease, rheumatologic conditions, bleeding, clotting disorders, or medications for the disorders mentioned above. Besides, none of the blood donors had an infection.

Cord blood and peripheral blood samples were freshly prepared for testing and stored at room temperature. Peripheral blood was collected by ACD-A (blood:ACD-A = 9:1), followed by centrifugation at 500*g* for 8 min at 26 °C for platelet-rich plasma (PPR) preparation. The lower solution was adjusted to 4 mL with PBS and subject to Ficoll density gradient centrifugation to separate mononuclear cells. Briefly, 4 mL Ficoll was added to the centrifuge tube, and then the blood suspension was added drop by drop until distinct layers formed. The sample was centrifuged at 900g for 30 min at 26 °C (acceleration 9 and deceleration 0). The middle buffy coat layer was aspirated to obtain mononuclear cells.

2.2. ELISA

PRP lysate was prepared from PRP after repeated freezing and thawing (−80 °C/4 °C). The PF4 level in PRP lysate was measured using a Human Platelet Factor, PF4 ELISA kit (CUSABIO, CSB-E07882h) according to the manufacturer's protocol.

2.3. Realtime quantitative PCR

Isolated mononuclear cells were centrifugated at 400g for 5 min at 4 °C and cell pellet was dissolved in RNAiso plus (Takara, 9108). Total RNA was extracted and RT-PCR was performed using the PrimeScript™ RT reagent Kit (Perfect Real Time) (Takara, RR037A) according to the manufacturer's protocol. qPCR reactions were performed on a 7900HT Fast Real-Time PCR system (Thermo Fisher) using TB Green® Premix Ex Taq GC (Perfect Real Time) (Takara, RR071B). GAPDH was used as the housekeeping gene. Primers are listed in Supplementary Table 2.

2.4. Flow cytometry analysis

Mononuclear cells were stained with FITC anti-human CD183 (CXCR3) (Biolegend, 353703) and DAPI at 4 °C. After being washed with PBS, cells were resuspended in PBS for analysis on a BD FACSAria™ II (BD Biosciences). The gating strategy is shown in Supplementary Fig. 1.

2.5. RNA-sequencing

PRP was centrifugated at 1500g for 10 min at 26 °C to isolate platelets, which were then dissolved in Trizol (Invitrogen, 15596026). Platelet samples derived from Cord blood and Young/Old blood were used to generate Smart-Seq2 libraries at Suzhou Geekgene Technology Co., Ltd, and subject to RNA-sequencing analysis. DEG and GO analyses were performed with the Geekgene Bioinformatics

Service Portal/Platform (http://bioinfo.geekgene.com.cn/). Heatmaps ware generated in R (4.3.1) using the pheatmap package (1.0.12). Venn diagrams were created in R (4.3.1) using the VennDiagram package (1.7.3). Volcano plots were made in R (4.3.1) using the ggplot2 package (3.4.3). GSEA analysis was performed in GSEA software (4.3.2).

2.6. Data analysis

Data are presented as mean ± SD. The normality of data distribution in each set of experiments was assessed using either the D'Agostino–Pearson omnibus test or the Shapiro-Wilk test. Statistical analyses were conducted using Prism v.9.0 (GraphPad). *P < 0.05, **P < 0.01, and ***P < 0.001.

3. Results

3.1. PF4 and PF4-related elements exhibited age-related discrepancy

We collected peripheral blood (PB) samples from two age groups: young individuals (Young group, 23.40 ± 2.13 years, n = 15) and elderly individuals (Old group, 75.23 ± 4.19 years, n = 13), along with cord blood (CB) samples (CB group, n = 15), to investigate the alterations in PF4 and related elements during natural aging (Fig. 1A). Considering that PF4 is mainly synthesized by megakaryocytes and stored in platelet α-granules, PRP lysate was prepared via repeated freeze-thaw cycles, and PF4 level was quantified by ELISA.

