# Messenger RNA profiling of human platelets by microarray hybridization

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**Summary**

Platelets are generally believed to be inactive in terms of *de novo* protein synthesis. On the other hand, the presence of ribosomes and mRNA molecules is well established. Many studies have used reverse transcriptase (RT) -PCR for detection of gene transcripts in platelets. As RT-PCR is a very sensitive method, any leukocyte contamination of platelet preparations can lead to false results. We performed three filtration procedures to minimize leukocyte contamination of pooled buffy-coat platelet concentrates prior to RNA isolation. Furthermore, by applying a genomic PCR approach with 50 amplification cycles we demonstrated that nucleated cells were not detectable. Microarray hybridization was used to analyze 9,850 individual human genes in RNA from purified platelets. In total we identified 1,526 (15.5%) positive genes. The data were confirmed in six individual experiments each performed on a PC pooled from four individual blood donations. Genes specific for nucleated blood cells such as CD4, CD83 and others were negative and verified the purity of PC. Overrepresentation of positive genes was found in the functional categories of glycoproteins/integrins (22.6% vs. 15.5%, p=0.029) and receptors (20.7% vs. 15.5%, p<0.001). Gene transcripts encoding RANTES, GRO-α, MIP-1α, MIP-1β, and others were found at high levels of signal intensity and confirmed literature data. This work provides a mRNA profile of human platelets and a complete list of results can be downloaded from the website of our institute www.ma.uni-heidelberg.de/inst/iti/plt_array.xls. The knowledge about gene transcripts may have an impact on the characterization of novel proteins and their functions in platelets.

**Keywords**

Gene transcripts; platelet transcriptome; leukocyte depletion

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## Introduction

The main biological functions of blood platelets have been implicated in hemostatic, wound healing and inflammatory processes. The absence of a nucleus in platelets excludes mechanisms regulating gene activity at the level of transcription. The proteins needed for platelet function are preformed in megakaryocytes and stored in α-granules, dense bodies or vesicles of platelets. The release of growth factors, cytokines and other effective molecules is the consequence of platelet activation. If this is true, there is no need for *de novo* protein biosynthesis in platelets and, today, it is generally accepted that platelets do not actively synthesize proteins. On the other hand, many important prerequisites, like ribosomes (1) and mRNA, (2) can be found in platelets and the capacity for *de novo* protein biosynthesis was demonstrated (3, 4) It was shown that the majority of protein synthesis occurs in mitochondria (4). Further evidence was given by Weyrich et al. (5) demonstrating synthesis of Bcl-3 in thrombin-activated platelets. In addition, the presence of specific mRNA and *de novo* synthesis of an HLA membrane protein could be demonstrated (6).

The presence of a variety of mRNA molecules was already shown by different methods, such as Northern blot hybridisation (7, 8) and reverse transcription-PCR (RT-PCR) (9). A platelet-

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specific cDNA library was constructed and clones encoding the platelet specific glycoprotein IIb could be identified (10). The use of RT-PCR particularly increased the knowledge of gene transcripts, such as chemokines and receptors, in platelets (11, 12). The introduction of microarray-based technologies for gene expression analysis enables the simultaneous characterization of large numbers of genes (13, 14).

In the present study we provide a gene expression profile from total RNA isolated from purified platelet concentrates by using microarray hybridization analysis. The commercial glass slide based system used included 9,850 individual human genes.

# Materials and methods

### Blood donors

Standard leukocyte-depleted platelet concentrates (PC) in platelet additive solution (T-Sol®; Baxter, Munich, Germany) were processed after pooling of 4 buffy coats, each derived from 500 mL CPD-stabilized whole blood donations (15). The microarray analysis was performed on 6 individual PC preparations representing 24 individual blood donors, all Caucasians. The 13 male and 11 female blood donors had a mean age of 40 (21 to 60) years. Each pool had matched blood groups concerning the ABO and Rhesus (Rh) D antigens (Table 1).

### Platelet preparation

The standard procedure of PC preparation from 4 pooled buffy coats includes leukocyte reduction using one single filtration step (Autostop BC; Pall, Dreieich, Germany). In a pilot experiment two PC were pooled and 2 further filtration procedures for leukocyte depletion were performed. After each filtration step 100mL PC were collected for RNA/DNA isolation. For microarray hybridization experiments each PC was twice leuko depleted by filtration in addition to one filtration during the process of buffy coat pooling. After filtration the platelets were centrifuged for 15 minutes at 2,000 × g and the pellet was resuspended in 50mL phosphate buffered saline containing 0.5% EDTA (PBS/EDTA). After centrifugation (15min. at 2,000 × g) the platelets were again resuspended in 50mL PBS/EDTA and prior to the final centrifugation, the platelet count was determined by the means of a hematology analyzer (CellDyn 3200; Abbott, Wiesbaden, Germany). Six individual PCs were processed as described and the total numbers of platelets obtained after leukocyte depletion are given in Table 1.

### DNA/RNA extraction

Washed platelets were resuspended in 30mL TriZol® (Invitrogen, Karlsruhe, Germany). Extraction and precipitation of nucleic acids was performed according to the manufacturers' protocol. The procedure is designed to isolate total RNA but genomic DNA is co-purified as well. An aliquot of extracted nucleic acids was kept for PCR amplification of genomic DNA. Most of the DNA co-purified with RNA is of mitochondrial origin, which was further proven by using restriction enzymes. After RNase A (Roche Biochemicals, Mannheim, Germany) treatment and phenol/chloroform extraction, 2 µg DNA was used for *Eco*RI, *Hin*dIII and *Xba*I digestion. Restriction fragments were seperated on 0.8% agarose gels.

