**Supplemental Table 1:** Differentially expressed miRNAs in ITP patients in comparison to controls in the profiling study

|  |  |  |
| --- | --- | --- |
|  miRNAs | Fold change | p-Value |
| hsa-miR-374b-5p | -6,06289 | 0.000005536 |
| hsa-miR-26a-5p |  -4,4328 |  0.00001026 |
| hsa-miR-107 | -2,85058 | 0.000032711 |
| hsa-miR-766-3p |  -3,966 | 0.000035415 |
| hsa-miR-191-5p | -2,74259 | 0.000041586 |
| hsa-miR-339-5p | -3,40254 | 0.000048708 |
| hsa-miR-486-5p | 3,75111 | 0.000049864 |
| hsa-miR-629-5p | 4,10173 | 0.000056974 |
| hsa-miR-223-3p | -1,69245 | 0.000067143 |
| hsa-miR-222-3p | 1,88067 | 0.000073451 |
| hsa-miR-199a-5p | -7,55819 | 0.000078417 |
| hsa-miR-26b-5p | -3,10204 | 0.000091002 |
| hsa-miR-103a-3p | -3,15783 | 0.000124252 |
| hsa-miR-1260a |  2,6978 | 0,000133446 |
| hsa-miR-423-5p | 2,46442 | 0.000175675 |
| hsa-miR-30b-5p | -2,29031 | 0.000226734 |
| mmu-miR-378a-3p |  3,0258 | 0.000238977 |
| hsa-miR-151a-5p | -2,93835 | 0.000322222 |
| hsa-miR-324-5p | -2,45864 | 0.000333085 |
| hsa-miR-331-3p | -2,01781 | 0.000356624 |
| hsa-miR-335-3p | -9,14685 | 0.000506967 |
| hsa-miR-320b | 2,10411 | 0.000579344 |
| hsa-let-7d-5p | -3,35279 | 0.000652767 |
| hsa-miR-18a-5p |  -3,1637 | 0.000813198 |
| hsa-miR-532-3p | 2,81132 | 0.000870833 |
| hsa-miR-320a | 2,06208 | 0.001024857 |
| hsa-miR-142-5p | -2,01616 | 0.001110752 |
| hsa-miR-28-5p | -4,70531 | 0.001201727 |
| hsa-miR-140-3p |  2,50258 |  0.00134344 |
| hsa-miR-199a-3p | -2,25626 | 0.001347707 |
| hsa-miR-15b-5p |  -1,8717 | 0.001381635 |
| hsa-miR-301a-3p | -6,33445 | 0.001503588 |
| hsa-miR-127-3p |  -2,61007 |  0.00157194 |
| hsa-miR-27a-3p | -1,86446 | 0.001767358 |
| hsa-miR-660-5p | 2,48966 | 0.002055184 |
| hsa-let-7b-3p | 2,45133 | 0.002092278 |
| hsa-miR-374a-5p | -2,67267 | 0.002223502 |
| hsa-miR-18b-5p | -2,15927 | 0.002460224 |
| hsa-miR-16-2-3p | 2,30137 | 0.002843992 |
| hsa-miR-92a-3p | 2,07104 | 0.002994123 |
| hsa-miR-22-3p | 2,00495 | 0.003207889 |
| hsa-miR-132-3p | 1,98488 | 0.003214071 |
| hsa-miR-25-3p | 2,01042 | 0,003791702 |
| hsa-miR-27b-3p | -1,64841 |  0.00383061 |
| hsa-miR-365a-3p | 2,82387 | 0.004943076 |
| hsa-miR-23b-3p | -1,69748 | 0.005328816 |
| hsa-let-7f-5p | -2,47636 | 0.006014747 |
| hsa-miR-148a-3p | 2,02716 | 0.006134752 |
| hsa-miR-363-3p |  2,414 | 0.006320249 |
| hsa-miR-590-5p |  1,7768 | 0.008642941 |
| hsa-miR-142-3p | -1,84677 |  0.00992511 |
| hsa-miR-140-5p | -2,18887 | 0.010417673 |
| hsa-let-7d-3p | 1,68012 | 0.012343398 |
| hsa-miR-505-3p | 2,13269 | 0.014067643 |
| hsa-miR-320d | 1,86976 | 0.014757119 |
| hsa-miR-485-3p | -2,23323 | 0.014888664 |
| hsa-miR-151a-3p | -2,08339 | 0.014966233 |
| hsa-miR-92b-3p | 2,25823 | 0.019428664 |
| hsa-miR-532-5p | 1,71203 | 0.019824616 |
| hsa-miR-29a-3p | 1,77724 | 0.020426995 |
| hsa-miR-874-3p | 2,11612 |  0.02081456 |
| hsa-miR-33a-5p | -2,82949 | 0.022277307 |
| hsa-miR-30c-5p |  -1,8947 | 0.023386556 |
| hsa-miR-424-5p | 2,01017 | 0.023642073 |
| hsa-let-7c-5p | -2,07875 | 0.024636688 |
| hsa-miR-193a-5p | 2,34334 |  0.02710825 |
| hsa-miR-223-5p | 1,96272 | 0.031702581 |
| hsa-miR-125b-5p | 1,69747 | 0.032863323 |
| hsa-miR-652-3p | -1,71799 | 0.034648075 |
| hsa-miR-136-3p | -2,48907 |  0.03469106 |
| hsa-miR-376c-3p | -2,68748 | 0.035886611 |
| hsa-miR-543 |  -3,5483 | 0.036610469 |
| hsa-miR-30e-5p | -1,52948 | 0.038205483 |
| hsa-miR-106b-3p | -2,29839 | 0.038751542 |
| hsa-miR-126-3p | -1,48931 | 0.038883884 |
| hsa-miR-221-3p |  -1,811 | 0.039455599 |
| hsa-miR-144-3p | 2,37047 |  0.04207841 |
| hsa-miR-484 | 1,78164 | 0.042240603 |
| hsa-miR-342-3p |  1,6529 | 0.042494778 |
| hsa-miR-502-3p | 2,47358 | 0.042660115 |
| hsa-miR-382-5p | -1,79631 | 0.046612355 |

