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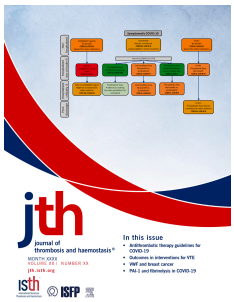

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RNA Sequencing Indicates Distinct Platelet Transcriptomic Changes in Immune Thrombocytopenia

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ABSTRACT

Background: In immune thrombocytopenia (ITP), platelet-intrinsic alterations and their potential contribution to disease pathogenesis remain incompletely understood. Since platelets, although anucleate, retain a functional transcriptome, transcriptomic profiling may provide insight into disease-associated changes.

Objectives: To investigate platelet-intrinsic transcriptomic changes in ITP and to determine whether these alterations are disease-specific compared with non-immune thrombocytopenia.

Methods: Total RNA sequencing was performed on purified platelets from patients with active ITP (n=6), chemotherapy-induced thrombocytopenia as non-immune thrombocytopenic control (n=6), ITP in treatment-free remission (n=6), and healthy controls (n=8).

Results: Platelets from patients with active ITP exhibited higher total RNA content, consistent with enrichment of young, recently released platelets. Transcriptomic analysis identified a profile dominated by platelet activation pathways, including integrin and calcium-dependent signaling, cytoskeletal remodeling, and vesicle trafficking. In contrast, genes involved in mitochondrial biogenesis and oxidative phosphorylation were downregulated, accompanied by a reduced proportion of mitochondrial-derived RNA. Although both active ITP and chemotherapy-induced thrombocytopenia showed elevated RNA

content relative to healthy controls, reflecting enhanced platelet turnover, the coordinated upregulation of activation-associated transcripts was unique to active ITP. Platelets from ITP patients in treatment-free remission showed partial normalization of the transcriptional profile, with loss of the activation signature and intermediate expression patterns between active ITP and healthy controls.

Conclusion: RNA sequencing data show distinct platelet transcriptomic changes in ITP marked by coordinated upregulation of platelet activation pathways and reduced mitochondrial gene expression. This profile is consistent with an activation-primed, metabolically altered platelet state and may reflect disease-related changes in platelet biology in ITP.

Keywords: Immune thrombocytopenia, RNA sequencing, platelet transcriptome, platelet activation

INTRODUCTION

Immune thrombocytopenia (ITP) is an autoimmune disease characterized by isolated thrombocytopenia due to increased platelet clearance and impaired megakaryopoiesis. Clinically, ITP manifests primarily through bleeding of variable severity [1].

The pathophysiology of ITP is driven by complex immune dysregulation, including antibody-mediated platelet destruction, alterations in megakaryocyte biology, and T cell-driven immune mechanisms [2]. Although immune dysregulation in ITP has been extensively characterized, the platelet-intrinsic contribution to disease pathogenesis remains insufficiently understood. Platelets in ITP have traditionally been considered primarily as targets of immune-mediated destruction rather than as active participants in the disease process. However, advances in platelet biology increasingly challenge this view, underscoring their pivotal roles not only in hemostasis but also in inflammation and immunity [3,4]. For example, platelets have been shown to directly interact with immune cells, including $CD4^+$ and $CD8^+$ T lymphocytes, and to participate in inflammatory signaling processes [5-8]. Expression of major histocompatibility complex class I by platelets enables modulation of T-cell activation and has been implicated in pro-inflammatory T-cell differentiation [5]. In addition, platelets contribute to immune signaling through the release of platelet-derived microparticles (PMP), which contain cytokines and autoantigens and can modulate immune responses [9,10].

At the molecular level, anucleate platelets retain a complex transcriptome inherited from megakaryocytes [11]. This RNA repertoire comprises coding, non-coding, and mitochondrial transcripts and reflects both megakaryocytic programming and regulatory influences during circulation [12]. In several disease contexts, transcriptomic analyses have revealed altered platelet RNA signatures, for instance in cardiovascular disease, cancer, and sepsis [13-18]. For ITP, however, such analyses are still scarce, presumably due to technical challenges associated with the limited amounts of starting material obtainable from thrombocytopenic patients.

