Toward Clinical Application of Leukocyte Counts Based on Targeted DNA Methylation Analysis

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BACKGROUND: Differential leukocyte counts are usually measured based on cellular morphology or surface marker expression. It has recently been shown that leukocyte counts can also be determined by cell-type–specific DNA methylation (DNAm). Such epigenetic leukocyte counting is applicable to small blood volumes and even frozen material, but for clinical translation, the method needs to be further refined and validated.

METHODS: We further optimized and validated targeted DNAm assays for leukocyte deconvolution using 332 venous and 122 capillary blood samples from healthy donors. In addition, we tested 36 samples from ring trials and venous blood from 266 patients diagnosed with different hematological diseases. Deconvolution of cell types was determined with various models using DNAm values obtained by pyrosequencing or digital droplet PCR (ddPCR).

RESULTS: Relative leukocyte quantification correlated with conventional blood counts for granulocytes, lymphocytes, B cells, T cells (CD4 or CD8), natural killer cells, and monocytes with pyrosequencing (r = 0.84; r = 0.82; r = 0.58; r = 0.50; r = 0.70; r = 0.61; and r = 0.59, respectively) and ddPCR measurements (r = 0.65; r = 0.79; r = 0.56; r = 0.57; r = 0.75; r = 0.49; and r = 0.46, respectively). In some patients, particularly with hematopoietic malignancies, we observed outliers in epigenetic leukocyte counts, which could be discerned if relative proportions of leukocyte subsets did not sum up to 100%. Furthermore, absolute quantification was obtained by spiking blood samples with a reference plasmid of known copy number.

CONCLUSIONS: Targeted DNAm analysis by pyrosequencing or ddPCR is a valid alternative to quantify leukocyte subsets, but some assays require further optimization.

Introduction

Leukocyte subsets are usually quantified with automated cell counting devices and—particularly for stratification of lymphocyte subsets—with flow cytometry (1, 2). Despite broad application, conventional methods have several limitations. First, the blood sample must be fresh and analyzed within 24 h after collection (3, 4). Second, particularly for multicolor flow cytometry, the required blood volume usually exceeds the volume that can be obtained by a finger prick (5). Third, white blood counts can be hampered by coagulation or ineffective antibody binding (6). Fourth, results vary in interlaboratory comparison because measurements are not fully standardized (7–9). These limitations may at least be partly overcome if leukocyte subsets are not characterized by cellular morphology or surface markers but rather by epigenetic means.

DNA methylation (DNAm) is a covalent modification of cytosine residues, mostly at CG dinucleotides (CpG sites) (10). By measuring the methylation level at CpG sites with cell-type–specific hypo- or hypermethylation, it is possible to quantify the composition of leukocyte subsets using statistical algorithms (deconvolution models) (11, 12). This analysis was initially performed by genome-wide DNAm analysis of Illumina BeadChip microarrays (12–15). However, microarray analysis is relatively labor-intensive, costly, and

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the regulatory requirements for an in vitro diagnostic test are difficult to implement. It has been recently demonstrated that cell-type–specific DNAm of leukocyte subsets can alternatively be analyzed by quantitative PCR, pyrosequencing, or methylation-specific digital droplet PCR (ddPCR) (16–18). These methods for targeted analysis of cell-type–specific CpGs appear to be better applicable for clinical use.

We previously identified CpGs with a characteristic hypomethylation in specific leukocyte subsets (17). These CpG sites were associated with the genes WD repeat domain 20 (*WDR20*; cg05398700) for granulocytes; FYN proto-oncogene (*FYN*; cg17587997) for lymphocytes; centromere protein A (*CENPA*; cg10480329) for monocytes; *CD4* (cg05044173) for CD4$^+$ T cells; *CD8A* (cg25939861) for CD8$^+$ T cells; WD repeat domain, phosphoinositide interacting 2 (*WIPI2*; cg02665297) for B cells; and solute carrier family 15 member 4 (*SLC15A4*; cg13617280) for natural killer (NK) cells. We demonstrated for a test set of 44 healthy donors that the estimated leukocyte counts—based on a nonnegative least square (NNLS) algorithm—correlated with conventional cell counts (17). This targeted DNAm analysis is subsequently referred to as "Epi-Blood-Count."

In this study, we optimized and validated Epi-Blood-Count based on pyrosequencing measurements on much larger cohorts of healthy donors and of patients with hematologic diseases. This was important since hematopoietic malignancies can have a particularly profound impact on DNAm patterns (19). We also describe a novel approach for absolute quantification of leukocyte subsets using ddPCR.

Material and Methods

BLOOD SAMPLES

All blood samples (332 venous and 122 capillary of healthy donors, and 266 venous of patients with hematologic diseases) were collected after informed and written consent according to guidelines specifically approved by the local ethics committee of the RWTH Aachen University (EK176/11, EK206/09, EK099/14). As a reference for healthy donors, we also used cryopreserved peripheral blood samples from the Health Effects in High-Level PCB program (20), and 36 blood samples from a ring trial (Referenzinstitut für Bioanalytik, Bonn, Germany). A detailed description of sample collection, sample processing, conventional blood counts, and cell sorting of leukocyte subsets is provided in online Supplemental Data file.

PYROSEQUENCING

DNA was isolated from 150 μL venous or 50 μL capillary blood and bisulfite-treated with the EZ DNA Methylation Kit (Zymo Research; Supplemental Data file). Bisulfite-converted DNA (10–20 ng) was then amplified using PyroMark PCR Kit (Qiagen) with primers designed with the PyroMark Assay Design 2.0 Software (Qiagen) as shown in Supplemental Table 1 (Metabion). PCR amplicons were sequenced on a PyroMark Q96 ID (Qiagen) and analyzed with PyroMark Q96 CpG 1.0.9 (Qiagen). Deconvolution of leukocyte subsets was performed as described in the Supplemental Data file.