**Supplemental Table 2**: Top 17 differentially expressed miRNAs in ITP patients in comparison to controls in the profiling study with relative quantification (PCR panel)

|  |
| --- |
| Down-regulated miRNAs  |
|  | **Fold change** | **p-Value** |
| miR-374b-5p | -6.0629 | 0.00000553 |
| miR-26a-5p | -4.4328 | 0.00001026 |
| miR-107 | -2.8506 | 0.00003271 |
| miR-766-3p | -3.9660 | 0.00003541 |
| miR-191-5p | -2.7426 | 0.00004159 |
| miR-339-5p | -3.4025 | 0.00004871 |
| miR-223-3p | -1.6924 | 0.00006714 |
| miR-199a-5p | -7.5582 | 0.00007842 |
| miR-26b-5p | -3.1020 | 0.00009100 |
| miR-103a-3p | -3.1578 | 0.00012425 |
| miR-30b-5p | -2.2903 | 0.00022673 |
| Up-regulated miRNAs |
|  | **Fold change** | **p-Value** |
| miR-486-5p | 3.7511 | 0.00004986 |
| miR-629-5p | 4.1017 | 0.00005697 |
| miR-222-3p | 1.8807 | 0.00007345 |
| miR-1260a | 2.6978 | 0.00013345 |
| miR-423-5p | 2.4644 | 0.00017567 |
| miR-378a-3p | 3.0258 | 0.00023898 |