For mRNA profiling the mitochondrial DNA content was minimized by treatment of the extracted total RNA with DNase I (Roche Biochemicals) for 30 minutes at 37°C. After phenol/chloroform extraction and ethanol precipitation the RNA pellet was dissolved in RNase-free water and the concentration was estimated from the absorbance at 260 nm. To visualize platelet RNA approximately 2 µg RNA were separated on 1% agarose gels containing 1% formaldehyde.

### PCR amplification of genomic DNA

To estimate the contamination of platelet RNA by genomic DNA from nucleated blood cells we performed PCR amplifica-

| Pool no. | Sex (M/F) | Mean age of donors | ABO/Rhesus D blood group of the pool | Total number of platelets[1] |
|---|---|---|---|---|
| # 1 | 2/2 | 32.8 | A/positive | $1.91 \times 10^{11}$ |
| # 2 | 1/3 | 44.8 | A/positive | $2.18 \times 10^{11}$ |
| # 3 | 4/0 | 44.0 | A/positive | $1.82 \times 10^{11}$ |
| # 4 | 1/3 | 43.8 | O/positive | $2.06 \times 10^{11}$ |
| # 5 | 2/2 | 39.8 | O/positive | $1.77 \times 10^{11}$ |
| # 6 | 3/1 | 35.3 | O/positive | $1.94 \times 10^{11}$ |
| All | 13/11 | 40.0 | | $1.95 \pm 0.15 \times 10^{11}$ |

[1]Total number of platelets after leukocyte depletion is given for each platelet pool and as mean ± SD of all pools in the bottom row.

**Table 1:** Characteristics of the blood donors represented by the pooled platelet concentrates.

tion of a 429 bp fragment of the human growth hormone HGH gene. The PCR was carried out in 20 µl reaction volume containing nucleic acids from $5 \times 10^8$ platelets, 1x PCR buffer (Qiagen, Hilden, Germany), 1 unit Taq DNA polymerase (Qiagen), 200 µM each dNTP (Amersham Pharmacia Biotech, Freiburg, Germany) and 1 µM of each primer (HGH-F: 5'-gccttcccaaccattccctta-3' and HGH-R: 5'-tcacgcatttctgttgtgtttc-3'). The cycling program was: 2 minutes at 95°C followed by 50 cycles with 95 C, 30 sec., 65 C, 30 sec., and 72°C, 30 sec. and was terminated after final extension at 72 °C for 5 minutes. Amplification products were separated on 2% agarose gels, stained with ethidium bromide and visualized under UV-light.

## Microarray hybridization

Approximately 10 µg total platelet RNA was used in each microarray experiment. Incorporation of a fluorescently labeled nucleotide (Cy3-dCTP; Amersham Pharmacia Biotech) was achieved by reverse transcription of platelet RNA to cDNA using the LabelStar Array Kit (Qiagen). After purification and vacuum drying, the Cy3-cDNA was resuspended in 25 µl microarray hybridization buffer (MWG-Biotech; Ebersberg, Germany). Prior to hybridization the cDNA samples were heat denaturated. The human 10K (MWG-Biotech) oligo microarray systems on glass slides were used for mRNA profiling. Hybridization of Cy3-cDNA was performed using cover slips and a hybridization chamber for 16 hours at 42°C in a water bath. After stringent washing of the glass slides according to the manufacturers specifications the hybridization signals were measured using a microarray laser scanner (GMS418; Affymetrix, MWG-Biotech AG, Ebersberg, Germany).

## Microarray data analysis and statistics

The ArrayVision (Imaging Research, Inc., St. Catharines, ON, Canada) software has been used for evaluation and calculation of signal intensities from the raw data images in 16-bit tagged-image-file (TIF) format. The 16-bit format encompasses grey scale values between 1 and 65,536 ($2^0 - 2^{16}$) for each pixel. For each individual gene spot the signal intensity was calculated as mean intensity of all pixels within the defined spot area. Certain spots on the array without any oligonucleotide on the glass surface were used to measure the background signal intensity of the hybridized array. Functional grouping of genes was performed on the basis of the database supplied by the array manufacturer. Computer-assisted evaluation of the raw data provides the mean signal intensity for each individual gene spot and the mean background intensity for each array experiment. The background signal intensities of the hybridized glass slides were in the range of 189 to 467 (297.5 ± 109.5). The signal-to-background (S/B) values were calculated by dividing the signal intensity for each spot with the background value of the array.

For evaluation of hybridization results we defined a negative range based on negative control spots (Arabidopsis-specific oligonucleotide probes) on the arrays. Taking all data from the six experiments together the mean signal intensity values of the 104 negative control oligos were in the range of 892.59 to 2,613.1 (1,513.5 ± 380.44). Thus, the maximum intensity of a negative spot was expected below 3,000 which was defined as the range of negative hybridization signals. Definition of the grey area (3,000 – 4,999) and the positive range (≥5,000) was based on our experience with microarray analysis using the method described above. Characterization of nucleated blood cells and in cells from *in vitro* culture revealed 25 to 35% positive genes (signal intensity ≥5,000) which can be expected from 'normal' and proliferating cells. Thus, for the microarray system used in the present study the defined signal intensity ranges seemed to be reasonable.

For statistical evaluation the mean signal intensity and standard deviation (SD) was calculated for each spot from the values obtained in the six individual experiments. The $X^2$-test was used to estimate the distribution of positive genes from different functional categories vs. the total number of positive genes. P-values $<0.05$ were regarded as significant.

