

Title: Sex-dependent membranopathy in stored human red blood cells

Running title: Sex-dependent membranopathy in stored human RBCs

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Data sharing statement

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Author Contributions

E. S-M. - study design, flow cytometry data collection, analysis and interpretation, writing and drafting of the manuscript; **T.M.** - ektacytometry data collection, analysis, interpretation and writing; **K.B.** - biochemical data collection, analysis and interpretation; **M.K.** - AFM data collection, analysis and interpretation; **A.W.** - preparation of samples for all measurements, biochemical and morphological data analysis; **K.M.M.** - concept, design, founding, writing and final approval of the version to be published.

Conflict of Interest Disclosure

The authors declare no competing interests.

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Research Limitation

The experimental setup was based on twelve healthy male (<30 years, N=3; 30-39 years, N=4; >40 years, N=5) and twelve female donors (<30 years, N=4; 30-39 years, N=4; >40 years, N= 4) so age was evenly distributed in the range of 18-60 years for fRBCs and mRBCs. However, conclusions regarding comparison of specific age groups presented in Supplementary Materials should be drawn in relation to relatively small N=(3-5). Observed processes may be an outcome of sex-related hormones action, which were not studied in this work. Major limitations of this study could be addressed in future research.

Over the past years, concern has been raised over the impact of red blood cell (RBC) alterations related to prolonged hypothermic storage on patient safety. Multiple reviews and works focus on presentation of a full list of changes characteristic for RBC storage lesions including alterations in RBC metabolomics, cytosol structure and membrane organization.¹⁻⁶ We have recently shown that a decrease in RBCs deformability was observed as a relatively late consequence of pRBCs storage, while the analysis of vesiculation observed on the RBC membranes in nanoscale with the application of atomic force microscopy (AFM) supported by analysis of biochemical parameters allowed for early changes detection.⁷ However, these results, as well as those of other groups were mainly obtained from pRBCs of male donors, or the sex of donors was not specified.^{5,8} Recently, it has also been suggested that RBCs from females are less prone to storage lesion and age slower than male erythrocytes^{9,10} but these reports were mainly focused on metabolic and functional RBC analysis.

In the present study, we investigated the influence of donor's sex on the sequence of changes observed during long-term storage of leukocyto-depleted pRBCs containing SAGM (Saline, Adenine, Glucose, Mannitol) additive solution and a trace amount of CPD (Citrate, Phosphate, Dextrose) preservative, which were purchased from the Regional Centre for Blood Donation and Haemotherapy in Krakow, Poland. According to the principles outlined in the World Medical Association Declaration of Helsinki, as well as a Bioethical Commission of the Jagiellonian University, venous blood for pRBCs was obtained from volunteers including men (mRBCs) N=12, aged <30 years (N=3), 30-39 years (N=4), >40 years (N=5) and women (fRBCs) N=12, aged <30 years (N=4), 30-39 years (N=4), >40 years (N= 4). All analyzes were carried out weekly throughout six weeks of pRBCs storage, while the seventh and eighth week's measurements were designed as additional time points exceeding pRBCs' expiration date (42 days), and were focused on the membranopathy on the level of pRBC membrane biochemistry, physical and mechanical properties (Fig. 1), as well nanoscale changes (Fig. 2).

Throughout the storage time, statistically significant differences between mRBCs and fRBCs in the kinetics of cholesterol and triglycerides' increase were observed (Fig. 1A,B). An increase between the first and eighth week was 2.27 times greater for cholesterol and 1.43 times greater for triglycerides in case of male donors. Such increase in lipid fraction in sheathing solution during pRBC storage is related to lipidome alterations and disruption of phospholipid asymmetry in the RBC membrane.¹¹ Our recent work and results of other groups suggest that release of lipids from RBCs membranes can be correlated with RMPs formation.¹² Our outcomes agree with previous reports showing that in the fresh sample cholesterol level is similar in both sexes, while triglyceride level is lower in women.^{13,14}