**Supplemental Table 3**: Pathways overrepresentation analysis in ITP patients *vs*. Controls (miR-199a-5p, miR-33a-5p & miR-195-5p). Pathways are sorted by fold enrichment.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **PANTHER Pathways** | **# Reference genes** | **# Input genes** | **# Genes expected** | **Fold Enrichment** | **rawp- value** | **p-value FDR** |
| **Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade (P00032)** | 33 | 21 | 5.85 | 3.59 | 1.70E-05 | 2.78E-04 |
| **Axon guidance mediated by netrin (P00009)** | 35 | 18 | 6.21 | 2.90 | 6.61E-04 | 5.13E-03 |
| **Insulin/IGF pathway-protein kinase B signaling cascade (P00033)** | 41 | 20 | 7.27 | 2.75 | 4.59E-04 | 3.94E-03 |
| **Hypoxia response via HIF activation (P00030)** | 33 | 16 | 5.85 | 2.73 | 1.92E-03 | 1.30E-02 |
| **Alzheimer disease-amyloid secretase pathway (P00003)** | 69 | 33 | 12.23 | 2.70 | 1.01E-05 | 2.06E-04 |
| **FGF signaling pathway (P00021)** | 123 | 54 | 21.81 | 2.48 | 2.14E-07 | 5.82E-06 |
| **Angiogenesis (P00005)** | 173 | 73 | 30.67 | 2.38 | 4.83E-09 | 2.62E-07 |
| **B cell activation (P00010)** | 69 | 29 | 12.23 | 2.37 | 2.97E-04 | 3.02E-03 |
| **PI3 kinase pathway (P00048)** | 55 | 23 | 9.75 | 2.36 | 1.18E-03 | 8.77E-03 |
| **Gonadotropin-releasing hormone receptor pathway (P06664)** | 237 | 99 | 42.02 | 2.36 | 1.56E-11 | 1.27E-09 |
| **Alzheimer disease-presenilin pathway (P00004)** | 123 | 51 | 21.81 | 2.34 | 1.77E-06 | 4.12E-05 |
| **VEGF signaling pathway (P00056)** | 68 | 28 | 12.06 | 2.32 | 4.55E-04 | 4.12E-03 |
| **Metabotropic glutamate receptor group III pathway (P00039)** | 69 | 28 | 12.23 | 2.29 | 5.00E-04 | 4.08E-03 |
| **Ras Pathway (P04393)** | 74 | 30 | 13.12 | 2.29 | 2.87E-04 | 3.12E-03 |
| **Endothelin signaling pathway (P00019)** | 85 | 34 | 15.07 | 2.26 | 1.50E-04 | 1.89E-03 |
| **CCKR signaling map (P06959)** | 174 | 69 | 30.85 | 2.24 | 1.03E-07 | 3.34E-06 |
| **T cell activation (P00053)** | 91 | 36 | 16.14 | 2.23 | 1.44E-04 | 1.95E-03 |
| **p53 pathway feedback loops 2 (P04398)** | 51 | 20 | 9.04 | 2.21 | 4.15E-03 | 2.60E-02 |
| **EGF receptor signaling pathway (P00018)** | 140 | 53 | 24.82 | 2.14 | 1.02E-05 | 1.84E-04 |
| **Muscarinic acetylcholine receptor 1 and 3 signaling pathway (P00042)** | 61 | 22 | 10.82 | 2.03 | 7.97E-03 | 4.33E-02 |
| **TGF-beta signaling pathway (P00052)** | 97 | 34 | 17.20 | 1.98 | 1.26E-03 | 8.90E-03 |
| **PDGF signaling pathway (P00047)** | 150 | 52 | 26.60 | 1.96 | 6.75E-05 | 1.00E-03 |
| **Wnt signaling pathway (P00057)** | 312 | 106 | 55.32 | 1.92 | 3.65E-08 | 1.49E-06 |
| **Interleukin signaling pathway (P00036)** | 89 | 29 | 15.78 | 1.84 | 6.32E-03 | 3.68E-02 |
| **Cadherin signaling pathway (P00012)** | 158 | 51 | 28.02 | 1.82 | 4.17E-04 | 4.00E-03 |
| **Parkinson disease (P00049)** | 103 | 33 | 18.26 | 1.81 | 5.24E-03 | 3.16E-02 |
| **Apoptosis signaling pathway (P00006)** | 120 | 38 | 21.28 | 1.79 | 3.42E-03 | 2.23E-02 |
| **Integrin signalling pathway (P00034)** | 190 | 60 | 33.69 | 1.78 | 1.74E-04 | 2.03E-03 |
| **Huntington disease (P00029)** | 145 | 42 | 25.71 | 1.63 | 7.26E-03 | 4.08E-02 |