## RT-PCR

Total RNA was isolated from white blood cells (WBC) using RNeasy Kit (Qiagen) and served as a positive control for leukocyte-specific genes. In order to obtain results comparable to microarray analysis, the same RT system (LabelStar; Qiagen) was used to synthesize cDNA from 2 µg total platelet RNA as well as from 2 µg total WBC RNA. Primers for PCR amplification of GAPDH,[16] FVIII,[17] FV,[18] GPVI,[19] and EPOR[20] as well as RANTES, MIP-1α and Mip-1β[21] were published previously. For other genes the primer sequences were as follows: P-selectin-sense (5'-accaacgaggctgagaactg-3'; exon 3) and -antisense (5'-ggaagctgcactgcgagtta-3';exon 5); vWf-sense (5'-tgctgagccttgtggacgtcat-3'; exon 30) and -antisense (5'-tctggctggcaagtcacggtgt-3'; exon 34); multimerin-sense (5'-cgatcgttgcctggtagtct-3'; exon 6) and -antisense (5'-caggataggaccaggtatag-3'; exon 8); CD15-sense (5'-gctgcgaagcctggcaagtaac-3'; exon 1) and -antisense (5'-gcgccagagcttctcggtgata-3'; exon 1); CD45-sense (5'-aagccaggtctggaacatgact-3'; exon 14) and -antisense (5'-caacaagctcctgctgttcatc-3'; exon 17). Each PCR amplification was performed as duplex reaction in a total volume of 20 µl with 0.1 µM each GAPDH primer, 0.5 µM each gene specific primer, 1 µl cDNA, 1 unit Taq DNA polymerase (Qiagen), 200 µM each dNTP and 1x PCR buffer (Qiagen). The cycling conditions were: initial denaturation for 2 minutes at 95 °C, followed by 40 cycles with 30 s at 94°C, 30s at 60°C and 1 minute at 72°C, followed by a final extension step for 5 minutes at 72°C. Amplification products were then separated on 2% agarose gels, stained with ethidium bromide and visualized under UV-light.

# Results

## Estimation of leukocyte contamination by genomic PCR

The standard procedure for the production of platelet concentrates (PC) from pooled buffy coats includes a single filtration for depletion of leukocytes. Using PCR primers for amplification of a genomic fragment of the HGH gene we found remaining leukocytes in the PC after the single filtration (Fig. 1, lane 1). In a pilot experiment additional filtration steps were performed once and twice. Nucleic acids were isolated from $10^{11}$ platelets after each filtration step and the amount of nucleic acids used in each PCR reaction corresponded to $5 \times 10^8$ platelets. We could demonstrate that the additional filtration steps further reduced the number of leukocytes (Fig. 1, lanes 2 and 3). After the three filtration procedures, a leukocyte specific PCR product was absent using 50 amplification cycles. Thus, for isolation and further characterization of nucleic acids from PC we performed a total of three leukocyte filtrations.

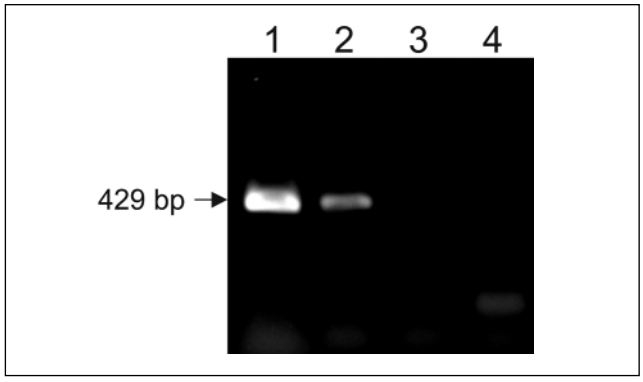


**Figure 1:** Evaluation of leukocyte contamination in buffy coat platelet concentrates by PCR amplification of a genomic part (429 bp in size) of the HGH gene. Nucleic acids were isolated from pooled buffy-coat platelet concentrates after single leukocyte filtration during the standard production process (lane 1). Additional leukocyte filtrations were performed once (lane 2) and twice (lane 3). The PCR with 50 amplification cycles was negative when using water as negative control (lane 4). Each PCR contained nucleic acids isolated from $5 \times 10^8$ platelets.

## Characterization of nucleic acids isolated from purified platelets

Nucleic acids isolated from PC were analyzed on denaturating agarose gels prior and post DNase I treatment (Fig. 2A). Mitochondrial DNA (mtDNA) could be removed by DNase treatment, whereas ribosomal RNA bands were visible in both samples. Further characterization of mtDNA was performed by using different restriction enzymes. The DNA fragments obtained from restriction digests revealed sizes as expected from the mtDNA sequence (Fig. 2B), whereas characteristics of digestion of genomic DNA (diffuse smear) was absent. In accordance with results from genomic PCR we could exclude the presence of genomic DNA in platelet RNA preparations. Thus, each RNA sample used for microarray hybridization experiments was isolated from leukocyte depleted PC and further purified by DNaseI treatment and phenol/chloroform