The levels of free iron, renown and specific indicator of hemolysis, were higher in mRBCs, suggesting greater hemolysis rate (Fig. 1D), what agrees with previous results.^{4,10} LDH levels in both

males and females below 40 years old do not differ, but higher average levels (Fig. 1C) of LDH in fRBCs could originate from higher skeletal muscle damage in post-menopausal women¹⁵ (age>40 years old, Suppl. Fig. 1E). Changes in glucose and lactic acid concentration (Fig.1 E,F), which are a natural consequence of glycolysis pathway, didn't show any sex-driven divergence. Additionally, some variations in individual values of glucose, lactate and free iron were observed in the youngest donors (< 30 years old), while cholesterol levels were most variable in patients aged 30-39 years (Suppl. Fig. 1C). The variation in the youngest blood donors may be related to previously reported 'healthy donor effect'.¹⁶

Anti-CD45 labeling proved the purity of pRBCs, showing only 0.1- 0.2 % of the whole cell population to be CD45-positive. From around fourth-fifth week of storage the expression of CD45 and CD71 (marker of reticulocytes) started to increase in mRBCs comparing to fRBCs (Suppl. Fig. 2). This could be a result of faster reticulocyte and/or leukocyte leftovers maturation in mRBCs during storage. In case of both CD45 and CD71, minor changes were observed regardless of donors' age. The level of CD47 ('don't eat me' signal) expression on the RBC membranes differed significantly between mRBCs and fRBCs throughout the storage period (Fig. 1G). Over time, it decreased age-independently in mRBCs, but did not change significantly in fRBCs (Suppl. Fig. 2). Exposure of CD47 was previously found as a result of storage-dependent proteolytic cleavage, oxidation and/or conformational changes caused by rearrangements of the phospholipid bilayer.¹¹ Our results of CD47 expression analysis confirm conformational changes of RBC membrane and phospholipid bilayer destabilization in mRBCs that lead to erythrocyte clearance¹⁷ as well as prove that basal composition of RBC membrane is sex specific.

Expression of phosphatidylserine (PS, a marker of senescence or damage) was found to increase during fRBCs and mRBCs storage¹⁸ but our results suggests that mRBCs are more prone to undergo membranopathies that result in higher PS exposure, at least at some point of their lifespan (Fig. 1H, Suppl. Fig. 2). In both sexes the values of maximum deformability were decreasing age-independently with the same speed during storage, whereas fRBCs retained higher deformability compared to mRBCs at every point of storage (Fig. 1I & Suppl. Fig. 3). These data agree with previous research¹⁹, which showed lower RBC deformability in males. RBC, HGB, HCT and MCHC were significantly higher in mRBCs compared to fRBCs throughout entire storage time (Fig.1). No differences in MCH values between mRBCs and fRBCs were detected. MCV (Fig.1J) was relatively stable up to the fifth week of storage, at which point we saw a significant increase in MCV only in mRBCs. This is indicative of greater swelling of mRBCs during long-term storage, which is due to higher alterations in their membranes, as reflected by higher leakage of membrane lipid and their lower deformability. Apart from MCV increase, a slight decrease in MCHC (Fig.1L) levels during storage of mRBCs confirms cell membrane loss.²⁰

In order to study the size of RBC microparticles (RMPs) appearing on the RBCs surface in mRBCs and fRBCs during storage, we applied our recently published AFM methodology⁷ (Fig. 2). Different changes in the RMPs size were observed in two clear-cut time intervals. In mRBCs during the first interval lasting from the first to fourth week, the fluctuation of the RMPs mean size, oscillating around 150 nm, was observed. In case of fRBCs, a different tendency was observed, where the values representing RMPs sizes significantly increased from around 150 nm in the first week, up to around 200 nm in the fourth week. During the second time interval, observed phenomena reversed in both sexes. In mRBCs, a significant increase in the values from around 150 nm up to 200 nm was observed, while in fRBCs the RMPs sizes stabilized on the level of ca. 160 nm until the end of the storage.