Notably, PF4 exhibited a significant elevation in the PRP lysates of the Young group compared to the Old group, while no significant disparity was observed between the CB and Old groups (Fig. 1B). We then investigated potential age-related discrepancies in PF4 gene expression within mononuclear cells (MNCs). qPCR analysis demonstrated that MNCs in the Young group had higher expression of PF4 compared with the CB group (Fig. 1C). However, the PF4 expression in MNCs from the Young group was only mildly, and insignificantly, higher than that in the Old group. Additional, there was no significant difference in MNC PF4 expression between the CB and Old groups. These results suggest that MNCs might have contributed to the augmented PF4 level in young peripheral blood to a certain degree.

CXCR3, a chemokine receptor for PF4, is reported to be widely expressed across various blood cell types and plays a pivotal role in mediating the rejuvenating and immune regulatory effects of PF4 [20,22,23]. We measured the surface expression of CXCR3 by flow cytometry and found that CXCR3 expression was mildly, but insignificantly, higher in the Young group than that in the CB or Old group

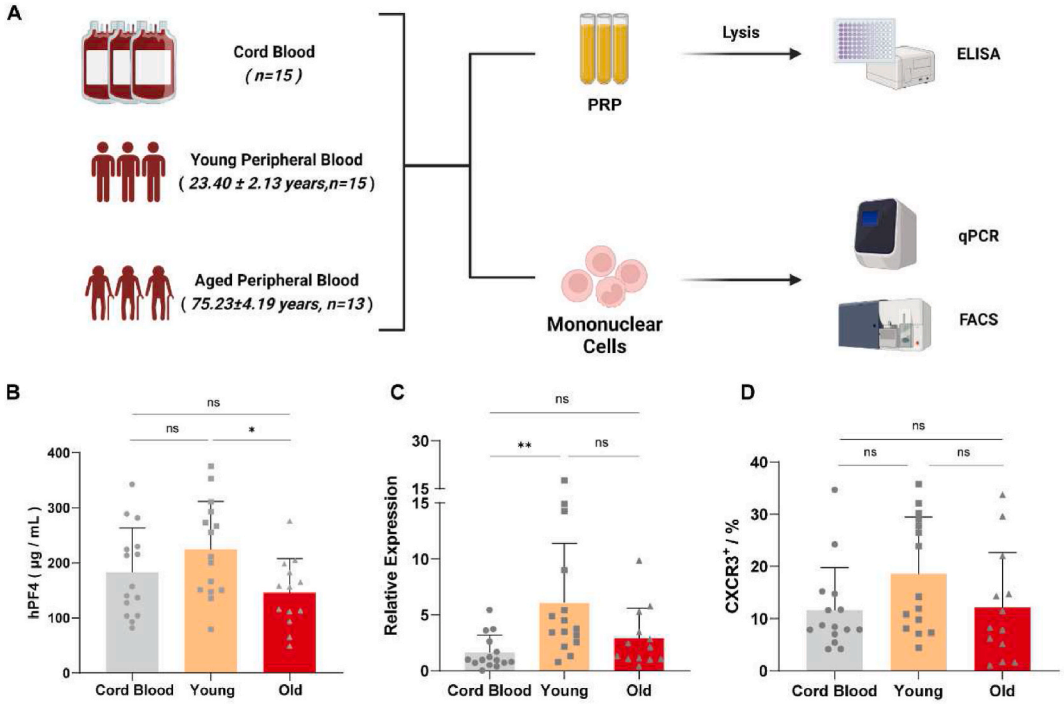

Fig. 1. The expression of PF4 and CXCR3 varied in different age groups. (**A**) Young and Old peripheral blood, as well as Cord Blood samples, were processed and evaluated using ELISA, qPCR and FACS. (**B**) ELISA quantification of PF4 in PRP lysate of Cord Blood (n = 15), Young (23.40 ± 2.13 years, n = 15) and Old (75.23 ± 4.19 years, n = 13) peripheral blood samples. (**C**) Quantitative real-time PCR analysis of PF4 expression in Cord Blood (n = 15), Young (n = 15) and Old (n = 13) peripheral blood mononuclear cells. (**D**) Flow cytometry analysis of CXCR3 expression in mononuclear cells from Cord Blood (n = 15), Young (n = 15) and Old (n = 13) peripheral blood. Data are presented as mean ± SD and analyzed using One-way ANOVA. *P < 0.05, **P < 0.01, and ***P < 0.001.

