Bio-engineered and native red blood cells from cord blood exhibit the same metabolomic profile

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Supplemental Methods

1-1 Biological material and sample preparation

Cultured red blood cells

Umbilical Cord Blood (CB) from normal full-term deliveries were obtained with informed consent. Cord Blood $CD34^+$ cells were isolated by supermagnetic microbead selection using Mini-MACS columns (Miltenyi Biotech, Bergisch Glodbach, Germany) (94 ± 3 % purity). The cells were cultured in erythroid differentiation medium (EDM) based on IMDM (Iscove modified Dulbecco's medium, Biochrom, Germany) supplemented with stabilized glutamine, 330 µg/mL human holo-transferrin (BBI solutions, Sittingbourne, UK), 10 µg/mL recombinant human insulin (FEF, Denmark), 2 IU/mL heparin Choay (Sanofi, France) and 5% solvent/detergent virus inactivated (S/D) plasma (Etablissement Français du Sang, France).

The procedure comprised three steps. In the first step (day 0 to day 8), 10^4/mL $CD34^+$ cells were cultured in EDM in the presence of 100 ng/mL SCF (PeproTech, Neuilly-sur-Seine, France), 5 ng/mL IL-3 (PeproTech) and 3 IU/mL Epo (Eprex, Janssen-Cilag, Issy-les-Moulineaux, France). On day 4, one volume of cell culture was diluted in four volumes of fresh medium containing SCF, IL-3, Epo. In the second step (day 8 to day 11), the cells were resuspended at 10^5/mL and seeded onto MS-5 at $2x10^4$cells/cm^2 in EDM supplemented with Epo. In the third step (day 11 to day 15), the non adherent cells were recovered, centrifuged, diluted in fresh medium not containing cytokines (the volume of culture medium was doubled at day 11 as compared to day 8). Cells were re-seeded onto stromal cells. The cultures were maintained at 37°C in 5% CO_2 in air.

Stromal cells

The murine MS-5 stromal cell line was expanded in αMEM medium containing ribonucleosides, deoxyribonucleosides, Glutamax (Life Technology) and 10% fetal calf serum (FCS). At confluence, adherent cells were collected after treatment of the cultures for 7-10 min with trypsin-EDTA 1X (Life Technology) at 37°C. The recovered cells (usually 10^6/$25cm^2$) were washed and replated at 4000/cm^2 in αMEM medium supplemented with Glutamax and 10% FCS. Cultures were incubated at 37°C under 5% CO_2 and adherence was usually reached after one week.

Deleukocytation

The cell suspensions were purified by passage through a deleukocyting filter to eliminate the expelled nuclei and residual erythroblasts. The purity of the cRBC samples was 99.3 ± 0.2 % after filtration and the filtered suspensions were washed twice in PBS.

Maturation of native and cultured reticulocytes

To induce the maturation of the RETc, cells were washed, resuspended at 5-$7x10^6$/ml in IMDM + 5% human AB plasma without cytokines and co-cultured on a new stromal layer. The culture supernatants were renewed twice a week. Cultures were maintained at 37°C under 5% CO_2.

Reticulocyte separation

Native reticulocytes were isolated from peripheral blood by an immunomagnetic method (Miltenyi Biotec). Briefly, the cells were incubated with anti-CD71 microbeads

(Miltenyi Biotec) and the labeled cells were enriched on Mini-MACS columns. The reticulocyte content of the CD71-purified population was controlled by thiazole orange staining.

Flow cytometric analyses
Cells were labeled with unconjugated or fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated antibodies. Anti-CD235 (glycophorin A) -PE, anti-CD71-PE or –FITC, anti-CD36-FITC and anti-CD34-PE antibodies (Beckman Coulter, Marseille, France) were used for phenotyping. Analyses were performed on CYAN ™ ADP flow cytometer (Beckman Coulter) using Summit software.

Reticulocyte count
$3x10^5$ cells were washed in PBS (pH 7.4) and incubated with 300 µL of Retic-count solution (Retic-count/Thiazole-Orange, BD Biosciences, Le Pont-de-Claix, France) for 30 min at room temperature. A negative control was carried out by incubating the cells with PBS alone.

Mitochondrial Membrane Potential

Resdietraethylbenzimidazolyl-carbocyanine iodide (JC-1) was used to measure the mitochondrial membrane potential. When this cationic dye accumulates into the mitochondrial membrane it forms aggregates leading to a shift in fluorescence from green to orange. Briefly, $5x10^5$ cells were washed and resuspended at 10^6/mL in prewarmed EDM, and then incubated at 37°C under 5% CO_2 for 15 min with 20 ng/mL JC-1 (Life Technologies) or without JC-1 (for negative control). Cells were generously washed twice in prewarmed PBS and immediately analyzed on a CYAN ™ ADP flow cytometer (Beckman Coulter) using Summit software (Excitation 488nm; Emission 530-590 nm).