Our findings shed a new light on pRBC sex-derived differences in the performance during storage related to RBC membranopathy, which are summed up in Fig. 3. To the best of our knowledge, in this work for the first time we have presented significant sex-related differences between the kinetics of both cholesterol and triglyceride level changes observed in mRBCs and fRBCs. Our results show that increased kinetics of RBC membrane lipid leakage is related with shedding of RMPs of smaller size (ca. 150 nm) until the fourth week of storage and bigger RMPs (ca. 200 nm) starting from the fifth week. On the other hand, our results reveal that shedding of large RMPs until the fourth week of storage, seems to be an adaptation of fRBCs to adverse conditions and show their higher resistance in comparison to mRBCs. This is reflected by a slower membrane lipid leakage and higher deformability of fRBCs during the whole time of storage. A relation observed between membrane lipids leakage and RMPs formation suggests that the mechanism of RMPs shedding, RMPs composition and RBCs' response to storage conditions are all sex-related. These changes are accompanied by higher level of hemolysis and decreased deformability at each time point of pRBCs storage observed for male donors. Statistically significant increase in MCV and CD47 expression of mRBCs in comparison to fRBCs during prolonged storage confirmed stronger swelling of mRBCs, what originates from greater alterations of their membranes including rearrangements of the phospholipid bilayer. fRBCs were shown to be more resilient to unfavorable milieu what was reflected by their higher deformability, no changes in MCV, slower membrane lipid leakage and lower hemolysis. Cold storage seems to affect mRBCs in a different, yet more severe way, while fRBCs cope with adverse conditions more efficiently what should be consider by modern transfusion medicine. Results may have an impact on future studies regarding the need for greater control over the process of selection, assignment, and administration of blood labile products. Moreover, they might be a call for re-evaluation and setting new standards for storage of blood products.

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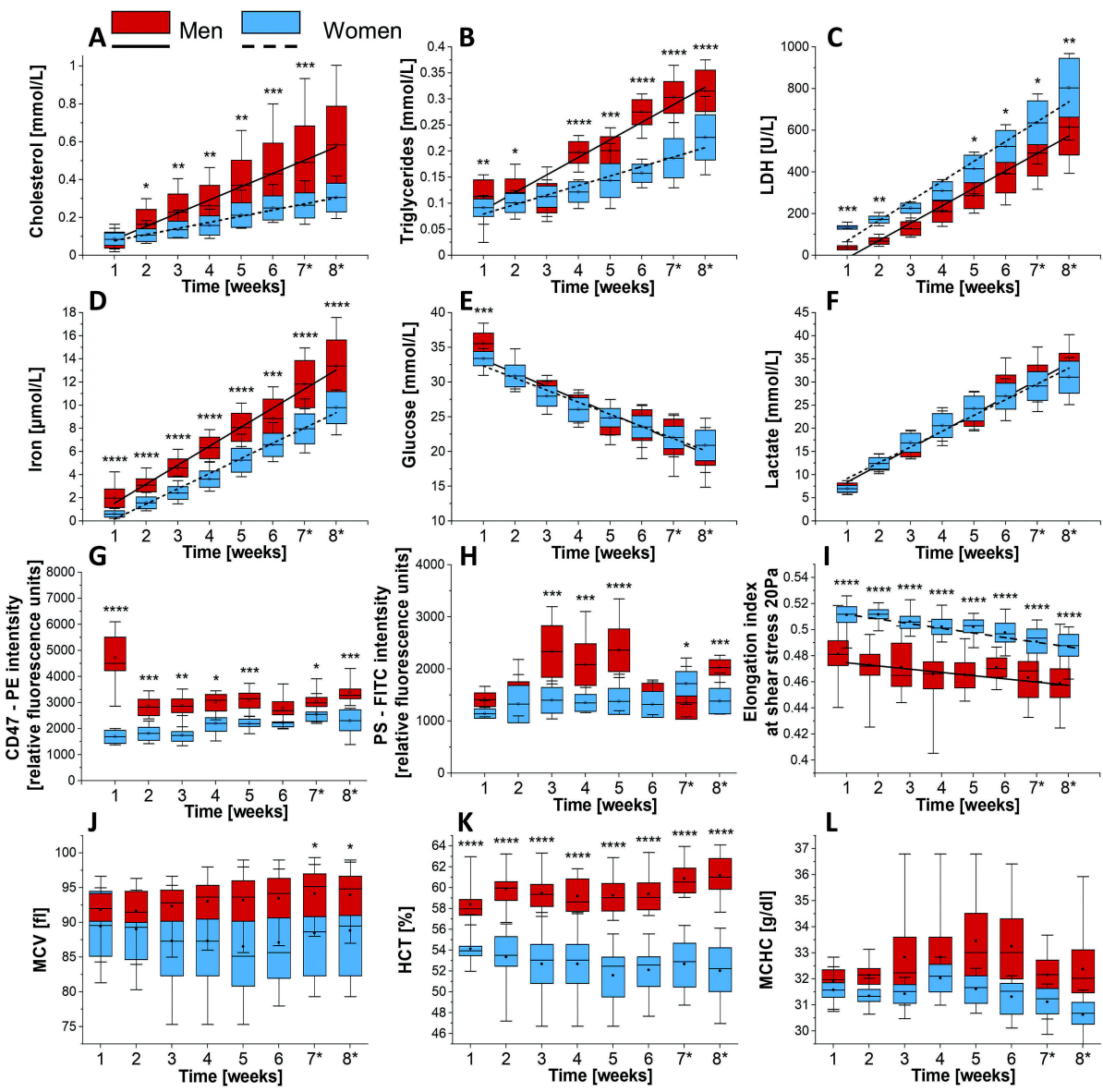
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Figures and figure legends