Here, we performed total RNA sequencing of highly purified platelets from patients with active ITP to enable transcriptome-wide characterization in this disease. We examined whether platelets from ITP patients display transcriptomic differences compared with healthy controls and evaluated the disease specificity of the identified signatures in comparison with chemotherapy-induced thrombocytopenia as a non-immune thrombocytopenic control.

METHODS

A total of 26 individuals were enrolled: six patients with active ITP without ITP-specific treatment at sampling (median PLT 35×10⁹/L); six patients with chemotherapy-induced thrombocytopenia during marrow recovery as platelet-matched non-ITP controls (median PLT 37×10⁹/L), six ITP patients in treatment-free remission (median PLT 178×10⁹/L); and eight healthy controls (median PLT 229×10⁹/L). Groups were balanced for age and sex (Supplementary Table 1).

ITP diagnosis was established according to current international consensus criteria [19]. All included patients had primary ITP. Active ITP patients had both newly diagnosed and persistent/chronic ITP. At the time of sampling, they were either under a watch-and-wait approach or before initiation of ITP-specific therapy; none had previously received any ITP-directed therapy other than corticosteroids. Patients in remission had stable platelet counts >100×10⁹/L for at least 3 months without ongoing ITP-directed therapy; previous treatments included corticosteroids and TPO-receptor agonists. Patients with chemotherapy-induced thrombocytopenia were sampled during marrow recovery at platelet counts approximating those of the active ITP cohort, resulting in comparable median platelet counts in both groups.

A minimum platelet count of 20×10⁹/L was required to ensure adequate RNA yield. Peripheral blood (36 mL) was collected in EDTA and processed immediately after venipuncture to avoid in vitro platelet activation. Platelet-rich plasma (PRP) was obtained by sequential centrifugation as described previously [20]. A platelet inhibition cocktail was prepared in Dulbecco's phosphate-buffered saline (DPBS, without Ca^{2+}/Mg^{2+}) containing 0.25 mM EDTA, 10 µM PGE_1, and 0.05 % (w/v) BSA. One ml of this cocktail was added per 10 ml of plasma during PRP preparation. Platelets were pelleted and further purified by a two-step leukocyte depletion protocol consisting of filtration (Acrodisc® WBC Syringe Filter) and CD45-targeted magnetic bead separation (Miltenyi Biotec). Total RNA was extracted using the mirVana™ miRNA Isolation Kit (Thermo Fisher Scientific). RNA concentration was measured using the Qubit™ RNA HS Assay. After CD45 bead depletion, final volumes were 50 µl with RNA concentrations ranging from 2 to 19.7 ng/µl. Platelet-specific transcripts (*P2RY1, ITGA2B*) and leukocyte/erythrocyte markers (*PTPRC, GYPA*) were assessed by qPCR to confirm sample purity and RNA yield. For library preparation, 50 ng of total RNA per sample were processed with the Watchmaker Genomics Total RNA-seq Library Prep Kit including Polaris ribosomal RNA depletion. Adapter ligation was performed with 1 µM truncated adapters (IDT), followed by double post-ligation clean-up. Libraries were amplified for 13 PCR cycles and sequenced on an Illumina NovaSeq 6000 (200 cycles, paired-end). Library preparation and sequencing

were performed in two batches, with samples from all four groups distributed across both batches to avoid batch-group confounding.

Reads were aligned to the human reference genome (GRCh38/hg38) using STAR (v2.7.10b) [21] with Ensembl release 104 splice annotations [22] and parameters optimized for unique alignments. Gene-level counts were generated with featureCounts (v2.0.1) [23] and normalized using DESeq2 (v1.38.3) in R (v4.2.2) [24] to account for differences in library depth and composition. Principal component analysis of the 500 most variable genes did not show clustering by batch (Supplementary Figure 1A). Differentially expressed genes (DEG) were identified with the Wald test including sex as a covariate, and p-values were adjusted for multiple testing with the Independent Hypothesis Weighting (IHW v1.26.0) method [25]. Genes with an adjusted $p \leq 0.05$ were considered significant. Functional enrichment of DEGs was evaluated using Gene Ontology (GO), KEGG, and Reactome frameworks implemented in a Python-based workflow (Python v3.10; gseapy v1.1.8 [26]; pandas v2.2.2 [27]).