Deformability measurements
The cell flexibility of cRBC and native reticulocytes was determined using a laser diffraction technique [LORCA (Laser-assisted Optical Rotational Cell Analyzer); R&R Mechanotrics, Hoor, The Netherlands] as extensively described previously[1] [2]The cell deformability was expressed as elongation index (EI) which was recorded continuously at various shear stresses in the range 0.3-30 Pa. The EI value at 30 Pa was referred to as EI_{max}.

Preparation of RBC lysates and metabolite extraction
10^9 packed native or cultured cells were pelleted and resuspended in qs 1ml Milli-Q water, and boiled for 3 minutes. After centrifugation at 1430*g* for 3 minutes at 4°C, the supernatant was ultrafiltered with centrifugal filter devices, molecular weight cutoff of 30 000 followed by 10 000 (Amicon Ultra-4 centrifugal filter devices) at 4000*g* for 20 minutes at 20°C. The final ultrafiltrates were divided into 4 aliquots and frozen at -80°C until mass spectrometry analysis.
Before injection into the chromatographic system, the samples were dried under nitrogen. Two aliquots of each sample were diluted in 105µL H_2O /External Standards mixture (ExS) in 105µL of H_2O/ACN/ExS, 40/60/5 µl for HILIC analysis . A quality control sample (QC) was obtained by pooling RBC samples subsequently diluted ½, ¼ and ⅛. A QC sample was injected after each dilution series and after each 10 samples to assess signal repeatability and stability.

1-2/ Instrumentation and LC-MS acquisitions:

LC-MS analyses were performed using a Dionex Ultimate chromatographic system (Thermo Fisher Scientific, Courtaboeuf, France) coupled to an Exactive spectrometer (Thermo Fisher Scientific, Courtaboeuf, France) fitted with an electrospray source operated in the positive and negative ion modes. The software interface was Xcalibur (version 2.1) (Thermo Fisher Scientific, Courtaboeuf, france). The mass spectrometer was calibrated before each analysis in both ESI polarities using the manufacturer's predefined methods and recommended calibration mixture provided by the manufacturer (external calibration).

The ultra-high performance liquid chromatographic (UHPLC) separation was performed on a hypersil GOLD C_{18} 1.9µm, 2.1mm x 150mm column at 30°C (Thermo Fisher Scientific, les Ulis, France). Hydrophilic interaction liquid chromatographic separation (HILIC) was achieved on a Sequant ZICpHILIC 5µm, 2.1 x 150mm at 15°C (Merck, Darmstadt, Germany). All chromatographic systems were equipped with an on line prefilter (Thermo). Experimental settings for each LC/MS condition are described below.

The Exactive mass spectrometer was operated with capillary voltage at -3 kV in the negative ionization mode and 5 kV in the positive ionization and capillary temperature at 280°C. The sheath gas pressure and the auxiliary gas pressure were set, respectively, at 60 and 10 arbitrary units with nitrogen gas. The mass resolution power of the analyzer was set to 50000 m/Δm, full width at half maximum (FWHM) at 200u, for singly charged ions. The detection was achieved from 50 to 1000 u in the positive ionization mode, from 95 to 1000u for reverse phase (RP) chromatography in the negative ionization mode and from 85 to 1000u for HILIC conditions.

Mobile phases for UHPLC were 100% water in A and 100% ACN in B, both containing 0.1% formic acid. Regarding HILIC, phase A consisted of an aqueous buffer of 10mM of ammonium carbonate in water with ammonium hydroxide to adjust basicity to pH 10.5, whereas acetonitrile was used as solvent B. Chromatographic elutions were achieved under gradient conditions as follows:

(i) RP based system: the flow rate was set at 500µL/min. The elution consisted of an isocratic step of 2 minutes at 5% phase B, followed by a linear gradient from 5 to 100% of phase B for the next 11 minutes. These proportions were kept constant for 12.5 min before returning to 5% B for 4.5 min.

(ii) HILIC based system: the flow rate was 200µL/min. Elution started with an isocratic step of 2 min at 80% B, followed by a linear gradient from 80 to 40% of phase B from 2 to 12 min. The chromatographic system was then rinsed for 5 min at 0% B, and the run ended with an equilibration step of 15 min.