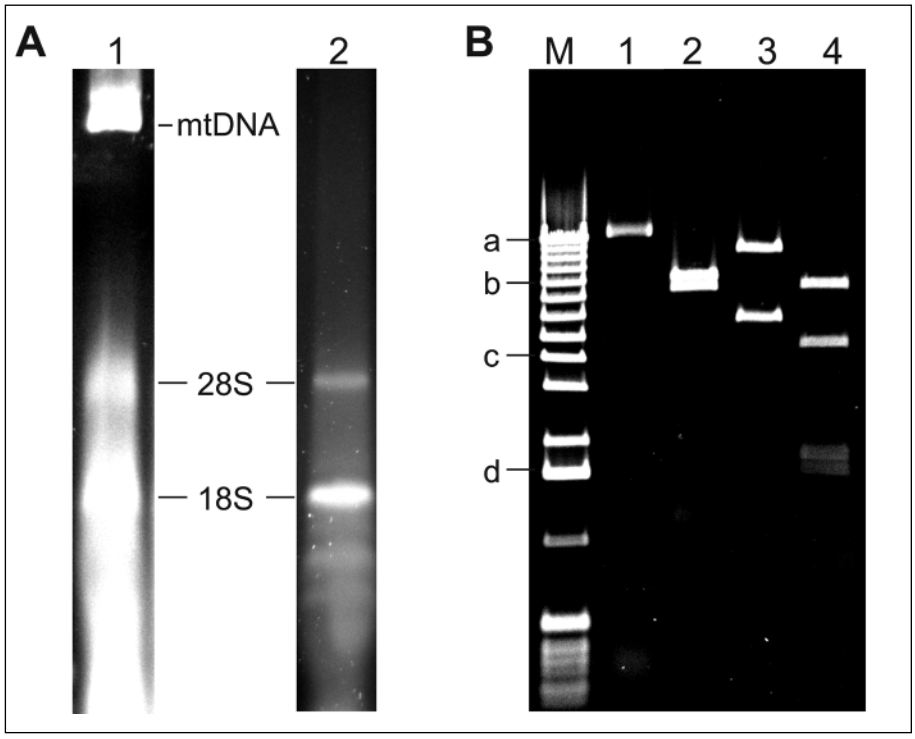


**Figure 2:** Characterization of nucleic acids isolated from platelets. A: Platelet RNA/DNA separated on denaturating agarose gels prior (lane 1) and post (lane 2) DNase I treatment. Mitochondrial DNA (mtDNA) is only visible in lane 1, whereas the bands representing the 28S and 18S ribosomal RNA can be seen in both lanes. B: Further characterization of the large DNA band (lane 1, undigested) by using restriction enzymes *Eco*RI (lane 2), *Hin*dIII (lane 3) and *Xba*I (lane 4). Marker (M) bands a, b, c and d correspond to 12,216, 8,144, 4,072 and 1,636 bp in size, respectively.

extraction. The quantity of RNA was estimated from the absorbance at 260 nm in all six RNA preparations. From $2 \times 10^{11}$ platelets we were able to isolate a mean quantity of 33.6 ± 4.4 µg total RNA (min. 28.3, max. 39.8).

## Microarray hybridization analysis of platelet RNA

The commercial microarray used in this experiment consists of specific 50mer oligonucleotide probes for 9.850 individual human genes. Different house keeping genes (glyceraldehyde-3-phosphate dehydroganase - GAPDH, β-actin, α-tubulin) served as positive controls. The microarray hybridizations were performed on six different RNA samples isolated from six pooled buffy-coat PC representing 24 individual blood donations. Based on the range definitions and excluding signals from the positive control genes we found 1,526 (15.5%) positive hybridization signals: 1,230 (12.5%) signals within the defined grey area and 7,094 (72.0%) negative hybridization signals (Fig. 3). The identified gene transcripts were grouped into different categories based on the knowledge of their function (Table 2). Most of the categories, like cytokines/growth factors, ribosomes/translation, kinases, phosphatases or transcription factors, reflected the number of positive genes as expected from all genes analyzed. The number of positive glycoprotein/integrin and receptor genes was higher compared to all positive genes: 22.6% vs. 15.5% (p=0.029) and 20.7% vs. 15.5% (p<0.001), respectively. Some examples of genes from different functional categories are shown in Table 3. A complete list of the 9,850 analyzed genes including all signal intensity and signal-to-background values can be downloaded from the website of our institute www.ma.uni-heidelberg.de/inst/iti/plt_array.xls.

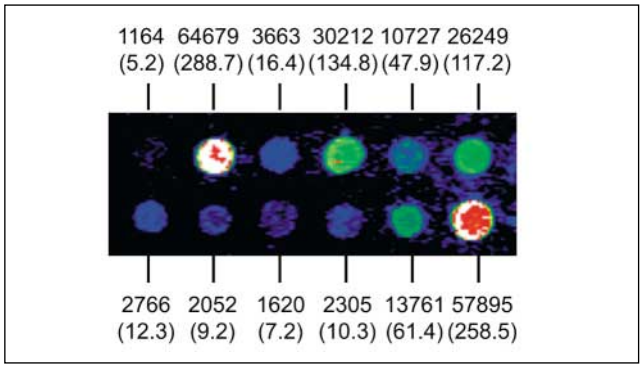


**Figure 3:** Representative hybridization signals from microarray analysis in pseudocolor formate. Mean signal intensity and signal-to-background (S/B) ratio is given for each individual spot. The signal intensity was calculated as mean intensity of all pixels within the defined spot area. S/B ratios (numbers in brackets) were calculated by dividing the signal intensity for each spot with the background value of the array.

**Table 2:** Results of microarray analysis of platelet RNA. Genes were grouped into different categories based on the knowledge of their function.

| Gene category | analyzed | Number of genes + | ± | - | % Positive genes | p-value[1] |
|---|---|---|---|---|---|---|
| Cytokines, growth factors | 66 | 11 | 11 | 44 | 16.7 | 0.792 |
| Interleukins | 22 | 0 | 2 | 20 | 0 | — |
| Glycoproteins, integrins | 62 | 14 | 10 | 38 | 22.6 | **0.029** |
| Receptors | 653 | 135 | 82 | 436 | 20.7 | **<0.001** |
| Ribosomes, translation | 166 | 23 | 16 | 127 | 13.8 | 0.560 |
| Kinases | 369 | 59 | 50 | 260 | 15.9 | 0.792 |
| Phosphatases | 137 | 19 | 14 | 104 | 13.9 | 0.599 |
| Transcription factors | 170 | 25 | 13 | 132 | 14.7 | 0.777 |
| All[2] | 9,850 | 1,526 | 1,230 | 7,094 | 15.5 | — |

[1] $X^2$-test; number of positive (+) genes vs. indifferent (±) and negative (-) genes in a functional category compared to the numbers of all genes analyzed; p-values <0.05 were significant (bold numbers).