A schematic overview of the workflow is provided in Figure 1.

This study was approved by the local ethics committee of the Technical University (TU) Dresden, Germany (approval number: BO-EK-572122021), and all patients and healthy control subjects provided written informed consent in accordance with the Declaration of Helsinki.

RESULTS

RNA yield and global transcriptomic profiling

Relative to peripheral platelet counts, median RNA yield was highest in active ITP and chemotherapy-induced thrombocytopenia, indicating enrichment of young, RNA-rich platelets in both thrombocytopenic groups (Supplementary Table 1). Sequencing produced high-quality libraries (mean 60.7 million paired-end reads per sample, >90 % unique mapping; median 5,864 transcripts at ≥1 CPM). Platelet purity was confirmed by high expression of PPBP and PF4 and very low expression of leucocyte and erythrocyte specific markers (PTPRC, CD14, HBB < 0.05 % relative to PPBP; Supplementary Table 2). Expression profiles of healthy control platelets closely matched reference platelet RNA sequencing datasets [28, 29], with the expected ranking of the most abundant platelet markers and high inter-sample Spearman correlation between healthy controls (ρ=0.93–0.97). Principal component analysis showed disease-associated separation, with ITP remission samples displaying an intermediate profile between active ITP and healthy controls (Supplementary Figure 1B). Differential expression analysis revealed distinct transcriptional signatures between groups (Figure 2; detailed results in Supplementary Tables 3–13).

Platelet activation pathways are upregulated in active ITP

To identify transcriptional changes in active ITP, platelet transcriptomes were first compared between active ITP patients and healthy controls. Differential expression analysis identified 3.643 significantly upregulated and 3.342 downregulated transcripts in ITP platelets. Pathway enrichment consistently revealed a pronounced platelet-activation signature across all annotation frameworks (Figure 3A+B). The top enriched categories involved *Platelet activation, signaling and aggregation* (adjusted $p=4.25 \times 10^{-21}$)

and *Hemostasis* (adjusted p=1.11x10^{-17}). Upregulated transcripts included key components of integrin-associated signaling (*AKT1, FERMT3, ITGA2B, ITGB3, SRC, TLN1*), as well as multiple genes involved in Calcium-dependent signal transduction (*ATP2A3, CALM1, ITPR1, ITPR2, PRKCB, SLC24A3*). In addition to classical platelet signaling pathways, enrichment analysis highlighted categories annotated with immune- or pathogen-associated terms (*Immune System, Innate Immune System, Infectious disease*), which showed substantial overlap in contributing transcripts with platelet activation and actin-dependent signaling processes.

In line with this platelet activation-related pattern, transcripts involved in cytoskeletal organization and adhesion, such as *ACTB, ARPC1B, FLNA, MAP1A*, *PARVB*, and *WASF2*, were significantly increased. Gene set enrichment analyses confirmed activation of Rho GTPase signaling and actin cytoskeleton regulation. In addition, pathways related to vesicle-mediated transport and intracellular trafficking were similarly upregulated (*Endocytosis, Lysosome, Autophagy*), supported by increased transcript levels of *ACTG1, DNM2, NBEAL2, PPP1CA, PRKCA,* and *RHOA.*

At the same time, several transcripts associated with cytoskeletal organization and vesicle recycling were reduced, including *PKN2, RAB11A, RASA1, RGS18, RHOBTB3, SLAIN2, TBC1D31,* and *TMSB4X*. This indicates selective, bidirectional transcript changes within vesicle- and cytoskeleton-associated pathways.

Reduced transcript levels were most prominent in mitochondrial and metabolic pathways. This included decreased RNA levels of several nuclear-encoded mitochondrial factors involved in RNA processing and translation (e.g., *NDUFV2, NDUFS6, MRPS9, MRPS14, MRPL3, ATP5ME, ATP5MK*) as well as lower abundance of all mitochondrial-encoded RNAs in ITP compared with healthy controls.