(Fig. 1D), indicating a potentially more robust responsiveness to PF4 in the Young group.

Taken together, young PB exhibited higher levels of PF4-related elements than old peripheral blood, coupled with heightened sensitivity to PF4. Conversely, cord blood does not demonstrate a discernibly "younger" composition in terms of PF4-related phenotype, at least within the scope of this assessment.

3.2. Distinct transcriptomes of platelets from young and old groups

To further delineate the distinctions in platelet characteristics during the natural aging process, we performed RNA-sequencing analysis of platelets isolated from young and elderly PB samples, as well as CB samples (Fig. 2A). Heatmap analysis, based on the 232 differentially expressed genes (DEGs) between the Young and Old groups, revealed significant changes in the platelet transcriptome during aging (Fig. 2B), and the CB group appeared to possess a transcriptome between the Young and Old groups. UMAP analysis showed good clustering of each sample group and further demonstrated that the CB group was in an intermediate state between the Young and Old groups (Fig. 2C). Besides, platelets in the CB vs. Young group exhibited less transcriptomic difference than in the CB vs. Old group and Young vs. Old group (Fig. 2D). The CB vs. Old and Young vs. Old groups shared similar transcriptome change trends (Fig. 2E and F).

In summary, the aging process significantly impacts the transcriptomes of platelets, and CB-derived platelets seems to possess a

