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Platelet Proteomics to Understand the Pathophysiology of Immune Thrombocytopenia: Studies in Mouse Models

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Abstract:
Immune thrombocytopenia (ITP) is an autoimmune disease characterized by enhanced platelet clearance and defective platelet production. Diagnosis by exclusion and "trial-error" treatment strategies are common practice, and despite the advancement in treatment options, many patients remain refractory. While the existence of different pathophysiological entities is acknowledged, we are still far from stratifying and understanding ITP. To deepen into this, we aimed to dissect the platelet proteome dynamics in the so-called passive and active pre-clinical ITP mouse models, which we propose to phenocopy respectively acute/newly diagnosed and persistent/chronic stages of ITP in human. We obtained the platelet proteome at the thrombocytopenic stage and upon platelet count recovery (reached naturally or upon IVIg-treatment, depending on the model). While most of the proteomic alterations were common to both ITP models, there were model-specific protein dynamics, which accompanied and explained alterations in platelet aggregation responses, as measured in the passive ITP model. Interestingly, the expression dynamics observed in Syk, may explain, extrapolated to human and pending validation, the increased bleeding tendency of ITP patients when treated with Fostamatinib as third-or-later-line, as opposed to second-line treatment. We propose that the platelet proteome may give diagnostic/prognostic insights into ITP, and such studies should be pursued in humans.

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Running Title: Platelet proteomics in preclinical ITP models

Key Words: Platelets, Bottom-Up Proteomics, Immune Thrombocytopenia, Pre-clinical Mouse Models

Data sharing statement: Data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD028814. The source code for the proteomics and subsequent WGCNA analyses is available on the GitHub repository: https://github.com/patmartinezb/ITP-mouse-proteomics. Please direct other inquiries to the corresponding author: gutierrezglaura@uniovi.es.

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Abstract

Immune thrombocytopenia (ITP) is an autoimmune disease characterized by enhanced platelet clearance and defective platelet production. Diagnosis by exclusion and “trial-error” treatment strategies are common practice, and despite the advancement in treatment options, many patients remain refractory. While the existence of different pathophysiological entities is acknowledged, we are still far from stratifying and understanding ITP. To deepen into this, we aimed to dissect the platelet proteome dynamics in the so-called passive and active pre-clinical ITP mouse models, which we propose to phenocopy respectively acute/newly diagnosed and persistent/chronic stages of ITP in human. We obtained the platelet proteome at the thrombocytopenic stage and upon platelet count recovery (reached naturally or upon IVIg-treatment, depending on the model). While most of the proteomic alterations were common to both ITP models, there were model-specific protein dynamics, which accompanied and explained alterations in platelet aggregation responses, as measured in the passive ITP model. Interestingly, the expression dynamics observed in Syk, may explain, extrapolated to human and pending validation, the increased bleeding tendency of ITP patients when treated with Fostamatinib as third-or-later-line, as opposed to second-line treatment. We propose that the platelet proteome may give diagnostic/prognostic insights into ITP, and such studies should be pursued in humans.

Key Points:

- The platelet proteome distinguishes platelets from two different pre-clinical ITP mouse models, and may be of use to profile human disease.
- The platelet proteomes suggest a slower turn-over of platelets in chronic ITP and basal degranulation in acute ITP due to hyporesponsiveness.

Introduction

Primary immune thrombocytopenia (ITP) is an autoimmune condition characterized by low platelet counts, due to both platelet impaired production and destruction. The causes behind its onset and progression are not yet fully understood.[1-2] The management of ITP comprises a number of treatment lines (immunosuppressants, thrombopoietin receptor agonists [TPO-RA´s] and splenectomy), which are indicated in a trial-error manner, alone or combined.[3] A deeper insight into the disease at the molecular level may aid in developing better, personalized treatment regimes, and platelet proteomics may provide valuable data.[4,5]

Here, we characterize the platelet proteome in two different ITP (passive and active) mouse models at the thrombocytopenic stage and upon platelet count recovery (reached naturally or upon IVIg-treatment, depending on the model). These two models could phenocopy, as we propose, the acute/newly-diagnosed and persistent/chronic stages of ITP in human, respectively.[6] Our results support the notion that the platelet proteome may be used to distinguish ITP models and thrombocytopenic stages and suggest that the alterations observed in the ITP platelet proteome might reflect potential fine-tuning of megakaryopoiesis and/or platelet priming in the circulation.