DIGITAL DROPLET POLYMERASE CHAIN REACTION (DDPCR)

Primers and fluorescently labeled probes for ddPCR were designed using Primer3Plus software (21) (Supplemental Table 2). Bisulfite-converted DNA (10–20 ng) were mixed with 2× Supermix for probes (no dUTPs, Bio Rad), 1 μM primers (each), and 0.25 μM TaqMan probes (each) in a 20 μL reaction. DNA was amplified on a C1000 Touch Thermal Cycler (Bio-Rad) using the following program: 1× 95°C for 10 min, 40× 94°C for 30 s and 56°C for 30 s, and 1× 98°C for 10 min and measured with a QX200 Droplet Digital reader (Bio-Rad). Data were analyzed with QuantaSoft analysis software (Bio-Rad).

ABSOLUTE LEUKOCYTE QUANTIFICATION

Absolute leukocyte quantification by pyrosequencing was performed as described previously (17). In short, 0.011 ng of nonmethylated LSM14B reference DNA plasmid was added to 150 μL blood sample and the ratio between unmethylated reference plasmid and methylated genomic *LSM14B* was determined (Supplemental Data file). For absolute leukocyte quantification by ddPCR, a reference plasmid was created containing a genomic region that is unique and conserved in the human genome, which was altered at 3 positions (Supplemental Table 3). Blood samples (150 μL) were mixed with 0.00389 ng of this reference plasmid and the number of genomic copies/μL was calculated as described in the Supplemental Data file.

DATA ANALYSIS AND STATISTICS

Data were analyzed and plotted in Windows Office 16 Excel (Microsoft) and GraphPad Prism Version 9 (Graph Pad Software Inc.). Heatmaps were generated with Multiexperiment Viewer v4.9 (TM4 Software Suite). Pearson correlation coefficient r and the mean absolute error (MAE) are provided. The Kolmogorov–Smirnov test (unpaired, nonparametric *t* test) was used to estimate significance of leukocyte predictions.

Results

VALIDATION OF EPI-BLOOD-COUNT WITH DIFFERENT DECONVOLUTION METHODS

Deconvolution of leukocytes might be improved by a more precise reference matrix of pyrosequencing measurements in purified leukocyte subsets. So far, the DNAm levels at the cell-type–specific CpGs were only estimated by available Illumina BeadChip data or calculated by a reverse NNLS model based on pyrosequencing of whole blood samples [17]. We have therefore used fluorescence-activated cell sorting to sort granulocytes, $CD4^+$ and $CD8^+$ T cells, B cells, NK cells, and monocytes from 6 healthy donors (Supplemental Fig. 1) and analyzed DNAm at the relevant CpGs by pyrosequencing (sorted NNLS). The results confirmed cell-type–specific hypomethylation in the genes *WDR20*, *CD4*, *CD8A*, *WIPI2*, *SLC15A4*, and *CENPA*, which was more pronounced compared to the Illumina BeadChip data [22] or reverse NNLS estimation (Fig. 1, A) [17].

Subsequently, we tested 4 different mathematical approaches to estimate the composition of leukocytes based on the DNAm levels: (i) reverse NNLS, (ii) single linear regression (SLIN), (iii) multiple regression, and (iv) sorted NNLS. To train these models, we used pyrosequencing data of 104 healthy blood samples from our previous work (Supplemental Fig. 2) [17], and for an independent validation, we analyzed frozen samples from 210 healthy donors. Deconvolution results from all 4 models correlated with conventional counts, using either a 3 CpG signature that discriminated granulocytes, lymphocytes, and monocytes (Fig. 1, B) or a 6 CpG signature that discriminated granulocytes, CD4 T cells, CD8 T cells, B cells, NK cells, and monocytes (Fig. 1, C). Pearson correlation values (r) were at a similar range for the 4 models. The MAE were overall lower for SLIN and multilinear regression. We decided to use the SLIN model for the remainder of the study.

EPI-BLOOD-COUNT IS APPLICABLE TO CAPILLARY BLOOD

Epi-Blood-Count is applicable to small blood volumes, and therefore we investigated applicability with 50 µL of capillary blood. We collected blood samples from 122 healthy donors from veins and finger prick. The estimated frequency of granulocytes (r = 0.78), lymphocytes (r = 0.84), and monocytes (r = 0.51) in capillary blood correlated with conventional measurements from venous blood (Fig. 2, A). Furthermore, Epi-Blood-Count measurements between capillary blood and venous blood correlated (granulocytes: r = 0.83; lymphocytes: r = 0.85; monocytes: r = 0.50) and revealed hardly systematic deviation. In contrast, conventional measurements of granulocytes varied between capillary and venous blood, indicating that there might be a discrepancy between the 2 cell counting devices (Abbott Cell Dyn Ruby and Abbott Cell Dyn Emerald, respectively; Supplemental Fig. 3).

BENCHMARKING OF EPI-BLOOD-COUNT IN RING TRIALS

To further evaluate the performance of Epi-Blood-Count, we enrolled in a ring trial in which roughly 400 laboratories measured the composition of granulocytes, lymphocytes, and monocytes of the same blood samples (n = 36). More than 20 different cell counting devices were used for analysis and revealed high variation in leukocyte counts (Fig. 2, B). Epi-Blood-Count predictions fell within this measurement range and correlated with the median of the ring trials measurements for granulocytes (r = 0.89), lymphocytes (r = 0.73), and monocytes (r = 0.75) (Fig. 2, C).

EVALUATION OF EPI-BLOOD-COUNT IN PATIENTS WITH HEMATOLOGICAL DISEASES

Several biological variations can impact DNAm, such as age, sex, and hematological diseases [23–25]. To determine if age or sex impacted our cell-type–specific CpGs, we stratified the test set into younger (<30 years) vs older adults (>60 years) or into male versus female samples. Analysis with the SLIN model did not reveal any notable effect of age or sex for any cell type tested (Supplemental Fig. 4).