The observed patterns were further supported by gene set enrichment analysis across the ranked transcriptome (Figure 3C).

In addition to protein-coding transcripts, RNA sequencing captured multiple classes of non-coding RNAs. Across samples, more than 4 000 annotated lncRNA loci were detected, with a subset showing differential transcript abundance between ITP and control platelets, including the most significantly altered lncRNAs *C6orf223, ZNF346-IT1, DLGAP1-AS2, MAP3K14-AS1*, and *SCIRT*. Small RNA species were also detected, including several miRNAs, as well as snRNAs and snoRNAs, some of which showed significantly different expression between ITP and controls. However, as rRNA-depletion protocols are sub-optimal for small-RNA recovery, these data do not permit reliable quantitative assessment.

Comparison with chemotherapy-induced thrombocytopenia suggests ITP-specific signatures beyond those of regenerative thrombocytopenia

To determine whether the transcriptional changes observed in active ITP platelets are disease-specific or reflect more general regenerative features of thrombocytopenia, we compared platelet transcriptomes from patients with ITP to those from individuals with chemotherapy-induced thrombocytopenia. The chemotherapy-induced thrombocytopenia cohort comprised patients with acute myeloid or lymphoid leukemia in documented complete remission who were undergoing marrow recovery after

consolidation chemotherapy. These patients had experienced profound chemotherapy-induced thrombocytopenia (nadir < 10×10^9/L) followed by physiological platelet regeneration without evidence of persistent marrow damage or immune-mediated platelet destruction.

Both ITP and chemotherapy-induced thrombocytopenia were associated with increased RNA yield relative to blood platelet counts, consistent with enrichment of RNA-rich, young platelets in both thrombocytopenic conditions. In line with this, both ITP and post-chemotherapy platelets showed increased abundance of transcripts associated with vesicle-mediated transport and membrane trafficking, with significant enrichment of categories including *Endocytosis* (KEGG) as well as *Membrane trafficking* and *Vesicle-mediated transport* (Reactome). A robust and coordinated platelet activation signature at the pathway level, however, was only observable in ITP. The Reactome term *Platelet activation, signaling and aggregation*, which ranked among the most strongly enriched categories in ITP, was not enriched in chemotherapy-induced thrombocytopenia; moreover, the KEGG *Platelet activation* pathway was significantly reduced in post-chemotherapy samples compared with healthy controls (adjusted $p=7.3\times10^{-4}$). Thus, coordinated activation-related transcriptional changes were specific to ITP, whereas chemotherapy-induced thrombocytopenia did not show enrichment of platelet activation pathways (Figure 4A).

While both thrombocytopenic conditions showed similarly reduced abundance of mitochondrial-encoded RNAs, the nuclear-encoded mitochondrial biogenesis program (OXPHOS subunits and mitochondrial ribosomal proteins) was reduced specifically in active ITP and, conversely, elevated in chemotherapy-induced thrombocytopenia (Figure 4B).

Attenuated activation-related transcriptional changes in ITP in treatment-free remission

To assess whether the transcriptional profile of active ITP normalizes with restoration of platelet counts, platelet transcriptomes from ITP patients in treatment-free remission were analyzed. In platelets from remission samples, substantially fewer transcriptional differences to healthy controls were observed (1.093 DEGs compared with controls, versus 6.985 in active ITP). In particular, the strongly coordinated activation-related transcriptional signature observed in active ITP was largely attenuated. A subset of activation-related transcripts nevertheless remained elevated in remission compared with healthy controls, including the αIIbβ3 integrin components *ITGA2B* and *ITGB3* as well as selected calcium-signaling and cytoskeletal transcripts, while PI3K/MAPK signaling components and α-granule secretion markers had returned to control levels. Overall, remission samples showed expression profiles intermediate between active ITP and controls, with greater inter-sample variability (Supplementary Figure 2). In remission samples, expression profiles tended to vary with the degree of platelet recovery: samples from patients with platelet counts below 150×10^9/L resembled active ITP more closely and showed a more pronounced activation-related pathway profile (Supplementary Figure 3).