2-4/ Data processing:

All raw data were manually inspected using the Qualbrowser module of Xcalibur version 2.1 (Thermo Fisher Scientific, Courtaboeuf, France). Automatic peak detection and integration were performed using the XCMS software package.[3] Grouping of features was performed using CAMERA software.[4] Features were then annotated by matching their accurate measured mass at ± 10 ppm with theoretical ones contained in biochemical and metabolomic databases such as KEGG[5] HMDB[6], Metlin[7] by using an informatics tool developed in R language, and also by our spectral database according to accurate measured masses and chromatographic retention times.[8, 9]

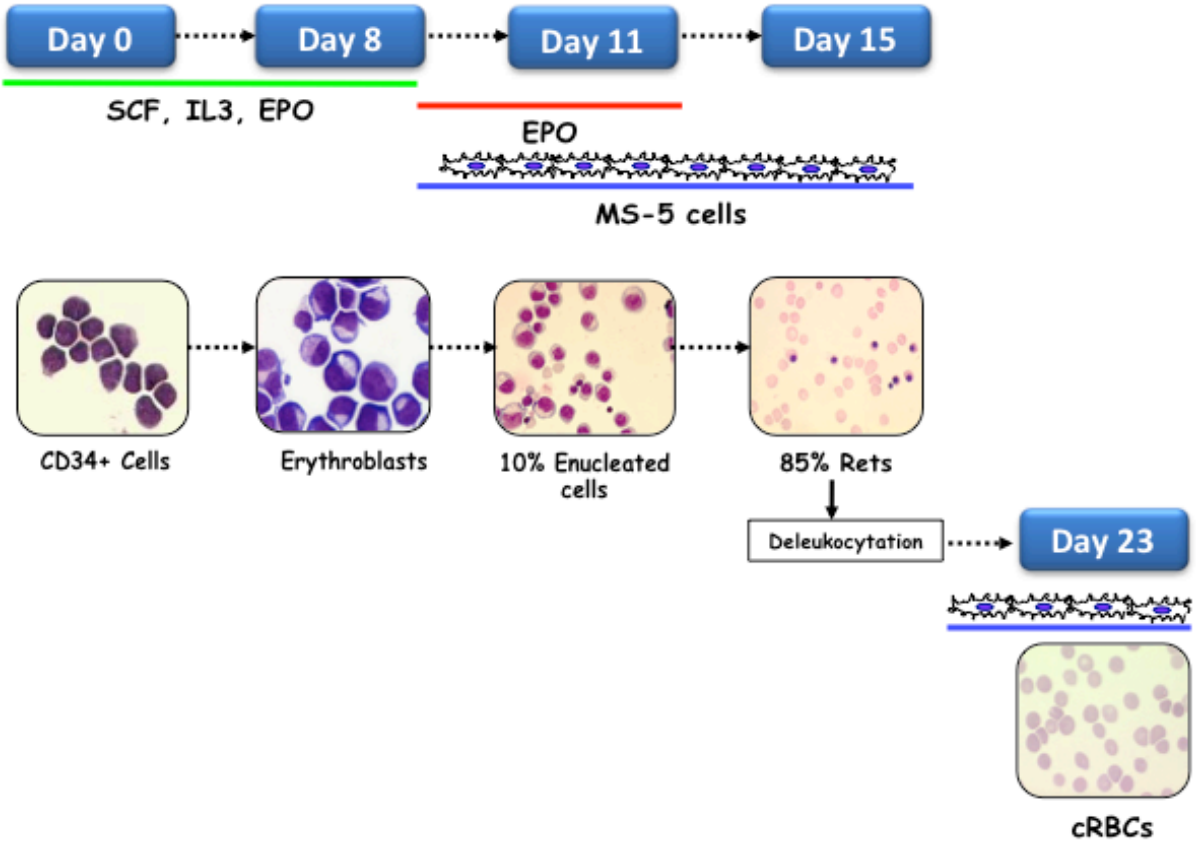
Signal Drift Correction and Batch Effect Removal

Within each peak table, intensities were corrected for signal drift and batch effect by fitting a locally quadratic (loess) regression model to the QC values.[10,11] The α parameter controlling the smoothing was set to 1 to avoid overfitting. Once the peak tables were normalized, metabolites with a coefficient of variation (CV) of their QC values >25% were filtered out.