Methods

Mice:

Mice were maintained in the animal care facilities of Lund University or the Netherlands Cancer Institute under specific-pathogen-free conditions. All animal experiments were approved by their respective animal ethics committees.

See Supplementary Methods.

Platelet functional assays, protein extraction, mass spectrometry (MS) and data analysis:

See Supplementary Methods. Data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD028814. The source code for the proteomics and subsequent WGCNA analyses is available on the GitHub repository: https://github.com/patmartinezb/ITP-mouse-proteomics.

Results and discussion

We obtained the platelet proteome from two ITP mouse models at the thrombocytopenic and recovered platelet count states to identify distinct protein signatures and dynamics in each model (Figure 1A). We excluded samples with > 35% missing values, as quality control.[7] Interestingly, ITP samples had more missing values, which could be considered as an ITP-specific trait (Figure S1A and Table S1). Principal Component Analysis (PCA) of the proteomes after data adjustment (Figure S1B), separated samples according to disease status (thrombocytopenia or recovery; PC1), and showed that day 3 and 7 controls (C D3 and C D7) clustered together, along with D7 passive ITP (P-ITP D7, recovered) samples (Figure 1B). Therefore, we joined C D3 and C D7 samples as healthy platelet control proteome for further analyses. Thrombocytopenic ITP samples (P-ITP D3 and A-ITP) separated from the rest (PC1), while the A-ITP IVIg samples positioned closer, but not overlapping, to controls or P-ITP D7 samples, suggesting that a normal platelet count may not necessarily reflect a complete proteome restoration after ITP induction (at the time points of study and in the active model). PC2 distinguished both ITP models at the thrombocytopenic state, showing that there are model-specific differences.

From the identified 1866 proteins, 544 were differentially expressed (DEPs) across all comparisons (Table S2). Weighted gene correlation analysis (WGCNA) of DE proteins after dynamic tree cutting identified 10 co-expression clusters, which were reduced to 7 modules after cluster merging (Figure S1C). Cluster profiling of each module showed different dynamics across groups (Figure 1C). The grey module (not depicted) comprises proteins that do not cluster to any other module. Sample clustering in the heatmap of the module eigenproteins (Figure 1D) followed the PCA clustering (Figure 1B), where ITP samples at the thrombocytopenic state separate from controls and platelet-count recovered respective samples, with the particularities mentioned above. The A-ITP IVIg samples, albeit closer to the control samples, have their own unique profile, pointing towards a partial recovery (or a persistent phenotype) of the platelet proteome after IVIg treatment. Furthermore, it showed that the ITP models possess common dynamics between them (black and brown modules), aside from their inherent, model-specific ones (green, pink, magenta and red) (Table S3). These results were substantiated with module-trait correlation analyses performed against the control group (Figure 1E).

Functional and pathway enrichment analyses were performed module- and model-wise (shown in Figures S2-3, and Table S4). Common to both models of ITP, the black

module represented downregulated proteins, involved in receptor signaling (Itgb1, Itgb3, Gp1ba, Gp1bb, Gp9, Clec1b, Lyn, Src, Rac1, Rap1a/b),[8] metabolism (Ndufs, Acads, Aldh2, Ak3),[9,10] and exo/endocytosis (Vamp3, Vps37b, Stx11/12, Snap23).[11] Upregulated proteins (brown module) were structural proteins, proteases (Serpins)[12] or immunomodulators (Orm1, C1ra).[13,14] It appears as if ITP platelets in general lose protein content through basal degranulation or vesiculation, and upregulated proteins are those of the structural components. Of note, upregulated proteins included Histones, which could derive (potentially) from neutrophil extracellular traps (NETs), or from megakaryocytes.[15,16]