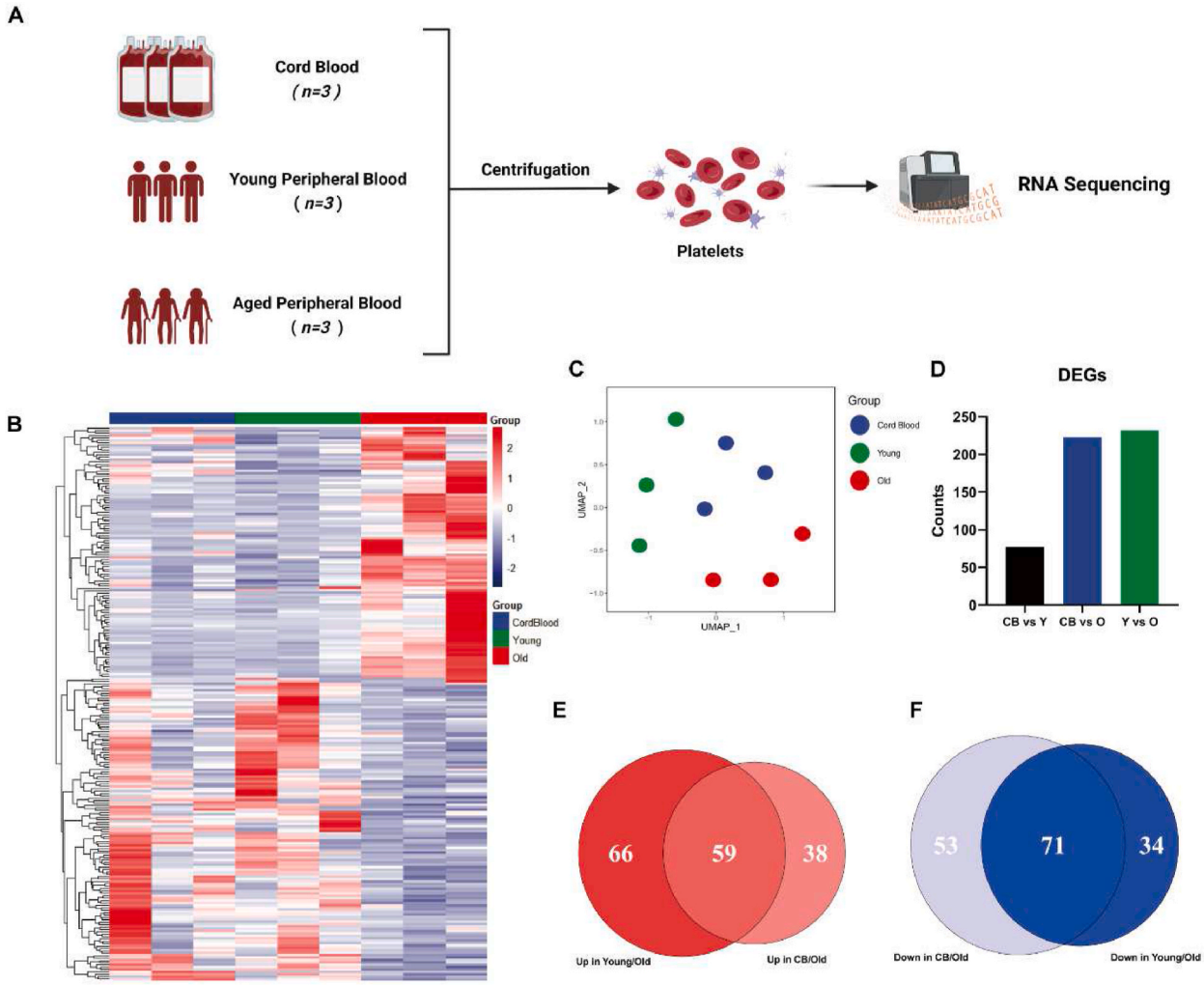

Fig. 2. RNA-sequencing revealed the intermediate state of Cord Blood-derived platelets between their young and old counterparts. (**A**) Platelets isolated from blood samples were used for RNA-sequencing. (**B**) Heatmap analysis of different sample groups after RNA-seq (Cord Blood, n = 3; Young peripheral blood, n = 3; Old peripheral blood, n = 3). Clustering genes were based on significant DEGs (p-value<0.05, n = 232) of the Young/Old group. (**C**) UMAP analysis of all sample clustering. (**D**) DEG statistics between different groups (Cord Blood/Young, Cord Blood/Old, and Young/Old). (**E**) Venn analysis of up-regulated DEGs in Cord Blood or Young peripheral blood groups compared with the Old group. (**F**) Venn analysis of down-regulated DEGs in Cord Blood or Young peripheral blood groups compared with the Old group.