Next, we evaluated the performance of Epi-Blood-Count in 266 samples from patients with various hematological and oncological disorders. These patients were categorized to different diseases, 14 of them to multiple categories (Supplemental Table 4). For most patients, Epi-Blood-Count measurements correlated with conventional cell counts (Fig. 3, A). Some outliers were observed, particularly in acute myeloid leukemia (AML) but also across other hematological diseases (Fig. 3, B).

For definition of outliers, we did not want to rely on comparison with conventional blood counts, which would not be available if Epi-Blood-Count is applied in a clinical setting. As each leukocyte subset was quantified independently by SLIN deconvolution, we checked whether the leukocyte predictions of the different subsets sum up to 100% (Fig. 3, C). Based on this, we identified 30 samples that deviated from the expected 100% by more than 1.5 times the interquartile range of all samples. Interestingly, 14 of these 30 (42%) samples were from patients with AML, which was significantly more than was expected (Chi-square test, $P = 0.0003$; Supplemental Fig. 5).

DIGITAL DROPLET PCR IS A SUITABLE ALTERNATIVE FOR EPI-BLOOD-COUNT

Next, we analyzed if DNAm measurement with ddPCR was also suitable for deconvolution of leukocyte subsets.
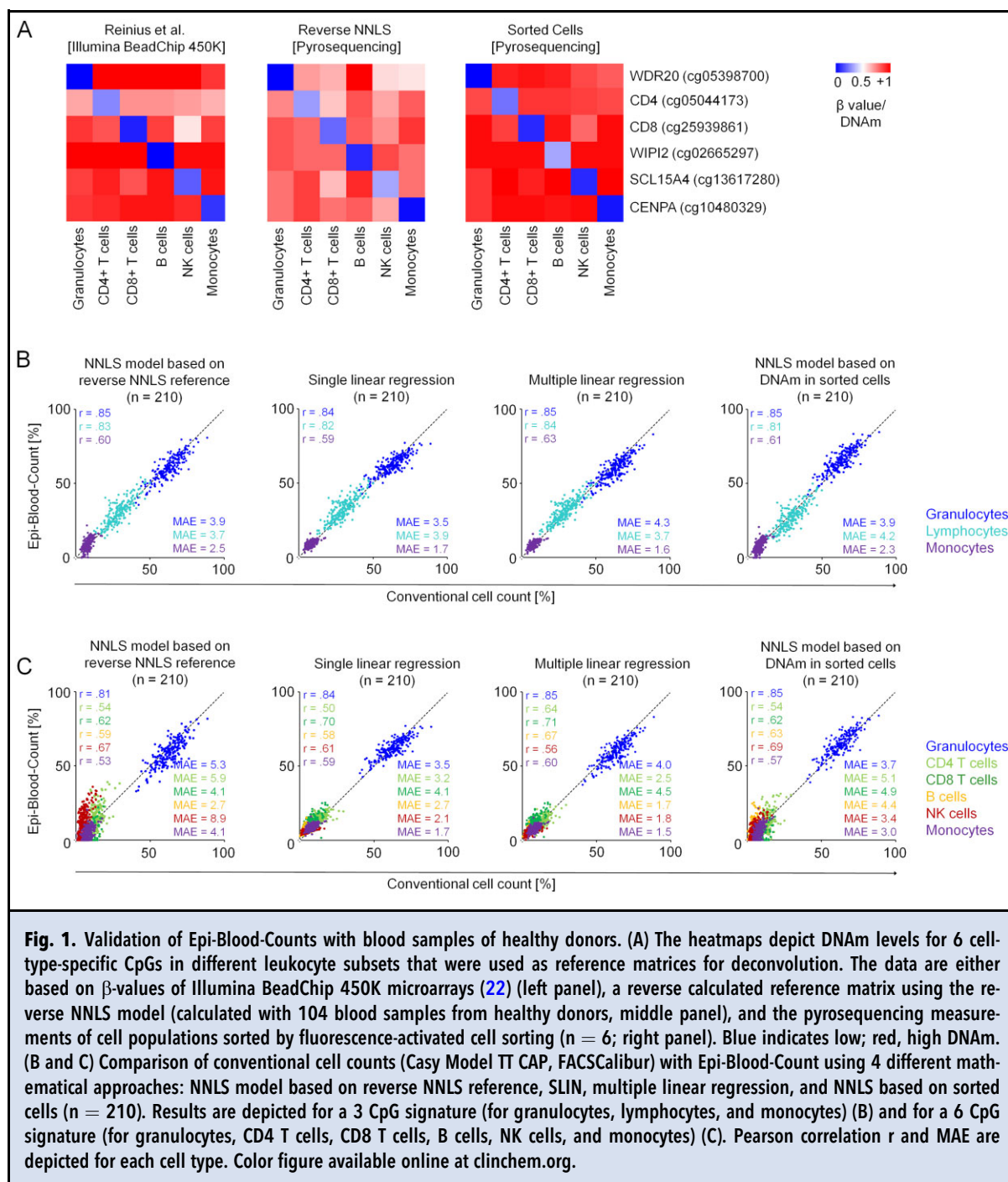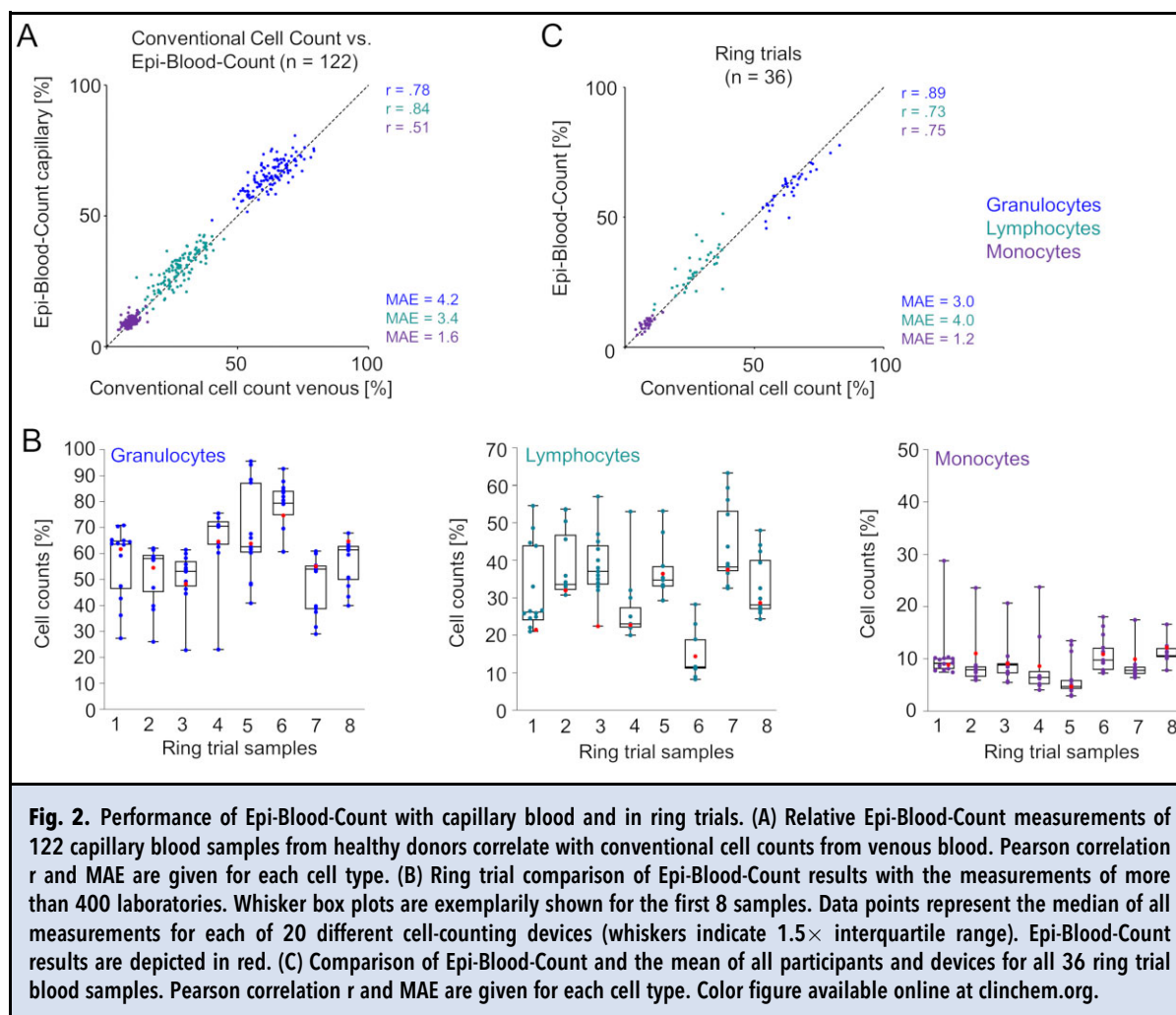
Fig. 1. Validation of Epi-Blood-Counts with blood samples of healthy donors. (A) The heatmaps depict DNAm levels for 6 cell-type-specific CpGs in different leukocyte subsets that were used as reference matrices for deconvolution. The data are either based on β-values of Illumina BeadChip 450K microarrays (22) (left panel), a reverse calculated reference matrix using the reverse NNLS model (calculated with 104 blood samples from healthy donors, middle panel), and the pyrosequencing measurements of cell populations sorted by fluorescence-activated cell sorting (n = 6; right panel). Blue indicates low; red, high DNAm. (B and C) Comparison of conventional cell counts (Casy Model TT CAP, FACSCalibur) with Epi-Blood-Count using 4 different mathematical approaches: NNLS model based on reverse NNLS reference, SLIN, multiple linear regression, and NNLS based on sorted cells (n = 210). Results are depicted for a 3 CpG signature (for granulocytes, lymphocytes, and monocytes) (B) and for a 6 CpG signature (for granulocytes, CD4 T cells, CD8 T cells, B cells, NK cells, and monocytes) (C). Pearson correlation r and MAE are depicted for each cell type. Color figure available online at clinchem.org.