[2] all genes analyzed excluding house keeping genes GAPDH, ß-actin and α-tubulin.

**Table 3:** Selected list of gene transcripts from different functional categories found in human blood platelets.

| Gene symbol | Gene description | Mean signal intensity ± SD* |
|---|---|---|
| Cytokines, chemokines, growth factors and receptors | | |
| SCYA5 | Small inducible cytokine A5 (RANTES) | **17,321.8 ± 15,650.8** |
| SCYA4 | Small inducible cytokine A4 (MIP-1ß) | **13,025.5 ± 2,449.9** |
| SCYA3 | Small inducible cytokine A3 (MIP-1α) | **10,963.1 ± 4,924.4** |
| SCYB6 | Small inducible cytokine B6 (granulocyte chemotactic protein 2) | **5,718.2 ± 6,487.7** |
| SCYB7 | Small inducible cytokine B7 (platelet basic protein) | **5,430.2 ± 2,631.4** |
| SCYA7 | Small inducible cytokine A7 (MCP-3) | 2,164.5 ± 1,122.5 |
| IL-17 | Interleukin 17 | *4,882.5 ± 2,536.1* |
| IL-12B | Interleukin 12b | *3,452.3 ± 853.4* |
| IL-1A | Interleukin 1α | 1,780.4 ± 362.8 |
| IL-1B | Interleukin 1ß | 1,509.4 ± 455.8 |
| IL-8 | Interleukin 8 | 1,305.5 ± 613.7 |
| PF4 | Platelet factor 4 | **33,272.8 ± 12,428.8** |
| GRO-α | Growth regulating oncogene α | **25,119.0 ± 5,626.1** |
| PDGFB | Platelet-derived growth factor ß | 1,698.7 ± 472.2 |
| TGFB1 | Transforming growth factor ß-1 | *4,257.0 ± 3,835.8* |
| TGFB2 | Transforming growth factor ß-2 | *3,100.5 ± 1,240.1* |
| TGFA | Transforming growth factor α | 2,452.1 ± 380.3 |
| VEGFB | Vascular endothelial growth factor B | **31,216.5 ± 18,178.1** |
| VEGF | Vascular endothelial growth factor | 1,325.1 ± 375.9 |
| FGF23 | Fibroblast growth factor 23 | **23,874.7 ± 11,281.7** |
| ECGF1 | Platelet-derived endothelial cell growth factor 1 | **19,008.8 ± 9,459.8** |
| IRF3 | Interferon regulatory factor 3 | **23,288.5 ± 20,730.6** |
| IRF1 | Interferon regulatory factor 1 | 2,094.1 ± 640.1 |
| CCR5 | Chemokine (C-C motif) receptor 5 | **13,912.3 ± 4,008.2** |
| IL-11RA | Interleukin 11 receptor α | **15,561.8 ± 7,256.6** |
| IL-10RA | Interleukin 10 receptor α | **7,328.6 ± 3,620.0** |
| IL-10RB | Interleukin 10 receptor ß | **5,149.5 ± 2,976.1** |
| HM74 | Putative chemokine receptor and GTP-binding protein | **11,968.5 ± 6,396.8** |
| EGFR | Epidermal growth factor receptor | **14,233.4 ± 11,805.4** |
| PDGFRB | Platelet-derived growth factor receptor ß | **5,986.2 ± 3,043.1** |
| PDGFRA | Platelet-derived growth factor receptor α | *3,553.7 ± 1,756.3* |
| Hemostasis, glycoproteins, integrins and selectins | | |
| F8B | Coagulation factor VIII, isoform B | **12,953.0 ± 5,950.0** |
| F8A | Coagulation factor VIII, isoform A | 1,599.2 ± 578.3 |
| F5 | Coagulation factor V | 2,153.7 ± 789.4 |
| GP1BB | Glycoprotein Ib, ß-polypeptide | **24,995.7 ± 20,525.5** |