Figure 1. Storage time-dependent changes in biochemical parameters: (A) cholesterol, (B) triglycerides, (C) LDH, (D) free iron of human pRBCs, (E) glucose, (F) lactate; in chosen protein expression (G) CD47 and (H) phosphatidylserine (PS); (I) pRBCs deformability and in the blood count including (J) Mean Corpuscular Volume- MCV, (K) Hematocrit- HCT, (L) MCHC. The blood was withdrawn from men (N=12) and women (N=12). Results A-F obtained from pRBCs supernatant with ABX Pentra 400 (Horiba Medical, Japan) are given as mean \pm SD, data distribution is presented as box plots (mean value, mean \pm SD, min-max whiskers). Results G-L presented as box plots (median, Q1, Q3, interquartile range, min-max whiskers). Q1, Q3 indicate 25th and 75th percentiles, respectively. Results G, H were obtained using a mixture of mouse anti-human antibodies carrying fluorescent markers: CD45 – APC-Cy7 (1:200, BD cat. no. 348815), CD71 – APC (1:200, BD cat. no. 551374), CD47 – PE (1:200, BD cat. no. 556046) and Annexin V – FITC (1:200, BD cat. no. 556547). Cellular analysis was performed with LSRII flow cytometer (BD Biosciences). RBC deformability was studied at 37 °C as elongation index at shear rates of 20 Pa with the use of ektacytometry RheoScanAnD 300 (RheoMeditech, Seoul, Korea) while parameters J-L with hematology analyzer Abc Vet (Horiba Medical, France). Data A-L normality was assessed using Shapiro-Wilk test. The significance of the differences between men and women in each week was evaluated by One-Way ANOVA with Tukey's test. If data distribution was not normal, non-parametric Kruskal-Wallis test with the Dunn's post-hoc test if appropriate; *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001 indicates significant difference between men and women in each week.