Proteins linked to a more severe downregulation in P-ITP D3 samples (red and magenta modules) indicate significant dysfunction in the cytoskeleton compartment (Actb, Myh9, Capza´s, Tubb´s),[17] general metabolism (Pdi´s, Alox12),[18,19] degranulation (PF4, Mmrn1, Emilin1, Nbeal2, vWF),[20] and integrin signaling (Itga2b, Fermt3, Tln1, Mylk; see also Figure 2A).[21] While these proteins are also downregulated in the A-ITP samples, they are markedly affected in the P-ITP D3 samples, suggesting that in chronic ITP there may be an accommodation or adaptation of the platelet phenotype as disease progresses. These results suggest that the level of hyporesponsiveness due to platelet pre-activation might be superior in P-ITP D3 platelets than on those form A-ITP mice.

Proteins linked to a more severe downregulation in A-ITP samples (green and pink modules) showed a strong association with mitochondrial metabolism, impaired in the case of the A-ITP group, but recovered (partially) upon IVIg-treatment. The reduced levels of mitochondrial electron transport chain enzymes may induce overproduction of reactive oxygen species (although there are no evident signs of platelet damage or apoptosis) compromising platelet ATP synthesis,[9] resulting in dysfunctional platelet activation. On the other hand, we cannot discard platelet mitochondria release, which has been reported as a bactericidal tool.[10] Furthermore, strong downregulation of key proteins in the lineage, such as Pear1, Pecam1 or Stim1,[8,22,23] appear unique to the “chronic” ITP state.

Our results suggest that in ITP, platelets may be generally dysfunctional, or hyporesponsive, probably due to the effects of basal activation and degranulation in the circulation. We performed platelet functional studies in P-ITP mice, which allow a better monitorization of the timing of platelet count recovery and measured the platelet aggregation capacity towards 5 different agonists at D4 and D7 after platelet depletion. Interestingly, results revealed a marked defect in the aggregation response upon

Aggretin A stimulation (Clec2-mediated) in P-ITP D4 samples (Figure 2B) which was largely recovered in P-ITP D7 samples. These results are supported by the proteomics dynamics observed, as key players in the Clec2-mediated platelet aggregation, including Clec2 itself, are downregulated at D3 and recovered in P-ITP D7 platelets (Figure 2A). Of note, not all P-ITP D7 mice displayed a balanced platelet aggregation profile at that time point, suggesting that a longer period might be required for full recovery of platelet function (Figure 2B), and that can be seen by the variation in the expression levels of other proteins such as the other hemITAM receptor, GP6, which may affect responses towards other agonists such as collagen or convulxin.

Furthermore, another level to consider is the "age" of the platelets analyzed in each model at the thrombocytopenic or recovered (PLT count) states. The pathological dynamics of platelet clearance and platelet production in ITP may affect their quality and function, as well as their turn-over. Interestingly, the mitochondrial defects we observed in active ITP are comparable to aging platelets.[24] Noteworthy, a slower turn-over of platelets has been reported in ITP patients,[25] which might be the reason behind the proteome differences identified in our active ITP model, characterized, hypothetically, by circulating older and hyporeactive platelets.