**Table 3:** Continued

| Gene symbol | Gene description | Mean signal intensity ± SD* |
|---|---|---|
| ITGA2B | Integrin α 2b, platelet glycoprotein IIb (CD41B) | **8,887.5 ± 4,945.8** |
| ITGB3 | Integrin ß 3, platelet glycoprotein IIIa (CD61) | **6,036.0 ± 1,636.5** |
| ITGB1 | Integrin ß 1 (CD29) | *3,670.0 ± 1,296.6* |
| GP5 | Glycoprotein V | *3,103.7 ± 1,472.0* |
| GP6 | Glycoprotein VI | 1,564.4 ± 537.8 |
| CLU | Clusterin, complement lysis inhibitor, sulfated glycoprotein 2 | **20,553.2 ± 21,511.8** |
| DNAM1 | Adhesion glycoprotein 1 | **12,652.5 ± 6,610.3** |
| SELL | L-selectin (CD62L) | 1,881.4 ± 517.2 |
| SELP | P-selectin (CD62P) | 1,692.6 ± 526.8 |
| MMRN | Prepromultimerin | 1,235.9 ± 351.1 |
| Protein biosynthesis, translation and ribosomal factors | | |
| EIF2B2 | Eukaryotic translation initiation factor 2b, ß-subunit | **11,349.2 ± 6,321.3** |
| EIF4E | Eukaryotic translation initiation factor 4E | **5,748.9 ± 1,604.0** |
| EIF4EBP1 | Eukaryotic translation initiation factor 4E binding protein 1 | 1,888.0 ± 411.8 |
| EIF4EBP2 | Eukaryotic translation initiation factor 4E binding protein 2 | *4,003.0 ± 784.4* |
| MRPS11 | Mitochondrial ribosomal protein S11 | **28,204.1 ± 8,695.3** |
| RPL28 | Ribosomal protein L28 | **15,408.0 ± 10,024.5** |
| RPL12 | Ribosomal protein L12 | **11,340.9 ± 6,636.9** |
| Blood group antigens, RBC genes | | |
| ABO | N-acetyl-galactosaminyltransferase, ABO blood group A antigen | **12,367.7 ± 11,526.6** |
| FUT3 | Fucosyltransferase 3, Lewis blood group antigen | **16,839.1 ± 9,829.5** |
| EPB3 | Erythrocyte membrane protein band 3, diego blood group antigen | **5,023.6 ± 1,511.1** |
| KEL | Kell blood group antigen | *3,393.5 ± 1,267.6* |
| FY | Duffy blood group antigen | 2,342.5 ± 841.2 |
| RHD | Rhesus blood group D antigen | 1,495.6 ± 601.8 |
| FTH1 | Ferritin, heavy chain 1 | **51,407.4 ± 24,372.5** |
| FTL | Ferritin, light chain | **19,231.1 ± 16,779.6** |
| HBD | hemoglobin delta | **11,824.6 ± 14,529.4** |
| EPOR | Erythropoietin receptor | **15,430.3 ± 9,390.0** |
| EPO | Erythropoietin | *3,732.1 ± 1,742.6* |
| GYPA | Glycophorin A | 2,711.0 ± 1,011.2 |
| EPB49 | Erythrocyte membrane protein band 4.9 | 1,858.2 ± 1,327.1 |
| EPB72 | Erythrocyte membrane protein band 7.2 | 1,615.9 ± 525.3 |

*mean signal intensity and standard deviation (SD) calculated from 6 individual experiments; bold numbers indicate values in the positive range, italic numbers represent indifferent values, and negative values are given as plain numbers.