DISCUSSION

This study provides, to our knowledge, the first transcriptome-wide RNA sequencing analysis of highly purified platelets in active primary ITP. Despite profound thrombocytopenia, all samples yielded high-quality RNA-seq data, demonstrating the feasibility of platelet transcriptomic profiling even at very low platelet counts. Platelets from patients with active ITP displayed a distinct RNA profile characterized by convergent upregulation of activation-related pathways across all annotation frameworks. Many of these activation-associated transcripts overlap with known markers of young, RNA-rich platelets, indicating that part of the observed variation reflects compositional differences in platelet age – specifically, an increased proportion of reticulated platelets. However, the magnitude and pathway coherence of the activation signature exceed patterns typically attributable to this immature platelet population [30, 31], supporting a contribution of disease-specific factors beyond compositional shifts. The comparison with chemotherapy-induced thrombocytopenia provides additional support for the disease specificity of these findings. Despite similar increases in RNA yield and cytoskeletal- and adhesion-related transcripts in both groups, only platelets from active ITP displayed a coordinated activation-related transcriptional program, while some canonical activation pathways were not enriched, and in some cases even reduced, in chemotherapy-induced thrombocytopenia. This indicates that disease-associated factors in ITP, rather than mere regenerative thrombopoiesis, may contribute to an activation-primed platelet state, consistent with prior functional studies demonstrating platelet hyperreactivity in ITP [32, 33].
As for the immune-related pathway categories that were found enriched in ITP platelets, many of the contributing transcripts have established dual functions in platelet activation and immune signaling, and the immune-related enrichment in our data is therefore not fully separable from the activation signature. The activated phenotype of ITP platelets may itself contribute to sustaining immune dysregulation. However, direct evidence for such a contribution or active immunoregulation by platelets cannot be derived from our transcriptomic data alone and requires functional studies of platelet-immune cell interactions.

In addition to the activation-related changes, ITP platelets showed a broad reduction in transcripts encoding mitochondrial and metabolic functions. This included multiple nuclear-encoded mitochondrial genes involved in RNA processing and mitochondrial dynamics as well as mitochondrial-encoded RNAs. Such concordant depletion of mitochondrial transcripts may indicate altered mitochondrial function in ITP platelets, aligning with prior reports describing mitochondrial depolarization, increased oxidative stress, and enhanced susceptibility to apoptosis [34-37].

When interpreting the results of this study, several biological and technical factors need to be considered. The most important limitation is the absence of functional or proteomic validation, which was beyond the scope of this study. Since RNA abundance does not directly equate to functional activity, the findings require experimental validation in future studies.

A further consideration is that the platelet transcriptome is physiologically dynamic and varies with platelet age and maturity even in healthy individuals [38]. A major interpretational challenge in platelet transcriptomics is therefore distinguishing disease-associated transcriptional changes from shifts in platelet population composition, since even in purified platelet preparations, heterogeneity in platelet age

and RNA content can substantially influence global transcript profiles [39]. To address this, chemotherapy-induced thrombocytopenia was included as a non-immune comparator with comparable degrees of thrombocytopenia and ongoing regenerative thrombopoiesis. This is, of course, not an ideal control condition, as cytotoxic therapy may specifically alter megakaryocyte gene expression and thereby introduce confounding transcriptional changes, consistent with the more pronounced separation of these samples in the PCA. Furthermore, parameters reflecting platelet maturity, such as the immature platelet fraction, may provide a more refined basis for comparison than platelet counts in future studies. In addition, inclusion of other regenerative or non-immune thrombocytopenic conditions, such as post-hemorrhagic thrombocytopenia or congenital thrombocytopenia, may further strengthen this approach.

A related question is at which level the observed transcriptional changes originate and by which mechanisms they are driven. Given that platelet RNA is largely inherited from megakaryocytes, disease-related alterations in megakaryopoiesis are likely to cause or at least contribute to the observed transcriptional landscape. In addition, circulating platelets may undergo further transcriptomic modulation through exposure to autoantibodies or immune effector cells, particularly affecting mitochondrial and stress-responsive genes. Because bulk RNA sequencing captures averaged transcriptional profiles, the analyzed samples represent composite molecular signatures that cannot be resolved at the level of individual platelet subpopulations. Future studies integrating single-platelet transcriptomics with megakaryocyte analyses may help address this issue [40].