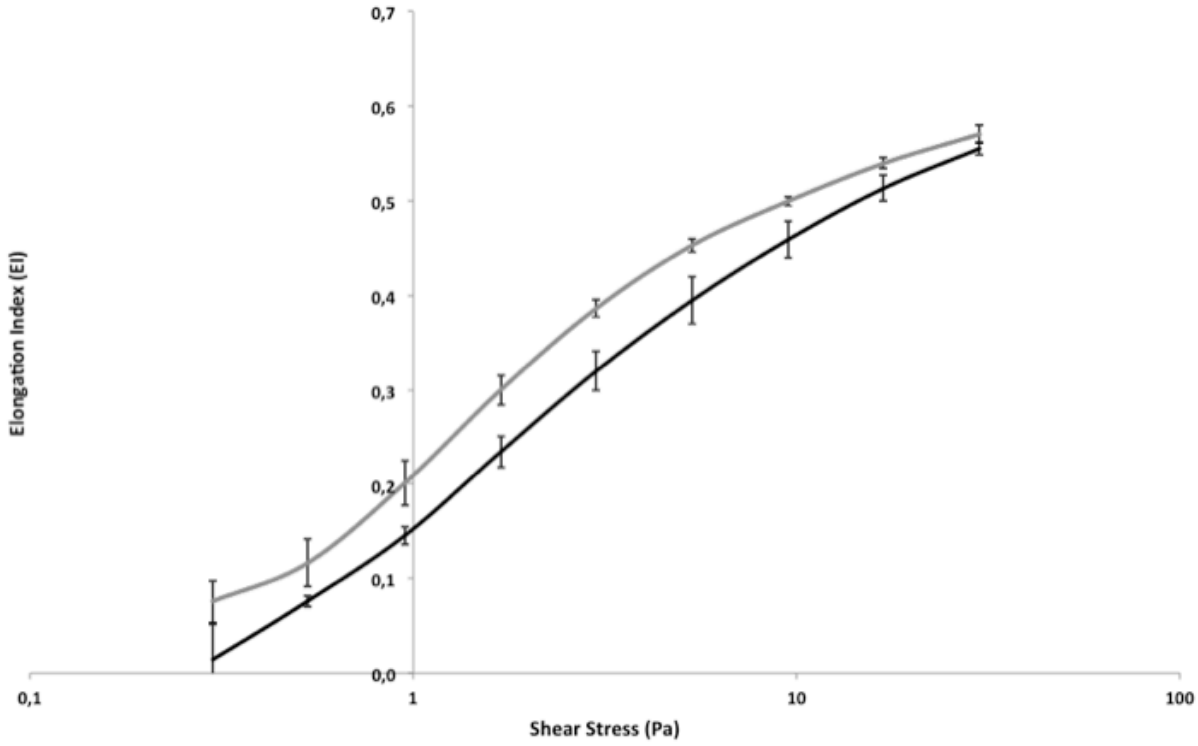
Supplemental references

1. Hardeman MR, Ince C. Clinical potential of in vitro measured red cell deformability, a myth? Clin Hemorheol Microcirc. 1999;21(3-4):277-84.
2. Giarratana MC, Rouard H, Dumont A, Kiger L, Safeukui I, Le Pennec PY, et al. Proof of principle for transfusion of in vitro-generated red blood cells. Blood. 2011 Nov 10;118(19):5071-9.
3. Smith CA, Want EJ, O'Maille G, Abagyan R, Siuzdak G. XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. Anal Chem. 2006 Feb 1;78(3):779-87.
4. Kuhl C, Tautenhahn R, Bottcher C, Larson TR, Neumann S. CAMERA: an integrated strategy for compound spectra extraction and annotation of liquid chromatography/mass spectrometry data sets. Anal Chem. 2012 Jan 3;84(1):283-9.
5. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 2000 Jan 1;28(1):27-30.
6. Wishart DS, Tzur D, Knox C, Eisner R, Guo AC, Young N, et al. HMDB: the Human Metabolome Database. Nucleic Acids Res. 2007 Jan;35(Database issue):D521-6.
7. Smith CA, O'Maille G, Want EJ, Qin C, Trauger SA, Brandon TR, et al. METLIN: a metabolite mass spectral database. Ther Drug Monit. 2005 Dec;27(6):747-51.
8. Boudah S, Olivier MF, Aros-Calt S, Oliveira L, Fenaille F, Tabet JC, et al. Annotation of the human serum metabolome by coupling three liquid chromatography methods to high-resolution mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2014 Sep 1;966:34-47.
9. Roux A, Xu Y, Heilier JF, Olivier MF, Ezan E, Tabet JC, et al. Annotation of the human adult urinary metabolome and metabolite identification using ultra high performance liquid chromatography coupled to a linear quadrupole ion trap-Orbitrap mass spectrometer. Anal Chem. 2012 Aug 7;84(15):6429-37.
10. van der Kloet FMB, I.; Verheij, E. R.; Jellema, R. H. Analytical Error Reduction Using Single Point Calibration for Accurate
and Precise Metabolomic Phenotyping. J Proteome Res. 2009;8(11):5132−41.
11. Dunn WB, Broadhurst D, Begley P, Zelena E, Francis-McIntyre S, Anderson N, et al. Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. Nat Protoc. 2011 Jul;6(7):1060-83.