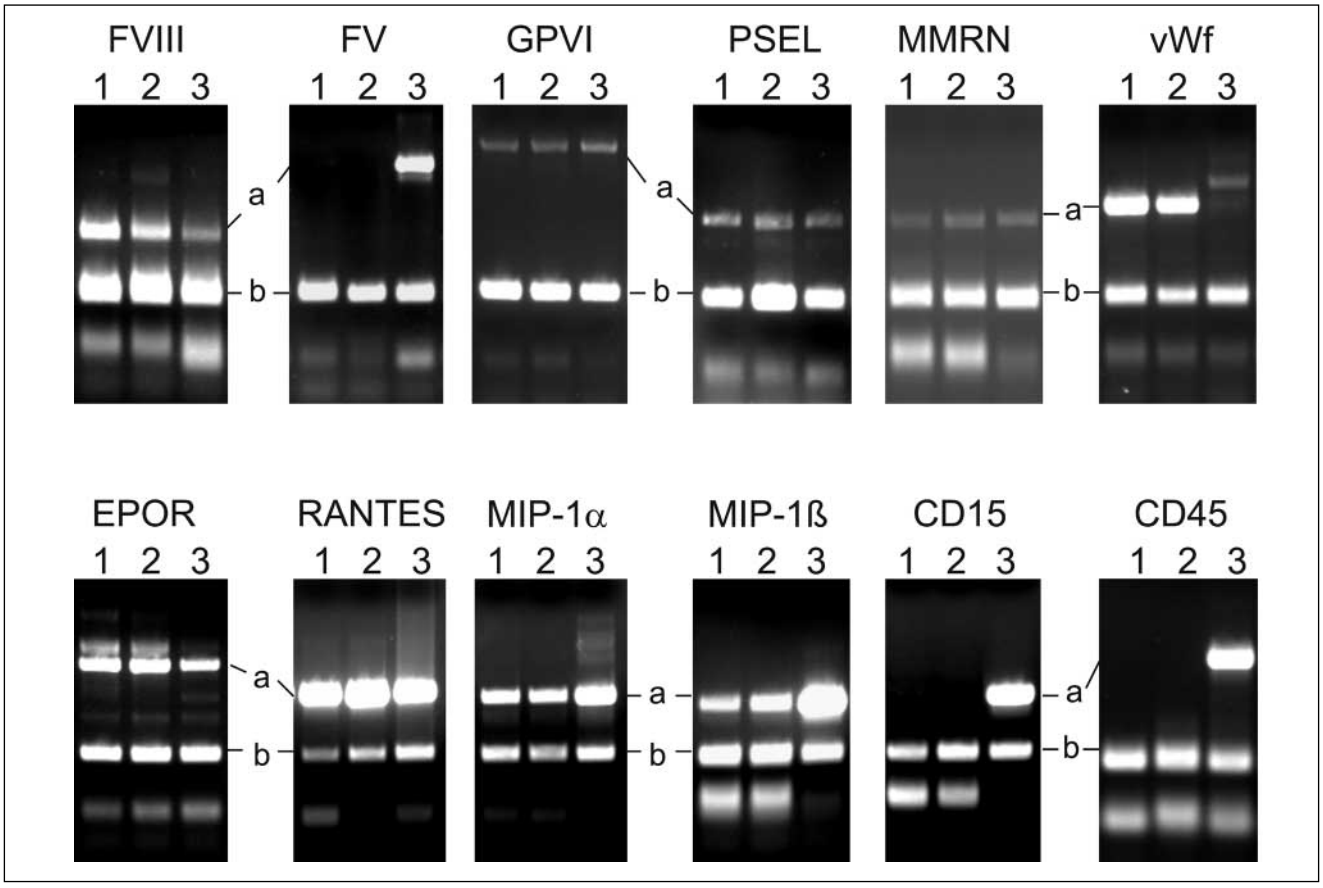


**Figure 4:** Representative results of RT-PCR analysis of selected genes to confirm microarray hybridization data. Total RNA from platelet pools #2 (each lane 1) and #4 (each lane 2) as well as from WBC (each lane 3) was reverse transcribed to cDNA and subsequently PCR amplified by using primers specific for the factor VIII (FVIII; 330 bp), factor V (FV; 777 bp), glycoprotein VI (GPVI; 830 bp), P-selectin (Psel; 355 bp), multimerin (MMRN; 393 bp), von Willebrand factor (vWf; 451 bp), erythropoietin receptor (EPOR; 485 bp), RANTES (352 bp), MIP-1α (333 bp), MIP-1β (310 bp), CD15 (370 bp) and CD45 (404 bp). Bands a correspond to the gene specific amplification products of different sizes, whereas band b indicates the 170 bp amplification product of the internal control (GAPDH).

Regarding platelet-derived, small inducible cytokines we found positive hybridization signals for the RANTES, platelet basic protein, MIP-1α and MIP-1ß gene transcripts. All interleukins (IL) analyzed were negative except IL-17 and IL-12b, with a weak signal intensity of 4,882.5 ± 2,536.1 and 3,452.3 ± 853.4, respectively. Interestingly, many genes for IL receptors were positive, like IL-10 or IL-11 receptors. Gene transcripts were also identified for the vascular endothelial growth factor B (VEGF-B) but not for VEGF. The mRNA coding for platelet factor 4 (PF4) and growth regulating oncogene α (GRO-α) could also be detected. Several glycoproteins that are known to be characteristic for platelets, like GPIbβ, GPIIb or GPIIIa, were found positive on the mRNA level, whereas GPVI was negative. The selectins (P-, L- and E-selectin) were negative as well. The platelet mRNA profile revealed a number of genes for ribosomal proteins and for translation factors which can be regarded as prerequisites of protein biosynthesis.

Genes encoding blood group antigens, such as Rhesus D, Kell and Duffy, were negative in all experiments. However, the galactosaminyltransferase of the ABO blood group and the fucosyltransferase 3 of the Lewis blood group revealed positive signals. Interestingly, we could identify gene transcripts encoding ferritin heavy and light chains and the erythropoietin receptor (EPOR) which are believed to be expressed predominantly in red blood cell (RBC). Most other RBC specific genes, such as glycophorin A, erythrocyte membrane protein bands 4.1, 4.9 and 7.2, were negative.

## Confirmation of array data by RT-PCR analysis

The microarray hybridization analysis indicated the presence of gene transcripts, such as coagulation factor VIII (FVIII) or EPOR, which have not yet been shown to be expressed in human megakaryocytes and platelets. Other genes, such as P-selectin, GPVI, and multimerin revealed signal intensities in the negative range but were expected to be positive. In order to confirm unexpected negative results and to reevaluate the array data we performed RT-PCR experiments on the same RNA sam-

ples used for the microarray hybridization. The von Willebrand factor (vWf) gene is regarded as a platelet-specific marker but was not represented on the microarray. The vWf gene was analyzed by RT-PCR and revealed high abundance of the mRNA in platelet RNA samples but not in RNA from white blood cells (WBC) (Fig. 4). The presence of FVIII and EPOR mRNA in platelets could be confirmed as well as the absence of FV mRNA. Investigation of the GPVI, P-selectin and multimerin genes revealed weak PCR bands of expected sizes after 40 amplification cycles. Those genes were negative in the array hybridization with signal intensities of 1,564.4, 1,692.6 and 1,235.9, respectively. RANTES, MIP-1α and MIP-1β were strongly positive in the microarray experiments which could be confirmed by RT-PCR. As expected, the leukocyte specific genes CD15 and CD45 were negative in platelet RNA but positive in the RNA sample from WBC.

## Discussion

This work provides a mRNA profile of human platelets obtained by microarray hybridization analysis in six individual experiments. We analyzed 9,850 human genes and repeatedly identified 1,526 (15.5%) positive transcripts. Genes grouped into different functional categories showed a similar percentage of positive genes (13.8 – 16.7%) apart from the glycoproteins/integrins (22.6%) and receptors (20.7%) which revealed higher numbers. The commercial microarray system was applied in other studies to characterize gene expression in nucleated blood cells and in cells from *in vitro* culture. The number of positive genes (signal intensity ≥5,000) in nucleated blood cells was ~25 to 28%; proliferating cells in culture revealed ~32 to 37% positive genes. Interestingly, more than 58% of genes in the protein biosynthesis category (ribosomal proteins and translation factors) can be found positive in nucleated cells compared to only 13.8% in platelets. This may reflect the limited capacity for *de novo* protein biosynthesis of platelets.