The technology is based on dispensing bisulfite-converted DNA into small droplets, which are then processed for PCR amplification separately, potentially reducing PCR bias, since for each droplet the methylated (HEX labeled) and/or unmethylated (FAM labeled) probes are detected on a yes/no bases, independent of amplification level (26, 27). We used ddPCR to analyze DNAm at our cell-type–specific CpGs in venous blood samples from 96 healthy individuals and 151 patients with hematological disorders. There was a high correlation between DNAm values obtained by pyrosequencing and ddPCR for healthy and diseased patients

Fig. 2. Performance of Epi-Blood-Count with capillary blood and in ring trials. (A) Relative Epi-Blood-Count measurements of 122 capillary blood samples from healthy donors correlate with conventional cell counts from venous blood. Pearson correlation r and MAE are given for each cell type. (B) Ring trial comparison of Epi-Blood-Count results with the measurements of more than 400 laboratories. Whisker box plots are exemplarily shown for the first 8 samples. Data points represent the median of all measurements for each of 20 different cell-counting devices (whiskers indicate 1.5× interquartile range). Epi-Blood-Count results are depicted in red. (C) Comparison of Epi-Blood-Count and the mean of all participants and devices for all 36 ring trial blood samples. Pearson correlation r and MAE are given for each cell type. Color figure available online at clinchem.org.
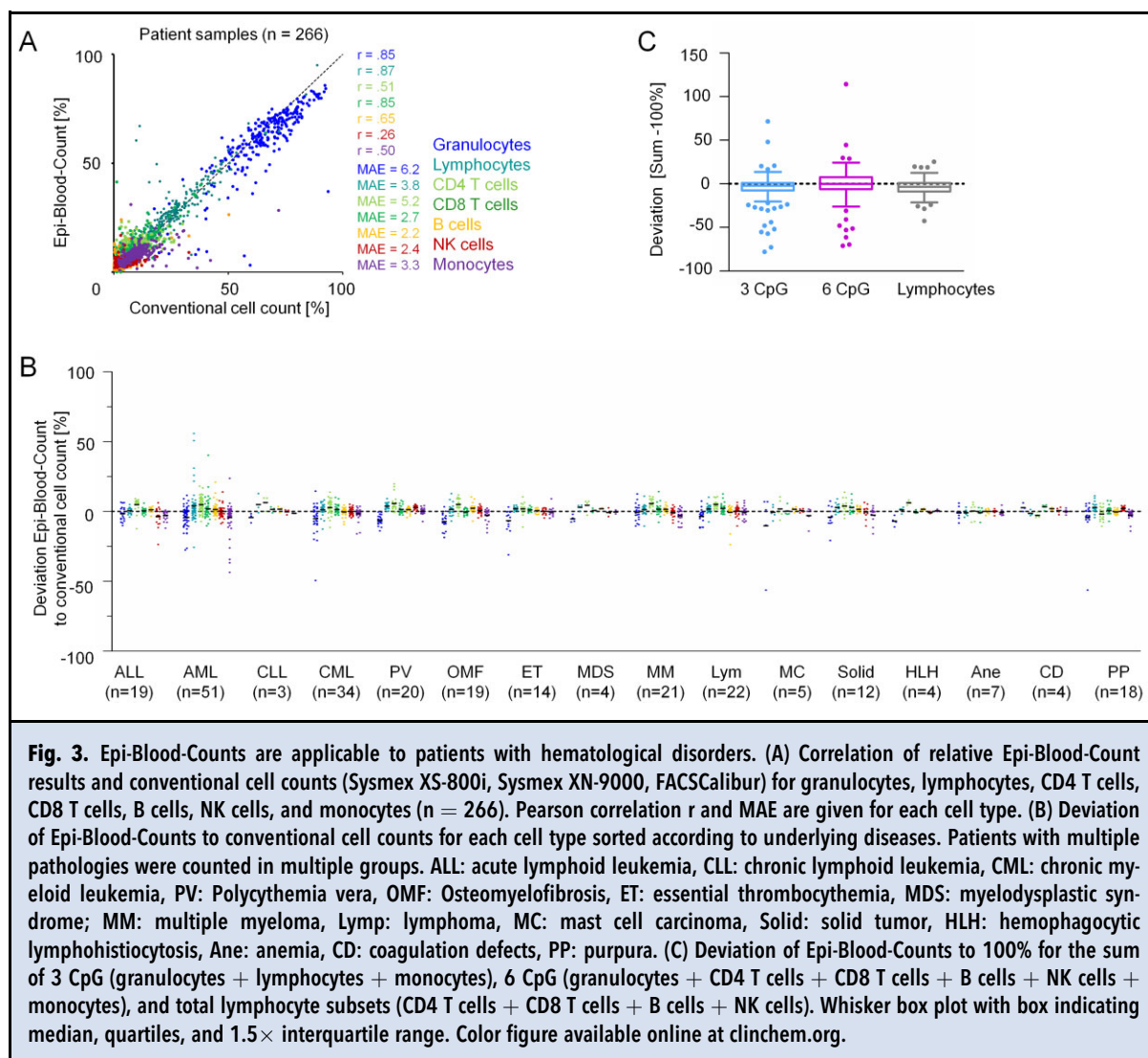
(Supplemental Fig. 6). In turn, leukocyte counts predicted by ddPCR measurements correlated with conventional leukocyte counts. In comparison to Epi-Blood-Count results with pyrosequencing, the correlations for healthy and diseased samples were slightly higher for CD4 T cells, CD8 T cells, and B cells but slightly lower for granulocytes and lymphocytes (Fig. 4).

ABSOLUTE QUANTIFICATION OF LEUKOCYTES VIA PYROSEQUENCING OR DDPCR

For absolute quantification of leukocytes, we tested 2 alternative approaches that are both based on a reference DNA plasmid that is spiked into the blood samples before DNA isolation. First, to determine cell numbers based on DNAm analysis with pyrosequencing, we focused on a genomic region in the gene *LSM14B*, which is generally methylated in leukocytes. The ratio between nonmethylated reference plasmid and methylated genomic LSM14B was determined after bisulfite conversion

to calculate the cells/µL (17). Second, alternatively, we generated a plasmid with a sequence variation as compared to the genomic sequence and the ratio of these sequences was determined by ddPCR without bisulfite treatment (Fig. 5, A and B). In total, 116 samples were spiked for pyrosequencing and 51 samples for ddPCR analysis. Conventional cell counts correlated with the estimates by pyrosequencing (r = 0.93) (Fig. 5, C) and ddPCR (r = 0.98) (Fig. 5, D).

Next, we combined the relative estimates of leukocyte subsets with the calculated absolute cell numbers, giving us absolute cell counts for all leukocyte subsets. The cell counts predicted by pyrosequencing (Fig. 5, E) and ddPCR (Fig. 5, F) correlated with conventional counts for granulocytes (r = 0.87 and r = 0.97) and monocytes (r = 0.72 and r = 0.76). Interestingly, absolute counts for B cells were better estimated by pyrosequencing (r = 0.98 vs r = 0.35), whereas CD8 T cells were better estimated by ddPCR (r = 0.69 vs r = 0.94).