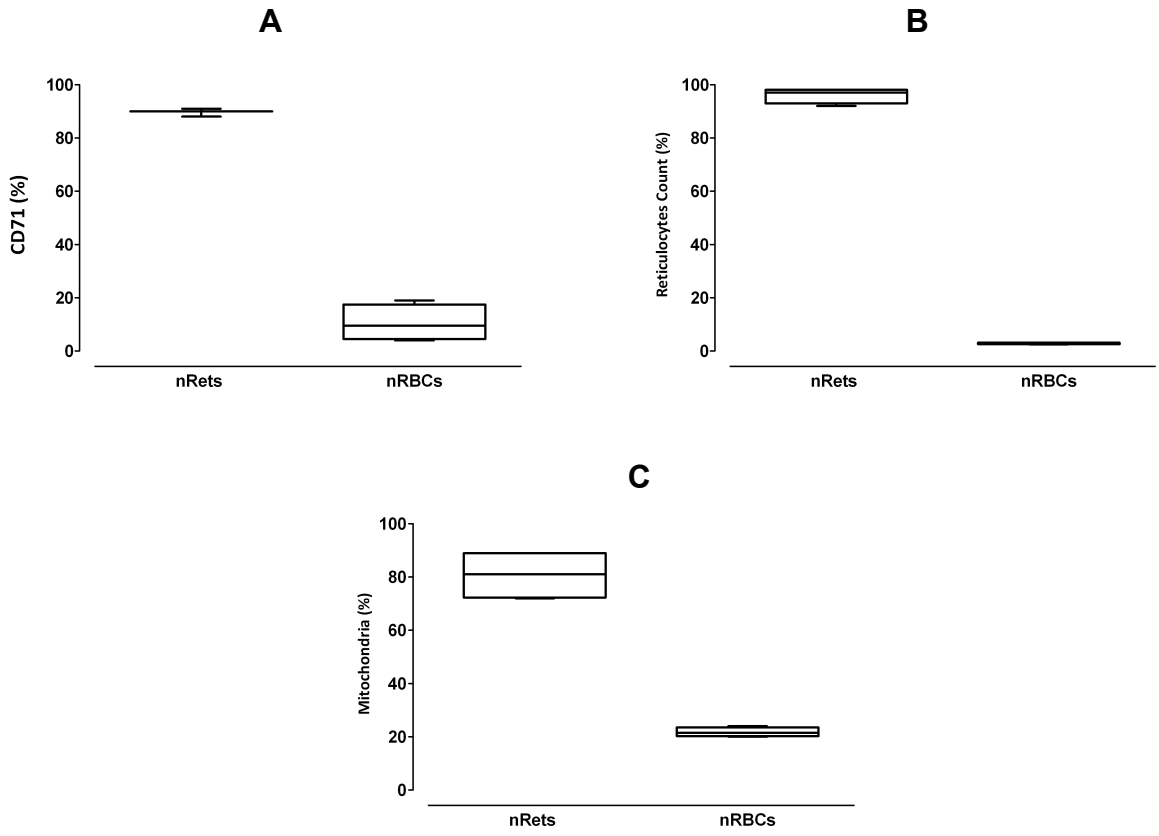
Supplemental Figures



Supplementary Figure 1: *In vitro* production of RBCs from cord blood CD34$^+$: The method used comprises three steps. In the first step, CD34$^+$ cells purified from Umbilical Cord Blood are grown for 8 days in medium supplemented with stem cell factor (SCF), interleukin-3 (IL-3) and erythropoietin (Epo). Then (second step), the cells are cultured on the murine MS-5 stromal cell line in the presence of Epo (day 8 to day 11). In the third step, all exogenous factors are withdrawn and the cells are grown further on MS-5 stromal cells without cytokines for up to 5 days. This protocol permits massive erythroid expansion and complete differentiation into mature cultured Red Blood Cells (cRBCs).



Supplementary Figure 2: Deformability profiles of cultured reticulocytes before and after maturation:
Cultured reticulocytes (cRets) were recovered at day 15 of culture and purified using a deleukocyting filter. Culturing the purified cRets for 8 supplementary days set up a maturation step. The deformability (elongation index, EI) of the cRets (n=3) was evaluated on LORCA over a range of shear stresses (0.3-30 Pa) before (black curve) and after the maturation step (grey curve).