Furthermore, the modest sample size limits statistical power to detect subtle transcriptional changes while potentially accentuating apparent differences, particularly given the known clinical heterogeneity of ITP. Although the present analysis was restricted to adult patients with primary ITP to reduce clinical heterogeneity, our cohort still included different disease phases, and patients in remission had received varied prior treatments. Transcriptomic patterns may also differ in pediatric or secondary forms of ITP, which were not addressed here. The predefined inclusion criterion of platelet counts >20 × 10^9/L precluded analysis of patients with more severe thrombocytopenia. Independent cohort validation will therefore be needed to confirm and extend these findings.

Within these limitations, this study identifies an ITP-specific platelet transcriptomic signature characterized by enrichment of activation-related pathways and downregulation of mitochondrial gene expression. This profile is consistent with previously described functional characteristics of platelets in ITP, including platelet hyperreactivity [3, 33] and procoagulant platelet phenotypes [41-43]. These features are particularly interesting in the clinical context of ITP, as many patients exhibit only mild bleeding symptoms despite profound thrombocytopenia [44].

Collectively, these findings highlight both the feasibility and interpretational complexity of platelet RNA sequencing, and provide molecular support for the concept that platelets may reflect, and potentially contribute to, the immune and hemostatic landscape of ITP.

FOOTNOTES

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

JAG, MH, and UAF performed data acquisition and analysis. KTG and CT supervised the study. JAG drafted the manuscript. All authors contributed to data interpretation, critically revised the manuscript, and approved the final version.

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Figures

Figure 1

Schematic overview of the platelet RNA-sequencing workflow. Platelet-rich plasma (PRP) was prepared from individuals with active ITP, ITP in remission, chemotherapy (CTx)-induced thrombocytopenia, and healthy controls. After leukocyte depletion, platelet RNA was extracted, quality-controlled, and subjected to rRNA-depletion-based library preparation followed by high-throughput sequencing. Differential gene expression and pathway enrichment analyses were performed.

Figure 2

Differentially expressed platelet transcripts across disease groups. Volcano plots for **(A)** ITP, **(B)** ITP in remission, and **(C)** chemotherapy-induced thrombocytopenia vs healthy donors, and **(D)** chemotherapy-induced thrombocytopenia vs ITP. X-axis: $\log_2$ fold change; y-axis: $-\log_{10}$ p value (y-axis scaling differs between panels). Transcripts with FDR < 5% are shown in orange (upregulated) or blue (downregulated); non-significant transcripts in grey. The number of DEGs per comparison is indicated.

Figure 3

Functional pathway and gene set enrichment analyses of platelet transcripts in ITP. (A) Functional enrichment analysis of differentially expressed platelet transcripts in ITP versus healthy controls. Shown are the top 20 enriched Reactome pathways and Gene Ontology (GO) Biological Process terms. Enrichment direction is indicated in parentheses. Dot size indicates the number of overlapping genes, color denotes statistical significance, and the x-axis shows the combined score. **(B)** KEGG enrichment map of the top significantly enriched KEGG pathways in ITP versus healthy controls **(**all of which were upregulated**).** Edges indicate gene overlap, and node size reflects enrichment significance. **(C)** Gene Set Enrichment Analysis (GSEA) of platelet transcripts in ITP compared with healthy controls. Enrichment plots show pathway-specific enrichment scores across the ranked gene list. Positive enrichment scores indicate enrichment in ITP, whereas negative scores indicate enrichment in healthy controls.

Figure 4

Group-level comparison of platelet activation and mitochondrial gene expression. Box plots of pathway expression scores from the different cohorts showing **(A)** platelet activation signature, based on the KEGG "Platelet activation" (hsa04611) and **(B)** mitochondrial OXPHOS and translation signature, based on KEGG "Oxidative Phosphorylation" (hsa00190) and GO "Mitochondrial Translation" (GO:0032543). Per-sample scores were calculated from DESeq2-normalised counts ($\log_2$-transformed and z-scored per gene). P values from two-sided Mann-Whitney U tests are indicated.