A previous proteomics analysis of bone marrow megakaryocytes showed the down-regulation of five apoptosis-related proteins (HSPA6, HSPA8, ITGB3, YWHAH, and PRDX6) in newly diagnosed ITP patients without treatment.[26] Four of those proteins (HSPA6 is not found in mice) are also differentially down-regulated in P-ITP D3, which we propose as equivalent to newly diagnosed ITP. These data support the notion that, in addition to proteomic alterations caused in the circulation, some of the protein dynamics observed may occur at the megakaryocyte level. Another proteomics study achieved similar results as us, but associated the resulting proteins to autophagy.[27]

Interestingly, treatment of ITP patients with Fostamatinib, a Syk inhibitor, is more effective in PLT count recovery and results in less bleeding events when implemented as second-line compared to third-or-later-line of treatment,[28] *i.e.* chronic ITP. We observed downregulation of Syk in P-ITP D3 platelets (*i.e.* acute), and partial restoration in A-ITP (*i.e.* chronic). Extrapolated to human, our results may explain why Fostamatinib is safer in terms of bleeding as second-line treatment: it does not affect platelet functionality through Syk inhibition, because it is already downregulated in newly diagnosed ITP. However, variation in Syk levels in chronic ITP may result in an increased risk of bleeding events, and reduced clinical responses.

Of note, differences related to blood collection methods and anti-coagulation amongst the models, despite stringent data adjustment, may still impact our analysis. Our results have not yet been validated with orthogonal assays or by performing mass spectrometry analysis with independent experimental sets (that could account for the differences mentioned above), which is a limitation to consider. However, our results highlight the potential of applying platelet proteomics to profile human ITP. Validation of our data in humans will broaden the knowledge of the disease and may result in the identification of biomarkers that will improve the tailored patient management.

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Author Contributions: P.M.-B. analyzed data and wrote the manuscript; M.M. and I.M.D.C. performed experiments; J.A.E. provided reagents and critically reviewed the manuscript; J.W.S. performed experiments and critically reviewed the manuscript; L.G. designed experiments, analyzed data and wrote the manuscript.

Disclosure of Conflicts of Interest: The authors declare no conflict of interest.

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FIGURE LEGENDS:

Figure 1. A) Schematic representation of the preclinical mouse models of ITP and their respective study groups, and the proposed parallelism with human ITP. **B)** Principal component analysis of the platelet proteomes (post-adjusted data) from both ITP models, at the thrombocytopenic and recovered normal PLT count state, and controls. **C)** Cluster profile for each expression module, grouped by their common dynamics. Each gray line represents one protein, and the thick colored line represents the average for all. **D)** Eigenprotein heatmap and dendrograms. Eigenproteins for each module are calculated by singular value decomposition and can be seen as linear combinations of the actual module proteins. **E)** Heatmap between the correlation between modules and each of the groups (except the control), where red and blue represent high and low correlations, respectively. Each cell is composed of the correlation coefficient and, in brackets, the corresponding P value. Active ITP samples shared the same most significant modules, green and pink, but on opposite directions, pointing to a recovery of the phenotype upon IVIg treatment. In addition, both ITP groups correlated with the black module, indicating a potential global ITP signature. Distinctively, the day 3 passive ITP group correlated with the magenta and red modules, and to a lesser extent, with the brown module. Lastly, the day 7 passive ITP group was only associated with the grey module, which comprises the set of proteins which have not been clustered in any module. P-ITP, passive ITP; D3, day 3; IV, intravenous; C, controls; PLT, platelets; Ig, immunoglobulin; SCID, severe combined immunodeficiency.

Figure 2. A) Protein expression across ITP preclinical models of selected proteins and key players in hemITAM-receptor (Gp6 and Clec2) signaling are represented. **B)** Flow cytometry based platelet aggregation assays (FCA) were performed with platelets of the P-ITP model, and respective controls, at day 4 and 7 after platelet depletion. We aimed at studying single-receptors by using the following agonists: PMA, botrocetin (Botro), Aggretin A (AggA), collagen (Coll) and convulxin (CVX). The area under the curve of each aggregation reaction was calculated and is plotted after setting the average in control mice to 100, for each condition. The values obtained from platelets from the same mouse are joined by lines. At D4, P-ITP platelets have a quite homogeneous platelet aggregation profile, characterized by impairment towards Agg A stimulation. At D7, platelets from most of the mice show a normal platelet aggregation profile, although not all mice have fully recovered platelets at this time point.