Supplementary Figure 3: Maturation of native reticulocytes into nRBCs :
After 8 days of maturation, native reticulocytes **(**nRets) exhibit a decrease in (A) Transferrin receptor (CD71) expression assessed by flow cytometry, (B) nucleic acids assessed by thiazole orange staining and (C) mitochondria assessed by JC-1 staining**.**

Supplemental Tables

Supplementary Table 1: List of ion observed in RBCs human samples, using UHPLC/Exactive and ZICpHILIC/Exactive

Identified metabolite	M	m/z (ESI-)	m/z (ESI+)	Molecular formula	Rt C18	Rt HILIC	Identification status
1,3-bis-P-Glycerate	265,95872	264,95089	nd	$C_3H_8O_{10}P_2$		9,50	a,d
1-Octanal	128,11956	nd	129,12739	$C_8H_{16}O$	10,10		a,b
1-L-Leucyl-L-Proline	228,1473924	227,14011	229,15467	$C_{11}H_{20}N_2O_3$	5,07	6,00	a,b,c
2-Deoxy-D-ribose-phosphate	214,02369	213,01586	nd	$C_5H_{11}O_7P$	0,90		a,d
2-Oxoglutarate	146,02097	145,01314	nd	$C_5H_6O_5$	1,20	8,10	a
3-Hydroxyglutaric acid	148,03662	147,02879	nd	$C_5H_8O_5$	1,40	8,20	a,b,c,d
4-Amino-deoxychorismic acid	225,06317	224,05534	nd	$C_{10}H_{11}NO_5$	5,99		a
5-Oxoproline	129,04204	128,03421	130.04986**	$C_5H_7NO_3$	0,80	4,50	a,b,c,d
Adenine	135,05394	134,04612	136,06177	$C_5H_5N_5$	1,30	3,40	a,b,c
Adenosine 3'diphosphate (ADP)	427,02886	426,02104	428,03669	$C_{10}H_{15}N_5O_{10}P_2$	1,05	8,25	a,b,c,d
Adenosine 5'diphophate (ATP)	506,99519	505,98737	508.00302*	$C_{10}H_{16}N_5O_{13}P_3$	1,00	8,80	a,b,c,d
Adenosine monophosphate (AMP)	347,06253	346,05471	348,07036	$C_{10}H_{14}N_5O_7P$	1,07	7,60	a,b,c,d
ADP ribose	559,07112	558,06329	560,07894	$C_{15}H_{23}N_5O_{14}P_2$	1,05	8,00	a
Alanine	89,04713	88.03931*	90,05495	$C_3H_7NO_2$	0,80	7,30	a,b,c
Asparagine	132,05294	131,04511	133,06076	$C_4H_8N_2O_3$	0,70	7,10	a,b,c
Aspartate	133,03695	132,02913	134,04478	$C_4H_7NO_4$	0,80	8,10	a,b,c
Benzoic acid/ Hydroxybenzaldehyde	122,03623	121,02840	nd	$C_7H_6O_2$	6,00	2,20	a,b,c
Benzoquinoneacetic acid	166,02606	165,01823	nd	$C_8H_6O_4$	5,60	7,50	a
R-Butyrylcarnitine	231,14650	nd	232,15433	$C_{11}H_{21}NO_4$	4,90	3,20	a,b,c,d
Caproic acid	116,08318	115,07535	nd	$C_6H_{12}O_2$		2,00	a,c
Carnitine	161,10464	nd	162,11246	$C_7H_{15}NO_3$	0,90	6,60	a,b,c,d
Choline	104,10699	nd	104,10699	$C_5H_{14}NO$	0,80	7,30	a,b,c
Citric acid	192,02645	191,01862	nd	$C_6H_8O_7$	1.09/1.65	9,60	a,b,c,d
Citrulline	175,09514	174,08731	176,10296	$C_6H_{13}N_3O_3$	0,80	7,50	a,b,c
Creatine	131,06892	130,06110	132,07675	$C_4H_9N_3O_2$	0,90	7,00	a,b,c,d
Cysteinylglycine (Cys-gly)	178,04066	177.03283**	179,04848	$C_5H_{10}N_2O_3S$	0,90	8,30	a,b,c