Fig. 3. Epi-Blood-Counts are applicable to patients with hematological disorders. (A) Correlation of relative Epi-Blood-Count results and conventional cell counts (Sysmex XS-800i, Sysmex XN-9000, FACSCalibur) for granulocytes, lymphocytes, CD4 T cells, CD8 T cells, B cells, NK cells, and monocytes (n = 266). Pearson correlation r and MAE are given for each cell type. (B) Deviation of Epi-Blood-Counts to conventional cell counts for each cell type sorted according to underlying diseases. Patients with multiple pathologies were counted in multiple groups. ALL: acute lymphoid leukemia, CLL: chronic lymphoid leukemia, CML: chronic myeloid leukemia, PV: Polycythemia vera, OMF: Osteomyelofibrosis, ET: essential thrombocythemia, MDS: myelodysplastic syndrome; MM: multiple myeloma, Lymp: lymphoma, MC: mast cell carcinoma, Solid: solid tumor, HLH: hemophagocytic lymphohistiocytosis, Ane: anemia, CD: coagulation defects, PP: purpura. (C) Deviation of Epi-Blood-Counts to 100% for the sum of 3 CpG (granulocytes + lymphocytes + monocytes), 6 CpG (granulocytes + CD4 T cells + CD8 T cells + B cells + NK cells + monocytes), and total lymphocyte subsets (CD4 T cells + CD8 T cells + B cells + NK cells). Whisker box plot with box indicating median, quartiles, and 1.5× interquartile range. Color figure available online at clinchem.org.
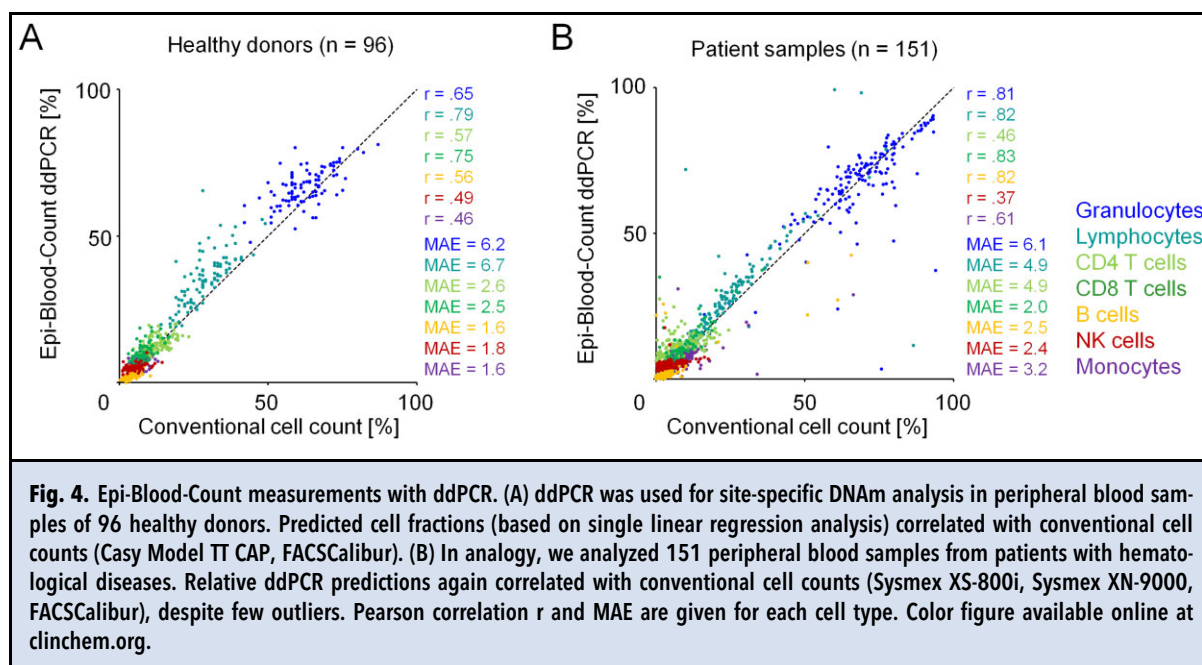
On the other hand, NK cell quantifications show a poor correlation for both pyrosequencing (r = 0.13) and ddPCR (r = 0.21). In terms of accuracy (estimated by MAE) ddPCR had a better performance than pyrosequencing.

Discussion

Translation of a new diagnostic method into clinical practice requires many steps. We previously provided proof of concept for Epi-Blood-Count in healthy donors (17). Our study validates these findings in much larger cohorts of healthy donors and additionally provided proof of concept for use in patients with hematopoietic malignancies as well as for use on small volumes of capillary blood obtained by finger prick. Furthermore, it

addresses several relevant points for further optimization to pave the way, according to regulatory requirements, for accreditation as an in vitro diagnostic procedure.