Figure 1

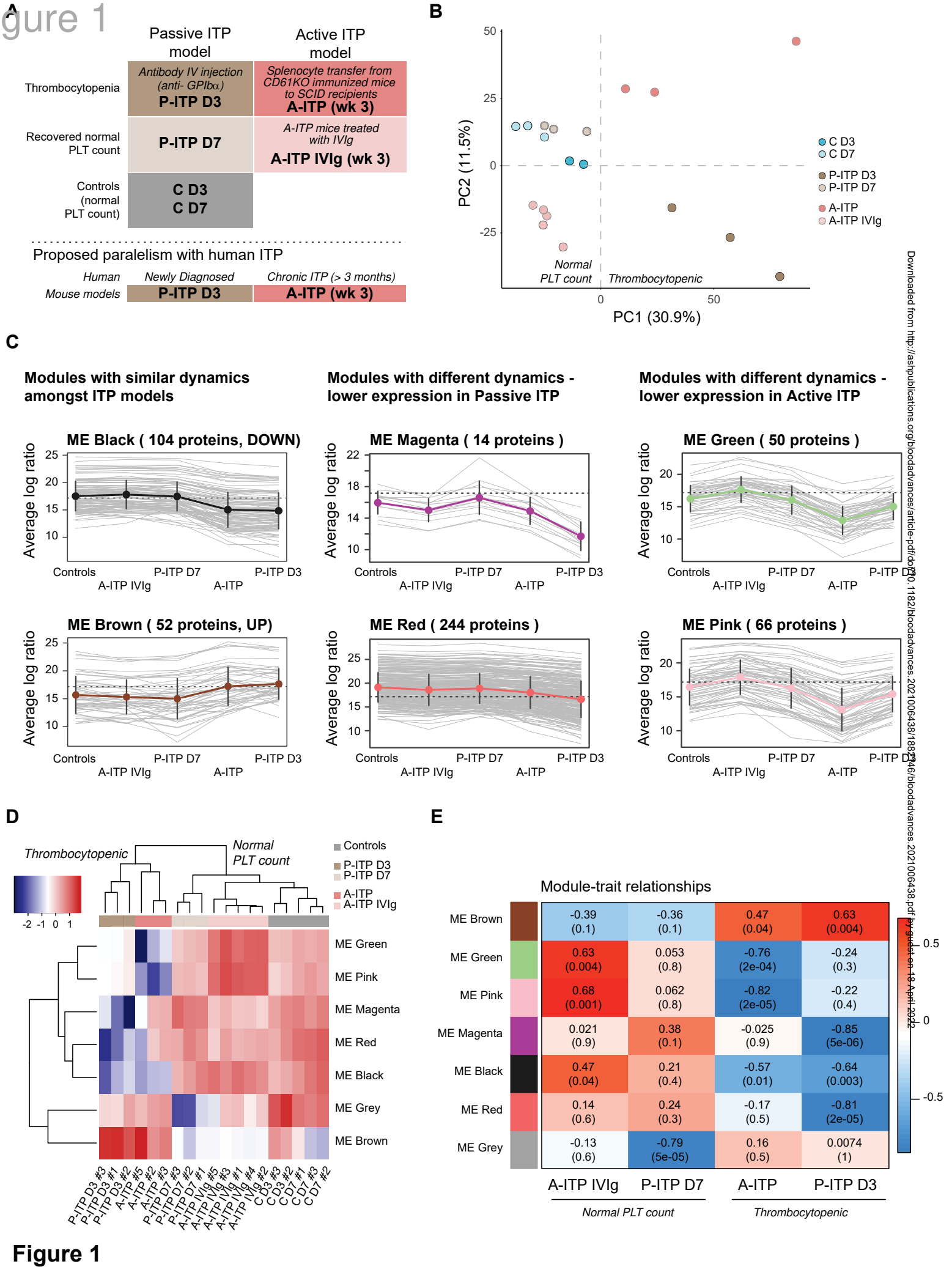