**Supplemental Table 4**: Pathways overrepresentation analysis in ITP patients before and after TPO-RA (miR-92b-3p, miR-221-3p, miR-26a-3p, miR-382-5p, miR-199a-5p & miR-33a-5p). Pathways are sorted by fold enrichment.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **PANTHER Pathways** | **# Reference genes** | **# Input genes** | **# Genes expected** | **Fold Enrichment** | **raw p- value** | **p-value FDR** |
| **Hedgehog signaling pathway (P00025)** | 23 | 11 | 3.91 | 2.82 | 6.74E-03 | 4.23E-02 |
| **Insulin/IGF pathway-protein kinase B signaling cascade (P00033)** | 41 | 19 | 6.96 | 2.73 | 6.61E-04 | 8.98E-03 |
| **Axon guidance mediated by netrin (P00009)** | 35 | 16 | 5.94 | 2.69 | 2.05E-03 | 1.76E-02 |
| **Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade (P00032)** | 33 | 15 | 5.60 | 2.68 | 3.00E-03 | 2.45E-02 |
| **Gonadotropin-releasing hormone receptor pathway (P06664)** | 237 | 97 | 40.24 | 2.41 | 6.33E-12 | 5.16E-10 |
| **p38 MAPK pathway (P05918)** | 42 | 17 | 7.13 | 2.38 | 4.48E-03 | 3.18E-02 |
| **PI3 kinase pathway (P00048)** | 55 | 22 | 9.34 | 2.36 | 1.62E-03 | 1.76E-02 |
| **Ionotropic glutamate receptor pathway (P00037)** | 50 | 20 | 8.49 | 2.36 | 1.99E-03 | 1.80E-02 |
| **Metabotropic glutamate receptor group III pathway (P00039)** | 69 | 27 | 11.72 | 2.30 | 6.48E-04 | 9.60E-03 |
| **CCKR signaling map (P06959)** | 174 | 67 | 29.55 | 2.27 | 9.42E-08 | 5.12E-06 |
| **VEGF signaling pathway (P00056)** | 68 | 25 | 11.55 | 2.17 | 1.69E-03 | 1.72E-02 |
| **Alzheimer disease-presenilin pathway (P00004)** | 123 | 45 | 20.89 | 2.15 | 3.56E-05 | 8.29E-04 |
| **Alzheimer disease-amyloid secretase pathway (P00003)** | 69 | 25 | 11.72 | 2.13 | 1.88E-03 | 1.81E-02 |
| **TGF-beta signaling pathway (P00052)** | 97 | 35 | 16.47 | 2.12 | 2.77E-04 | 4.52E-03 |
| **Muscarinic acetylcholine receptor 2 and 4 signaling pathway (P00043)** | 63 | 22 | 10.70 | 2.06 | 5.14E-03 | 3.49E-02 |
| **B cell activation (P00010)** | 69 | 24 | 11.72 | 2.05 | 4.44E-03 | 3.29E-02 |
| **Muscarinic acetylcholine receptor 1 and 3 signaling pathway (P00042)** | 61 | 21 | 10.36 | 2.03 | 7.16E-03 | 4.33E-02 |
| **Integrin signalling pathway (P00034)** | 190 | 65 | 32.26 | 2.01 | 3.92E-06 | 1.28E-04 |
| **Angiogenesis (P00005)** | 173 | 59 | 29.38 | 2.01 | 1.28E-05 | 3.47E-04 |
| **PDGF signaling pathway (P00047)** | 150 | 49 | 25.47 | 1.92 | 1.58E-04 | 3.21E-03 |
| **Apoptosis signaling pathway (P00006)** | 120 | 39 | 20.38 | 1.91 | 9.20E-04 | 1.07E-02 |
| **Endothelin signaling pathway (P00019)** | 85 | 27 | 14.43 | 1.87 | 6.67E-03 | 4.35E-02 |
| **Cadherin signaling pathway (P00012)** | 158 | 50 | 26.83 | 1.86 | 2.32E-04 | 4.20E-03 |
| **EGF receptor signaling pathway (P00018)** | 140 | 44 | 23.77 | 1.85 | 6.91E-04 | 8.67E-03 |
| **Wnt signaling pathway (P00057)** | 312 | 98 | 52.98 | 1.85 | 4.04E-07 | 1.65E-05 |
| **FGF signaling pathway (P00021)** | 123 | 37 | 20.89 | 1.77 | 3.25E-03 | 2.52E-02 |

Supplementary Methods Droplet digital PCR

Total RNA was extracted from 200 µl plasma using the miRCURY™ RNA isolation Kit for Biofluids (Cat no 300112, Exiqon) according to the manufacturers’ protocol. Synthetic spike- ins were included to monitor RNA extraction efficiency. RNA was eluted in 50 µl nuclease- free water and stored at -80 °C until use.