For deconvolution of the cellular composition by targeted DNAm analysis, it is crucial to choose CpGs that are consistently hypomethylated in 1 cell type and hypermethylated in the rest. We have analyzed leukocyte subsets sorted by fluorescence-activated cell sorting to validate the cell-type–specific hypomethylation and the results were in line with estimates from our previous work (17). Therefore, we have chosen to continue with this selection of cell-type–specific CpGs and to rather address other critical points of the procedure. However, since the correlations of Epi-Blood-Count with conventional counts were lower for monocytes, T cells, and NK cells, alternative CpGs might be considered for
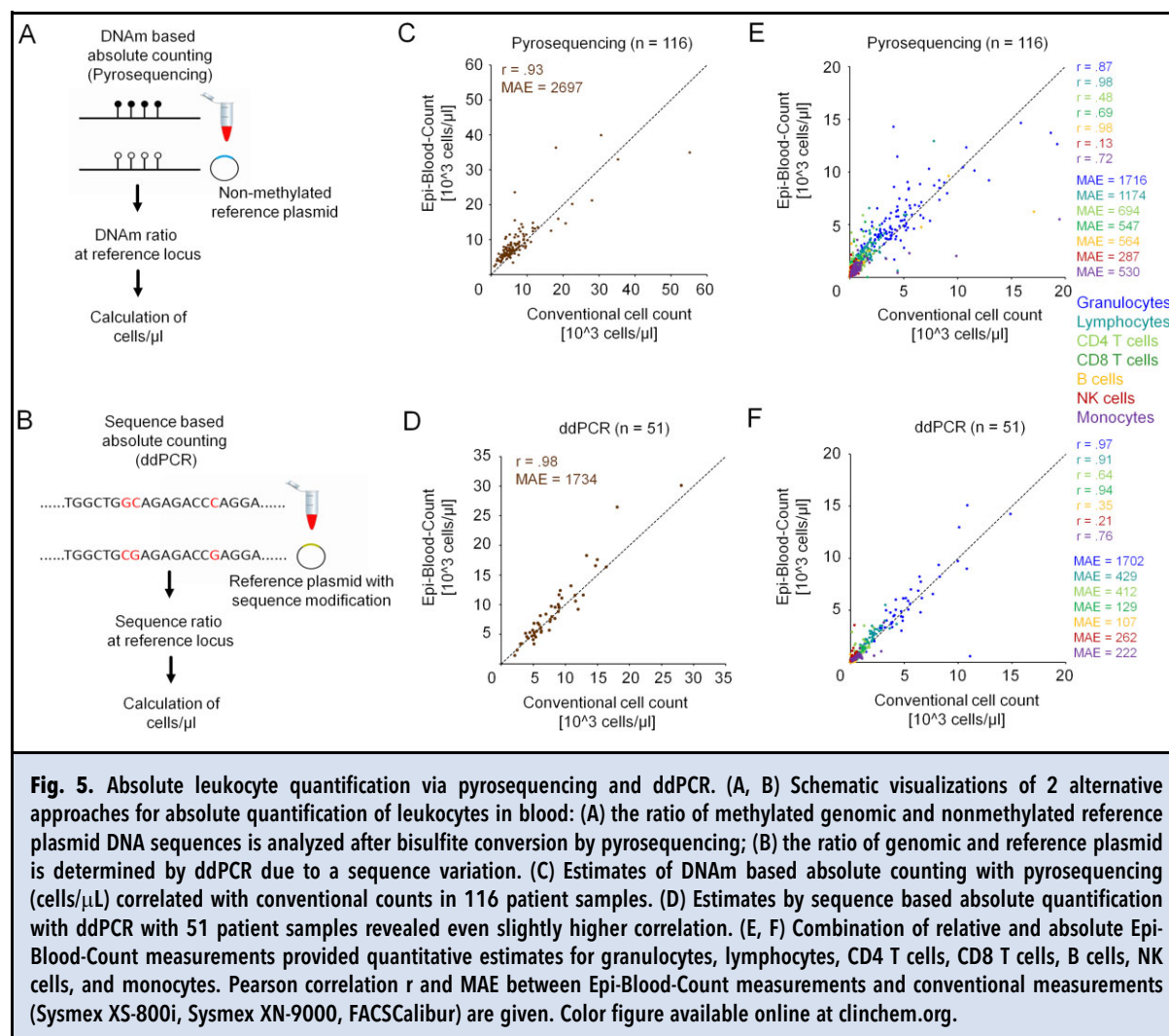
Fig. 4. Epi-Blood-Count measurements with ddPCR. (A) ddPCR was used for site-specific DNAm analysis in peripheral blood samples of 96 healthy donors. Predicted cell fractions (based on single linear regression analysis) correlated with conventional cell counts (Casy Model TT CAP, FACSCalibur). (B) In analogy, we analyzed 151 peripheral blood samples from patients with hematological diseases. Relative ddPCR predictions again correlated with conventional cell counts (Sysmex XS-800i, Sysmex XN-9000, FACSCalibur), despite few outliers. Pearson correlation r and MAE are given for each cell type. Color figure available online at clinchem.org.

these cell types in the future. Such selection of alternative CpGs could, for instance, be based on the combination of larger publicly available data sets of DNAm profiles of sorted subsets. Furthermore, the selection should consider that specific gene regions, such as promoters in oncogenes, are more likely to have aberrant DNAm patterns in malignancies. For example, it is conceivable that lymphocytes were overestimated in some of the AML outliers due to aberrant DNAm in the FYN proto-oncogene. Optimized selection of the target regions might increase the precision of measurements for monocytes, T cells, and NK cells.

It is also conceivable that some of the deviation is attributed to misclassification by conventional measurements based on surface marker expression: monocytes are normally detected as CD14^{high} cells, but there are also intermediate and nonclassical subsets with CD14^{low} expression, which may not be identified with conventional gating strategies (28). A highly variable fraction of gamma delta T cells ($\gamma\delta$ T cells) does not always express CD4 or CD8 surface markers (29). On the other hand, NK cell counting is usually based on the CD56^{+}/CD3^{-} subset, which does not include the natural killer T cells (30). Thus, the choice of gating strategies and unclear assignment to the cellular categories may contribute to the discrepancy between leukocyte counts based on either surface markers or epigenetics.

Subsequently, we focused on mathematical models for deconvolution of the cellular composition. The corresponding software, which would also be part of an accredited procedure, needs to provide robust and reliable results. Deconvolution of leukocyte subsets in

Illumina BeadChip data sets is usually based on a reference matrix of purified leukocyte subsets (11, 31, 32). We anticipated that, for our targeted approach, the reference matrix based on sorted subsets (sorted NNLS) would clearly outperform the reference matrix that was estimated on blood counts (reverse NNLS), yet the results did not reveal a clear advantage. Alternatively, we tested single and multiple linear regression models, which do not require a reference matrix. Overall, the 4 methods obtained similar results for correlation or MAE across the different cell types. We reasoned that the single linear regression model was best suited, since it does not require simultaneous measurement of all leukocyte subsets, making it more effective for tailored analysis of specific leukocyte subtypes. Furthermore, our results demonstrate that Epi-Blood-Count measurements may have offsets in hematopoietic diseases, which can be flagged by conflicting results when individual cell-type predictions on SLIN model are summed up. The other 3 models cannot identify the offsets because their individual cell type predictions take all CpGs into account.