Figure 1

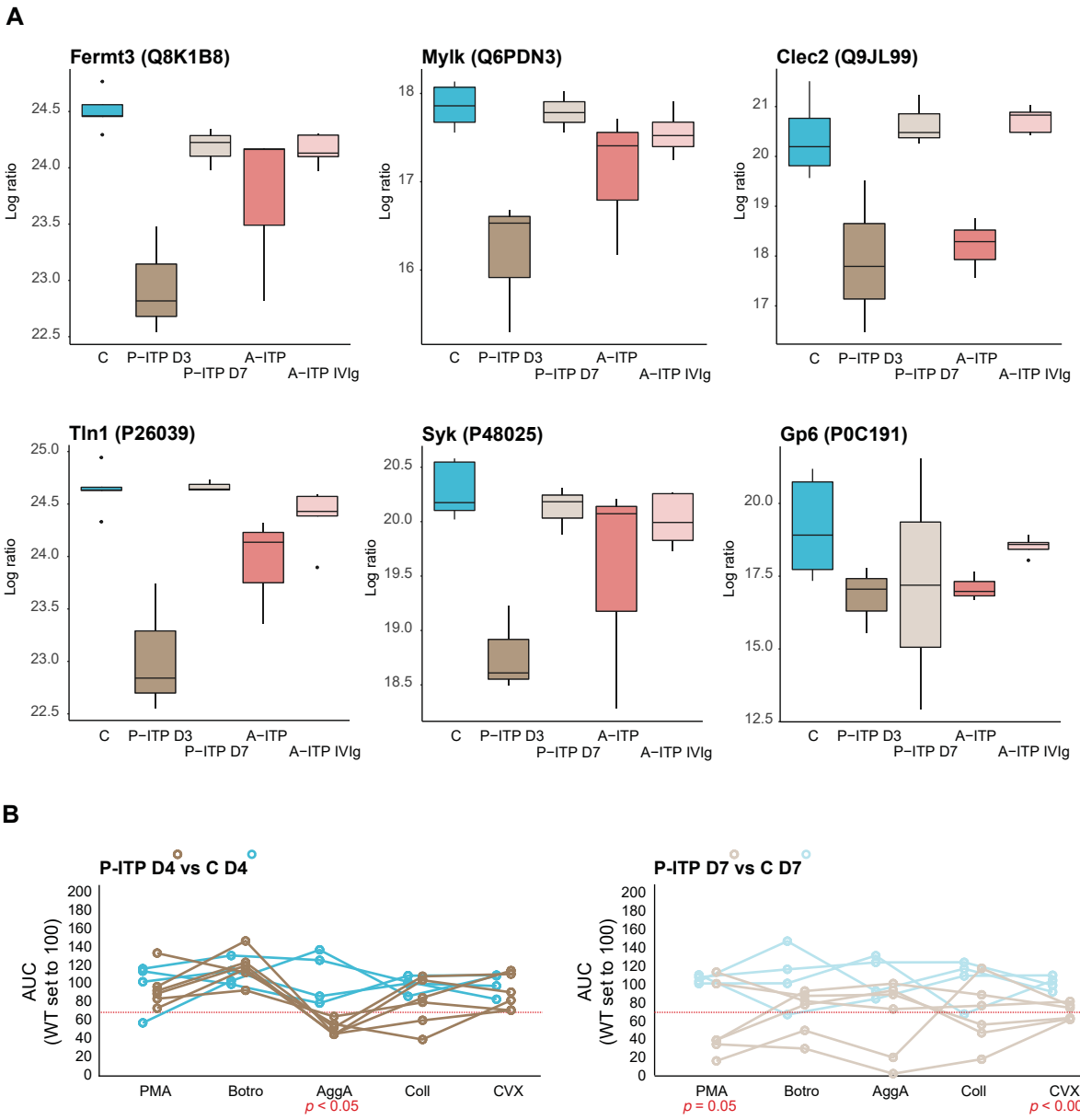

Figure 2