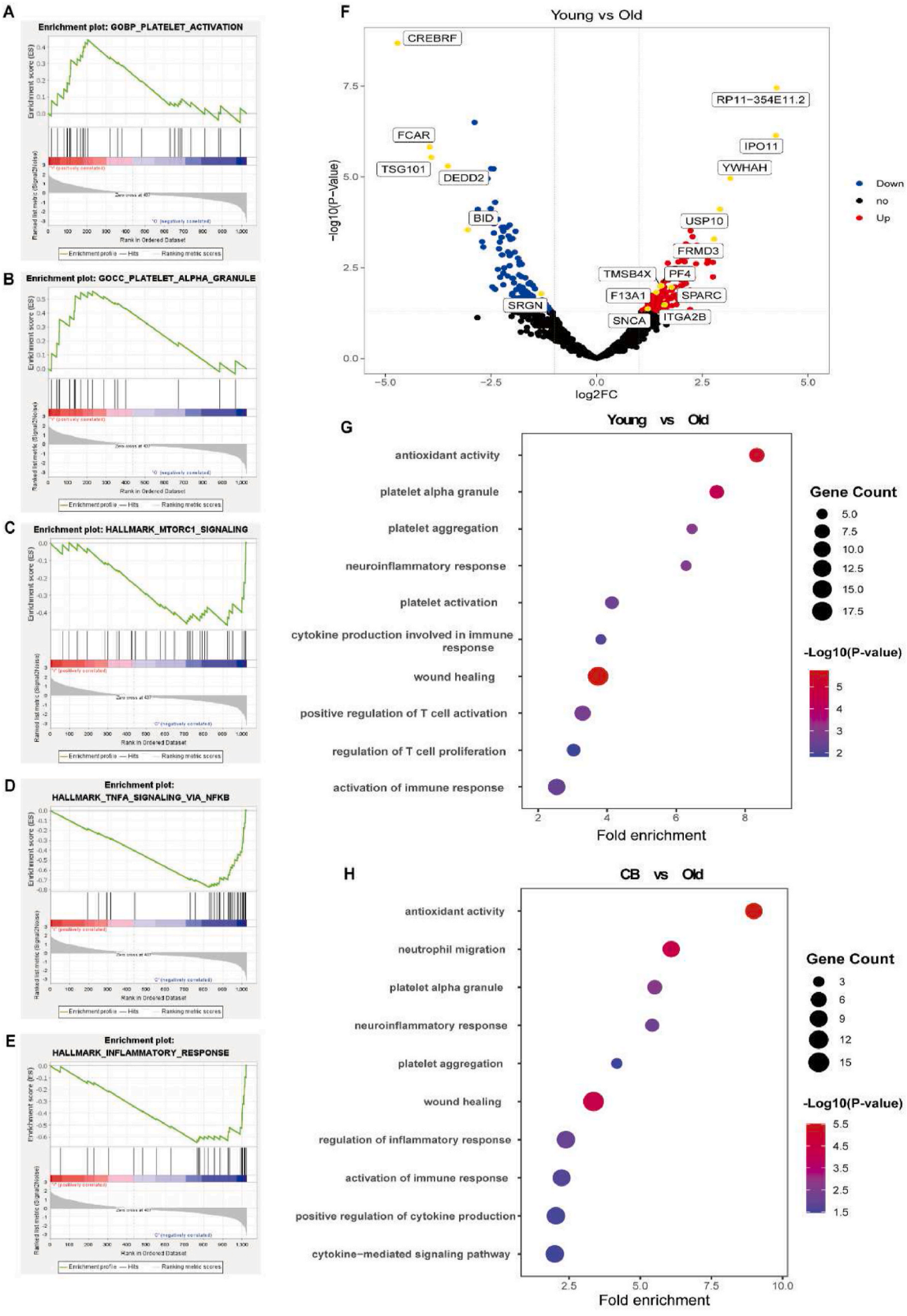

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Fig. 3. Platelets actively responded to aging and contributed to antioxidant activity. (**A-E**) GSEA analysis of Young/Old sample groups. (**F**) Volcano plot analysis of DEGs between Young and Old peripheral blood groups. Red and blue dots indicate DEGs identified by p-value <0.05 and $|\log_2$ (Fold Change)$| > 1$. Gene of interest were marked as yellow dots. (**G**) GO analysis of Young vs. Old groups. (**H**) GO analysis of CB vs. Old groups.

transcriptome that is intermediate between the young and old PB-derived platelets.

3.3. Platelets were sensitive to natural aging and contributed to antioxidant activity

Further analysis of the RNA-sequencing data using gene set enrichment analysis (GSEA) revealed significant findings regarding platelet characteristics during the natural aging process. Specifically, the platelet activation (GO:0030168) and platelet α-granule (GO:0031091) gene sets were enriched and upregulated in the Young group compared with the Old group, indicating potential differences in platelet activation at the functional level during aging (Fig. 3A and B). The mTORC1 signaling pathway, known for its role in cellular senescence and organismal aging [24–26], show down-regulation of related gene sets in the Young group (Fig. 3C). Additionally, aging-related gene sets, including TNFalpha signaling pathway which contributed to aging-associated platelet hyperactivity and thrombosis [27], were enriched and down-regulated in the Young group (Fig. 3D and E).

Interestingly, several platelet-α-granule-related genes (GO:0031091), in addition to PF4, were also found to be differentially expressed between the Young and Old groups (Fig. 3F). Notably, Gene Ontology (GO) enrichment analysis highlighted significant enrichment of antioxidant activity (GO:0016209), neuroinflammatory response (GO:0150076) and wound healing (GO:0035313) in comparisons between Young vs. Old and CB vs. Old groups. Several immune-related and platelet-related pathways were also significantly enriched (Fig. 3G and H).