Many hematopoietic diseases affect the epigenetic makeup (33, 34). Our scoring system could discern almost all patient samples that revealed high deviation between conventional blood counts and Epi-Blood-Count measurements. In healthy samples, we hardly observed such outliers. The fraction of outliers was particularly high in AML, which is characterized by frequent epigenetic modifications and high blast counts in peripheral blood (19, 35, 36). Such measurements could be flagged for further analysis with conventional counts or manual inspection. In fact, also with automated cell counters, a

Fig. 5. Absolute leukocyte quantification via pyrosequencing and ddPCR. (A, B) Schematic visualizations of 2 alternative approaches for absolute quantification of leukocytes in blood: **(A)** the ratio of methylated genomic and nonmethylated reference plasmid DNA sequences is analyzed after bisulfite conversion by pyrosequencing; **(B)** the ratio of genomic and reference plasmid is determined by ddPCR due to a sequence variation. **(C)** Estimates of DNAm based absolute counting with pyrosequencing (cells/μL) correlated with conventional counts in 116 patient samples. **(D)** Estimates by sequence based absolute quantification with ddPCR with 51 patient samples revealed even slightly higher correlation. **(E, F)** Combination of relative and absolute Epi-Blood-Count measurements provided quantitative estimates for granulocytes, lymphocytes, CD4 T cells, CD8 T cells, B cells, NK cells, and monocytes. Pearson correlation r and MAE between Epi-Blood-Count measurements and conventional measurements (Sysmex XS-800i, Sysmex XN-9000, FACSCalibur) are given. Color figure available online at clinchem.org.

relatively large proportion of samples are flagged for manual review of blood smears, and the criteria for this selection are largely dependent on machine settings and users (37–39).

In addition, we have provided proof of principle that Epi-Blood-Count is applicable to capillary blood. This holds the perspective that white blood cell counts can be performed by a simple finger prick for self-sampling, for example on elderly people who may not easily visit a clinician. These samples could then be shipped at room temperature or stored at −20°C for later analysis. Despite the small sample volume of only 50 μL, the Epi-Blood-Count results correlated with conventional measurements. Furthermore, the Epi-Blood-Count results were similar in venous and capillary blood, albeit other studies suggested that granulocytes were in tendency higher in capillary blood than venous blood (40, 41). Thus, a more systematic comparison of

capillary and venous blood is needed, also regarding other leukocyte subsets that can so far hardly be analyzed in capillary blood.

Various methods can be used for targeted DNAm analysis (42). It has been demonstrated that cell-type–specific DNAm of leukocyte subsets can be analyzed by pyrosequencing, quantitative PCR, and ddPCR (16–18). Particularly, quantitative PCR and pyrosequencing may have a bias due to variation in PCR efficiency between the methylated and nonmethylated strands. At least some of this bias might be circumvented by ddPCR because individual droplets are either scored as positive or negative independent of the PCR efficiency (27, 43, 44). Yet, for relative quantification of different leukocyte subsets, the precision of Epi-Blood-Count measurements was similar in ddPCR and pyrosequencing. Another advantage of ddPCR is that Bio-Rad's Droplet Digital systems are already approved for use in

a regulatory environment in several in vitro diagnostics applications.

Costs are also a critical issue for implementing a new method in the clinical routine. Analysis with automated cell counters is fast and cost-effective, whereas flow cytometry of lymphocyte subsets necessitates more expensive instrumentation, antibodies, and a specifically trained operator. For Epi-Blood-Count, the costs for consumables—including DNA isolation, bisulfite conversion, and analysis of 6 CpGs—would currently sum up to about 40€ per sample. This is similar for pyrosequencing and ddPCR. Additional costs for personnel are relatively high with a hands-on working time of about 10 h. Yet, personnel costs decline significantly when many samples can be processed in parallel. In the future, the time and costs for the Epi-Blood-Count procedure might be further reduced by implementation of liquid handling units and fully automated instruments.

Lastly, we compared 2 approaches for absolute quantification of leukocyte counts based on spiking blood samples with a reference plasmid: (i) pyrosequencing of DNAm levels at the *LSM14B* locus, which is consistently methylated in leukocytes [17], and (ii) ddPCR of sequence variation between genomic and plasmid DNA. Both approaches necessitate an empirically determined correction factor for each plasmid preparation. The absolute quantification with pyrosequencing provided lower correlation with conventional leukocyte counts compared to ddPCR, which might be due to some hypomethylation in the genomic locus of LSM14B, the need of additional bisulfite conversion, and to the fact that the method is most sensitive if the reference vs genomic DNA is spiked at a 50/50 ratio [17]. We anticipate that the reference-plasmid based approach for cell counts will also be useful for other experimental settings, for cell-free DNA, or in very small blood volumes.

Taken together, targeted DNAm analysis is suitable for leukocyte counts of clinical samples. The accuracy of our Epi-Blood-Count measurements was particularly high for granulocytes, lymphocytes, CD4 T cells, and B cells, whereas predictions for NK cells, CD8 T cells, and monocytes showed higher deviation from conventional cell counts. We anticipate that the accuracy can be increased with an alternative selection of cell-type–specific CpGs. While it is unlikely that Epi-Blood-Count replaces conventional methods due to the turnaround time (about 2 days), relatively high costs, and inability to estimate erythrocyte or thrombocyte numbers, it may become a valuable alternative to answer specific clinical questions. In addition, it could enable self-sampling by a finger prick, retrospective analysis of frozen blood, and leukocyte quantification in samples that provided unclear results in conventional analysis.

Ethics Approval and Consent to Participate

All blood samples were taken after informed and written consent, in accordance with the Declaration of Helsinki, and stored at RWTH cBMB, the central biobank of the medical faculty of RWTH Aachen University (Ethic approval number EK 206/09). The current study was subsequently approved by the Ethic Committee of the Use of Human Subjects at the University of Aachen (permit numbers: EK176/11 and EK099/14).

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.*

S. Sontag and L. Bocova performed and coordinated experiments and analyzed the data; S. Nüchtern, M. Schnitker, and T. Look performed pyrosequencing and ddPCR measurements; V. Tharmapalan designed the ddPCR assay for absolute quantification; B. Plümäkers performed lymphocyte subset quantification by flow cytometry; K.M. Schröder, M. Wessiepe, T. Kraus, L. Rink, and S. Koschmieder provided blood samples and analyzed them by conventional methods; J. Kramer supported experimental design and acquisition of funding; W. Wagner conceived and supervised the study; S. Sontag, W.H.G. Hubens, L. Bocova, and W. Wagner wrote the manuscript.

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