Numerous gene transcripts, including IL-1β and IL-10, were identified in an arrayed cDNA library with 588 cDNAs (22). In our microarray hybridization experiments we were not able to identify IL-1ß or IL-10 transcripts. Only weak hybridization signals were found for IL-17 and IL-12b. The absence of IL-1β, IL-2, IL-6 and IL-8 mRNA in leukocyte depleted platelet concentrates was shown by RT-PCR analyses (16, 23). The discrepancies may reflect the different procedures of platelet purification. Many published data were obtained from platelets prepared by centrifugation and washing. Counting the cells revealed values of 1 leukocyte in approximately 3,000 to 50,0000 platelets (5, 6, 9, 10, 24). Based on these counts, one can expect 20,000 to 330,000 leukocytes in $10^9$ platelets used for RNA isolation and subsequent RT-PCR. We performed three filtration procedures for leukocyte depletion and by applying a genomic PCR approach with 50 amplification cycles we could demonstrate that leukocytes were removed efficiently. Christensen et al. (16) and Hartwig et al. (23) already pointed out the importance of leukocyte depletion, especially for the detection of platelet mRNA by PCR. Taking into account that the amount of total RNA in platelets is much lower compared to nucleated cells, the removal of leukocytes prior to platelet RNA isolation is essential. Residual contamination by leukocytes can not be excluded but regarding the mRNA data we presume that the contamination is not reasonable since leukocyte specific genes such as CD4, CD15, CD45, CD83 and others were negative. Based on the microarray data a contamination with RNA from red blood cells (RBC) is also unlikely since most RBC specific genes, such as glycophorin A, erythroid membrane proteins (bands 4.9, 7.2 and others) and blood group antigens (Rhesus D, Kell, Duffy and others), were negative or in the grey area range. The absence of these blood group antigens has been shown for human platelets (25). In contrast, expression of ABO and Lewis blood group antigens on platelets is well known (26, 27). Our microarray data confirmed the presence of gene transcripts encoding the corresponding N-acetyl-galactosaminyltransferase and the fucosyltransferase. Interestingly, the erythropoietin receptor (EPOR) and the δ-hemoglobin genes were strongly positive at the mRNA level. These proteins have not been demonstrated in human platelets so far.

The microarray results confirmed published data about the presence of various gene transcripts in platelets such as amyloid precursor proteins (APP), (28) osteonectin (29) and clusterin (30). In contrast to published data about positive detection of mRNA for VEGF (vascular endothelial growth factor) and VEGF-C (31), we could not find the gene transcripts in our experiments. However, the VEGF-B gene revealed strong hybridization signals. Coagulation factor XI mRNA was PCR amplified and characterized from platelets (24). The gene proved to be negative in our experiments. A number of platelet-specific genes such as GPVI, P-selectin, multimerin and FV were expected to be positive at the mRNA level. Microarray analysis though revealed negative results for the genes. Using 40 cycles of RT-PCR amplification we could identify gene transcripts for GPVI, P-selectin and multimerin. Obviously, the hybridization-based microarray technology showed a lower sensitivity than the technique based on enzymatic amplification. Concerning the FV gene RT-PCR, analysis confirmed the negative microarray result. It is an ongoing controversy whether synthesis or uptake of FV in human megakaryocytes is the major mechanism (32). Our data tends to support the proposed uptake of FV in megakaryocytes and platelets. On the other hand, the mRNA may be degraded after the protein has been synthesized in megakaryocytes and stored in α-granules of platelets. Multimerin is also synthesized in megakaryocytes and stored in α-granules of platelets (33). From the negative microarray results we would assume that the gene transcript is degraded in the megakarycytes after the protein was synthesized. Platelet-

specific genes with high signal intensity values in the microarray experiments such as RANTES, MIP-1α and MIP1β also showed strong PCR signals. We conclude that the genes indentified in the positive range of microarray results are reasonably expressed in platelets and megakaryocytes. However, negative microarray results may reflect low amounts of mRNA as seen in GPVI, P-selectin and multimerin or really absent mRNA, as seen in the FV gene. Discrepancies between published data and our microarray data may also reflect the variable stability of mRNA. The platelet concentrates used in our experiments were processed one day after the whole blood donation. In particular, unstable mRNA molecules may be degraded during that time period.

The gene expression profile obtained from platelets has an impact on different research fields and might give new insights into platelet functions, i.e., the identification of FVIII mRNA by microarray hybridization and RT-PCR may provide evidence for megakaryocytes and platelets as a source for FVIII (34). Interestingly, the interferon regulatory factor 3 (IRF3) gene was positive, whereas IRF1 was negative. Both genes are activators of genes with an ISRE (interferon-stimulated response element) in their promoter (35). IRF3 but not IRF1 is part of a transactivator complex that is induced in response to DNA damage and CMV infection (36, 37). The RANTES, arginase type II, and other genes are activated by IRF3 (38). In our platelet microarray data, RANTES and arginase type II were positive. Thus we assume that IRF3 is expressed in megakaryocytes and is involved in transactivation of a number of genes.

In conclusion, the mRNA profile provides important information about 1) gene activities in megakaryocytes and platelets, 2) novel proteins (232 hypothetical proteins with unknown function were positive in the mRNA profile) and 3) proteins with known function which were not described for megakaryocytes and platelets so far. The question as to whether mRNA is capable for *de novo* protein synthesis in platelets can not be answered by gene expression profiling and has to be studied at the protein level. A number of studies gave evidence for *de novo* protein synthesis in platelets, e.g. when thrombin-activation leads to Bcl-3 synthesis (5). The signal transduction pathway regulating protein synthesis was partly identified and included integrin $\alpha_{IIb}\beta_3$, translation initiation factors (eIF-4E and eIF2α) as well as the inhibitory protein 4E-BP1 (39, 40). Interestingly, we could identify gene transcripts for Bcl-3 and a number of translation initiation factors, including eIF-4E and eIF2β. The mRNA encoding 4E-BP1 was not detectable in our study. Again, leukocyte contamination of platelet concentrates used in protein biosynthesis studies is problematic and should be considered for future studies.

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