deoxyadenosine	251,10184	250,09379	252,10875	$C_{10}H_{13}N_5O_3$	1,16	6,50	a,b,c
Deoxyribose	134,05736	133.04953*	135.06518**	$C_5H_{10}O_4$	0,99	3,50	a,b,c
DHA	174,01588	173,00806	175.02371**	$C_6H_6O_6$	1,80	9,50	a,b,c
Dihydrothymine	128,05802	nd	129,06585	$C_5H_8N_2O_2$	0,80		a,b
D-Ribose	150,05227	149,04444	nd	$C_5H_{10}O_5$	0,90	6,30	a,b,c,d
D-Ribose-1-P	230,01860	229,01078	nd	$C_5H_{11}O_8P$	0,80	8,30	a,b,c
Ergothioneine	229,08794	228,08012	230,09577	$C_9H_{15}N_3O_2S$	1,00	7,30	a,b,c
Erythritol/ meso-Erythritol	122,05736	121,04953	nd	$C_4H_{10}O_4$		5,20	a,c
Fumarate	116,01041	115,00258	nd	$C_4H_4O_4$	1.00/1.80	8,50	a,b,c,d
GABA (g-Aminobutyric acid)	103,06278	nd	104,07060	$C_4H_9NO_2$		7,50	a,c
Glutamate	147,05260	146,04478	148,06043	$C_5H_9NO_4$	0,83	8,00	a,b,c,d
Glutamine	146,06859	145,06076	147,07641	$C_5H_{10}N_2O_3$	0,80	7,00	a,b,c,d
Glutathione oxidized	612,15141	611,14358	613,15923	$C_{20}H_{32}N_6O_{12}S_2$	3,00	9,20	a,b,c,d
Glutathione reduced	307,08325	306,07543	308,09108	$C_{10}H_{17}N_3O_6S$	1,47	8,30	a,b,c,d
Glyceraldehyde 3 phosphate	169,99747	168,98965	nd	$C_3H_7O_6P$		9,50	a
Glycerol-3-phosphate	172,01312	171,00530	nd	$C_3H_9O_6P$	0,80	8,00	a,b,c,d
Glycine	75,03148	nd	76,03930	$C_2H_5NO_2$	0,80	7,50	a,b,c
Hexose	180,06283	179,05501	nd	$C_6H_{12}O_6$	0,85	6,90	a,b,c,d
Hexose P	260,02916	259,02134	nd	$C_6H_{13}O_9P$	0,80	8,20	a,b,c,d
Hexose-bisphosphate	339,99550	338,98767	341.00332**	$C_6H_{14}O_{12}P_2$	0,90	9,40	a
Hippurate	179,05769	178,04986	nd	$C_9H_9NO_3$	7,13	1,79	a
Hydantoin-5-propionic acid	172,04785	171,04003	173,05568	$C_6H_8N_2O_4$	0,90		a
Hydroxyisovaleric acid	118,06244	117,05462	nd	$C_5H_{10}O_3$	4,00	2,60	a,b,c
Isovaleric acid/ valeric acid	102,06753	101,05970	103,07535	$C_5H_{10}O_2$		2,30	a,c
Acetylcarnitine	203,11520	nd	204,12303	$C_9H_{17}NO_4$	1,50	4,90	a,b,c,d
Lactate	90,03114	89,02332	nd	$C_3H_6O_3$	1,10	4,10	a,b,c
Leucine/ Isoleucine/ Norleucine	131,09408	nd	132,10190	$C_6H_{13}NO_2$	1.00/2.20	4,00	a,b,c
Lysine	146,10497	145,09715	147,11280	$C_6H_{14}N_2O_2$	0,70	9,30	a,b,c,d
Malate	134,02097	133,01314	nd	$C_4H_6O_5$	1,00	8,40	a,b,c,d
Malonic acid	104,01041	103,00258	nd	$C_3H_4O_4$	1,08	8,30	a,b,c
N-Acetyl-4-Aminosalicylic acid	195,0531577	194,04588	196,06044	$C_9H_9NO_4$	4,80	7,00	a,b,c
N-Acetyl-Glucosamine	221,08994	220,08266	222,09722	$C_8H_{15}NO_6$	0,83	5,70	a,b,c
N-Acetyl-L-aspartic acid	175,04752	174,03969	nd	$C_6H_9NO_5$		7,80	a,c
N-Carbomoyl-L-Aspartate	176,04277	175,03494	177,05059	$C_5H_8N_2O_5$	1,00	8,60	a