Following the protocol for microRNA PRC profiling with the QX200™ Droplet Digital ™ PCR system from Exiqon, reverse transcription was performed using the Universal cDNA Synthesis Kit II. The resulting cDNA was diluted 1:50 for all assays before droplet generation and amplification for all miRNA assays. Each PCR assay was mixed in a 20 µl volume containing 10 µl 2X Eva Green supermix (Cat no 1864034; Bio-Rad Laboratories GmbH, Munich, Germany), 8 µl diluted cDNA and 1 µl of one of the miRCURY LAN PCR primer sets (Exiqon) (Supplementary Table 1). Droplets were generated using the QX200™ Automated droplet generator (Bio-Rad), and PCR was performed with the following thermal cycling conditions: 95°C for 5 min, 40 cycles of 95°C for 30 seconds, and 58 °C for 1 min (Ramp-rate 1,6°C/s), followed by three final steps; 4°C for 5 minutes, 90°C for 5 minutes and a 4 °C indefinite hold. Droplets were read in the QX200™ droplet reader (Bio-Rad) and the amount of miRNA (miRNA copies/µl) was generated by the Quanta Soft software (Bio-Rad).

Data analysis

The relative quantitative data generated from the profiling PCR panel were processed and analyzed using GenEX v.6 software (MultiD, Gothenburg, Sweden) according to the “Data Analysis Guide” (version3, Feb 2014). Global mean normalization was used1. Changes in relative quantification of miRNAs using the comparative cycle threshold (Ct) values by qPCR were calculated and expressed as fold-change (FC) between the groups (prior to treatment, 3 time points post initiation of treatment, and control group).

QuantaSoft™ software was used for the analysis of ddPCR data (absolute quantitative data) generated from droplet reader (Bio-Rad). The threshold was manually set between the positive and negative droplet populations, and varied between the different miRNAs. The target miRNA concentrations were calculated using the Poisson statistics. All samples with a concentration higher than the upper 95% Confidence Interval for the merged NTCs were considered positive. The absolute transcript levels were computed in copies/20µL, results presented in this study are copies/µL plasma.

Pathway analysis

The differentially expressed miRNAs identified in the evaluation of ITP patients *vs.* controls, and in the assessment of ITP patients before and after TPO-RA treatment were analyzed using mirDIP, which integrates several miRNA target databases, to determine miRNA target genes with high confidence2. For increased stringency, the analysis was performed by focusing only on top 1% targets, that were further analyzed by Panther (Annotation Version and Release Date: PANTHER version 13.1 Released 2018-02-03) to identify enriched biological pathways. The comparative analysis was performed by a Fisher's Exact Test in combination with a robust False Discovery Rate (FDR) correction for multiple testing.

Statistics

Statistical analysis for the relative quantitative screening data were performed using GenEX

v.6 software (MultiD) and SPSS 23.0 software. T-test was used to assess the differentially expressed miRNAs in ITP patients before TPO-RA treatment compared to matched controls, and paired t-test was used for comparison between two time points (2 weeks, 6 weeks and 12 weeks after treatment) with pre-treatment values as reference level. Repeated measures one- way ANOVA was used to evaluate the significance of the changes in the sequential samples. For the absolute quantitative validation data (non-parametric data), Mann-Whitney test was used to evaluate the differences between ITP patients and controls using SPSS 23.0 software. Friedman test was used to evaluate the significance of the changes before and sequentially after the initiation of treatment and Wilcoxon signed-rank test was run thereafter to find where significant changes were. Spearman correlation and linear regression were used to evaluate the correlation between levels of miRNAs and platelet count using GraphPad prism

* 1. which also was used to create figures and plots.

For identification of potential diagnostic miRNA for ITP, Receiver-operator characteristic (ROC) curves were generated by R (pROC Package)3 using the absolute quantitative levels of the three differentially expressed miRNAs (miR-195a-5p, miR-33a-5p, and miR-199a-5p). Area Under Curve (AUC) was used to determine the sensitivity and specificity of these three individual miRNAs in discriminating ITP patients prior to treatment from healthy controls. Logistic regression analysis was used to find the best combination of miRNAs to generate ROC curves with highest discrimination ability between disease and controls.

Supplementary References

* + 1. Gevaert AB, et al. MicroRNA profiling in plasma samples using qPCR arrays: Recommendations for correct analysis and interpretation. PLoS One. **13(2):** e0193173.
		2. Tokar T, Pastrello C, Rossos AEM et al. mirDIP 4.1-integrative database of human microRNA target predictions. *Nucleic Acids Res* 2018; **4**: 46(D1).
		3. Robin X, Turck N, Hainard A, Natalia, et al. pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinformatics* 2011; **12**: 77.