In summary, RNA-sequencing analysis of platelets from individuals of different age groups revealed substantial changes in the platelet transcriptome during natural aging. These findings underscore potential correlations between aging-related shifts in platelets and their roles in antioxidant activity and immune response mechanisms.

4. Discussion

In this study, we have discovered age-related disparities in PF4 levels during natural aging and found mononuclear cells in young peripheral blood exhibited elevated expression of CXCR3. RNA-sequencing analysis highlighted platelets as a particularly sensitive element within peripheral blood during natural aging. Moreover, our findings revealed a disparity in antioxidant activity between platelets from Young and Old groups according to GO analysis, and several age-related gene sets were significantly enriched in platelets from the Old group.

The peripheral blood system is susceptible to natural aging. Previous studies have demonstrated significant age-related cellular changes in T cells, natural killer cells, and myeloid cells [28–32]. Furthermore, hematopoietic stem cells (HSCs) gradually lose their self-renewal and regenerative potential during aging, accompanied by an increased myeloid differentiation tendency [33]. Here we found that PF4 and platelets also showed aging related changes. Since PF4 is mainly stored in platelets, which are non-nucleated cell elements, the expression disparity of PF4 may already exist in platelet progenitors, such as megakaryocytes, megakaryocyte progenitors, and HSCs Investigating specific molecular changes in these cells during aging is needed to further explain the multi-level alterations in platelets. Furthermore, it has been reported that neutral blood exchange in aged mice could increase PF4 level in peripheral blood, indicating that central-peripheral communication may affect the peripheral environment, leading to the age-related disparity in PF4 [34]. However, it remains unclear if it also occurs in human and whether this regulation begins with HSCs or downstream cells.

It is noteworthy that the transcriptome of CB-derived platelets closely resembles that of young peripheral blood-derived platelets. However, CB-derived platelets appear to occupy an intermediate state between young and aged platelets. It has been reported that after cord blood transplantation, platelet recovery is significantly slower compared to that after peripheral blood transplantation. In addition to the lower level of proliferating stem cells in cord blood, this phenomenon is likely attributed to immature megakaryocyte (MK) compartments in cord blood, as evidenced by reduced CFU-MK colony formation capacity and incomplete functionality in terms of polyploidization [35–37]. Previous studies have also elucidated the inherent sluggishness in platelet development in cord blood [36], further supporting the intermediate state observed in the transcriptome of CB-derived platelets.

Considering the established role of PF4 in rejuvenating the neural system by regulating the peripheral immune system, it is conceivable that other platelet-related factors might also have undiscovered connections to systemic aging. Additionally, the decreased level of PF4 in elderly individuals indicates an avenue for intervening in the aging process through PF4 supplementation. However, it must be noted that several studies have demonstrated that PF4 supplementation influenced thrombosis and susceptibility to heparin-induced thrombocytopenia (HIT) [38,39]. Besides, a mouse model of atopic dermatitis also showed elevated levels of PF4 in the spleen. Therefore, the intricate role of PF4 in systemic health requires further investigation to facilitate the clinical use of PF4 supplementation for aging and age-related diseases.

Funding

This work was supported by National Natural Science Foundation of China (82100140), the STI2030 project (STI2030-SQ2021AAA010745) and the 111 project (B21024).

Ethic and consent

This study was approved by the Ethics Committee of the Women's Hospital, Zhejiang University School of Medicine (IRB-20230319-R). We obtained written informed consent from participants and provided the Human participant declaration form.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Ruopeng Weng: Writing – review & editing, Resources, Project administration, Methodology. **Jiaqing Liu:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Qingtan Yu:** Writing – review & editing, Supervision, Methodology. **Haitao Yuan:** Writing – review & editing, Investigation. **Yun Qiu:** Investigation. **Huicong Liu:** Writing – review & editing, Investigation. **Lingna Wang:** Writing – review & editing. **Zijie Mei:** Writing – review & editing. **Fangfang Zhu:** Writing – review & editing, Supervision, Methodology, Funding acquisition.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e34923.

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