Niacinamide	122,04746	121,03963	123,05528	$C_6H_6N_2O$		2,50	a,c
Nicotinamide adenine dinucleotide (NAD)	664,11639	662,10074	664,11639	$C_{21}H_{28}N_7O_{14}P_2$	1,07	7,70	a,b,c
Nicotinamide adenine dinucleotide phosphate (NADP)	744,08272	742,06707	744,08272	$C_{21}H_{29}N_7O_{17}P_3$	1,00	8,80	a,b,c
O-propanoyl-carnitine	217,13085	nd	218,13868	$C_{10}H_{19}NO_4$	3,50	3,75	a,b,c,d
O-Phospho-L-serine	185,00892	184,00164	186.01620*	$C_3H_8NO_6P$		8,02	a,c
Ornithine	132,08932	131,08150	133,09715	$C_5H_{12}N_2O_2$	0,80	8,75	a,b,c
Oxalic acid	89,99476	88.98693*	91.00258**	$C_2H_2O_4$	0,90	8,90	a,b,c
P-Glycerate	185,99239	184,98456	nd	$C_3H_7O_7P$	1,03	8,80	a,b,c,d
P-Enolpyruvate	167,98182	166,97400	nd	$C_3H_5O_6P$		9,00	a,c,d
Phenylalanine	165,07843	164.0706*	166,08625	$C_9H_{11}NO_2$	4,20	3,25	a,b,c
Proline	115,06278	nd	116,07060	$C_5H_9NO_2$	0,90	6,00	a,b,c,d
Pyruvate	88,01549	87,00767	nd	$C_3H_4O_3$	0,90	3,10	a,b,c
Quinic acid	192,06283	191,05501	nd	$C_7H_{12}O_6$	0,90	6,70	a,b,c
Serine	105,04204	104.03421*	106,04986	$C_3H_7NO_3$	0,80	7,20	a,b,c
Sorbitol	182,07848	181,07066	nd	$C_6H_{14}O_6$	0,90	6,90	a,b,c
Spermidine	145,15734	nd	146,16517	$C_7H_{19}N_3$	0,70		a,b,d
Spermine	202,21519	nd	203,22302	$C_{10}H_{26}N_4$	0,70		a,b
Stachydrine (Proline betaine)	143,09408	nd	144,10190	$C_7H_{13}NO_2$	0,95	4,50	a,b,c
Succinate	118,02606	117,01823	nd	$C_4H_6O_4$	2,00	7,90	a,b,c,d
Taurine	125,01411	124,00629	126,02194	$C_2H_7NO_3S$	0,80	6,80	a,b,c
Threonic acid	136,03796	135,03013	nd	$C_5H_4N_4O$	0,80	6,30	a,b,c,d
Threonine	119,05769	118,04986	120,06551	$C_4H_9NO_3$	0,80	6,60	a,b,c
Trimethylamine oxide	75,06786	nd	76,07569	C_3H_9NO	0,85	4,60	a,b,c
Tyrosine	181,07334	180,06551	182,08116	$C_9H_{11}NO_3$	2,30	5,30	a,b,c,d
Urate	168,02779	167,01996	169.03561**	$C_5H_4N_4O_3$	1.07/1.60	6,40	a,b,c
Valine	117,07843	116,07060	118,08625	$C_5H_{11}NO_2$	1,00	5,00	a,b,c,d

* nd in C18
** nd in HILIC
(a) Identification based on accurate mass
(b) Identification based on C18 retention time
(c) Identification based on HILIC retention time
(d) Identification based on MS spectra

Supplementary Table 2: **Immunophenotypic and functionality caracterisation of cultured reticulocytes (cRets) and red blood cells (cRBCs)**

	cRets	cRBCs
Retic Count (%)	89±10,4	8±1,6
CD 71 (%)	89±10,4	26± 7
GlycoA (%)	100±0	100±0
CD 36 (%)	37±34,8	3±3,3
VGM (fL)	128±11,4	114±7,4
TCMH (pg)	29±2,8	30±3,8
CCMH (g/dL)	22±0,24	26±2,2

The results are expressed as the mean ± SEM

Supplementary Table 3: Genetic correlation for the 11 metabolites differentially expressed in native (nRBCs) and culured red blood cell (cRBCs) using coefficient of variation (CV, %)

	Stachydrine	R-butyryl-carnitine	Taurine	Carnitine	O-propanoyl-carnitine	Trimethylamine Oxide	Acetyl carnitine	Sorbitol	DHA	Hexose-P	1,3-Bis-P-D-Glycerate
cRBCs	14.6	95.9	12.0	29.9	59.1	103.2	10.5	28.6	23.3	28.0	39.3
nRBCs	9.3	32.9	7.4	27.8	60.9	84.5	28.3	7.3	50.8	25.8	22.1

No correlation is associated with CV below 25-30%.