Figure 2. Time sequence of the appearance of microparticles (RMPs) on the surface of human pRBCs isolated from: (A) men, (B) women. Examples of atomic force microscopy (AFM) images with the use of WITec confocal CRM alpha 300 in non-contact mode (AC) (WITec, Ulm, Germany) and dry Zeiss objective (ECEPIPLAN 20x/0.4). AFM images of 256x256 lines and 512x512 lines were collected from areas of 25x25 μm^2 , 8x8 μm^2 and 1.5x1.5 μm^2 , which were performed at room temperature on dried smears of (A) fRBCs and (B) mRBCs fixed with 1% glutaraldehyde (10 min). (C) **Sex-specific, time-dependent changes of the RMPs sizes observed on the surface of human pRBCs.** Data distribution is presented as box plots (median, Q1, Q3, interquartile range, min-max whiskers). Q1, Q3 indicate 25th and 75th percentiles, respectively. * Weeks 7 and 8 are additional measurements exceeding expiration date. Statistical significance of the obtained (N>35) was tested with Kruskal-Wallis ANOVA nonparametric test followed by Tukey's post-hoc (NS - not significant; *p < 0.05; **p < 0.01, ***p < 0.001, ****p < 0.0001).

Figure 3. Schematic summary of the sex-specific, time-dependent changes in pRBCs derived from men (N=12) and women (N=12). PRBCs were cold- stored for 8 weeks (weeks 7 and 8 being additional timepoints exceeding expiration date). Physiological parameters were assessed with use of biochemical analysis, morphological analysis, flow cytometry, ektacytometry and atomic force microscopy (AFM).



Storage-related changes

lactate ↗

glucose ↘

LDH ↗

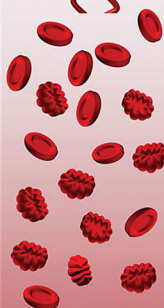
triglycerides ↗

RBC deformability ↘

free iron ↗

cholesterol ↗

hemolysis ↗



Initial stage of storage

Late stage of storage

Sex-dependent changes related to storage

♀ fRBCs

RBC deformability, LDH,
RMPs size

free iron, triglycerides, CD47
cholesterol

size of RBC microparticles ↘

RBC deformability, LDH
free iron, CD47

RMPs size, cholesterol,
triglycerides

lipids leakage

relation



♂ mRBCs

RBC deformability, LDH,
RMPs size

free iron, triglycerides, CD47
cholesterol

size of RBC microparticles ↗

RBC deformability, LDH
free iron, CD47

RMPs size, cholesterol,
triglycerides

lipids leakage

SUPPLEMENTARY INFORMATION

Title: Sex-dependent membranopathy in stored human red blood cells

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Men

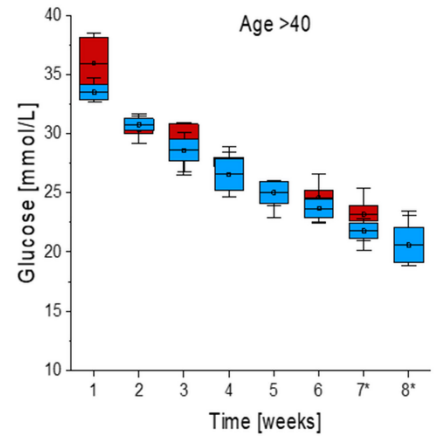
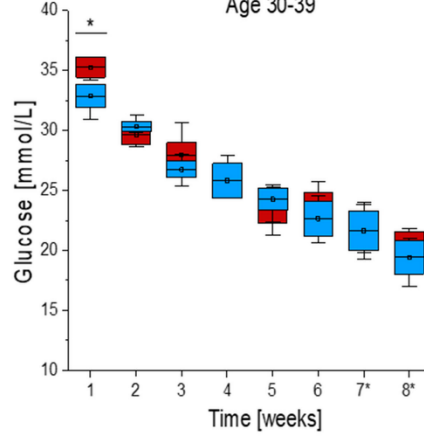
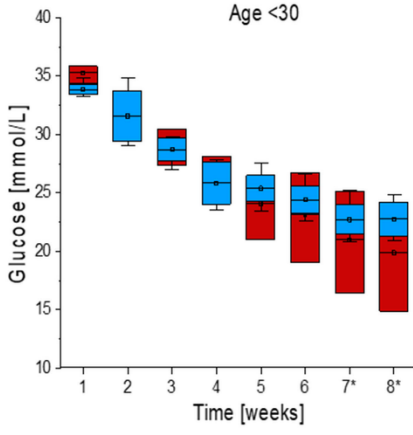
Women

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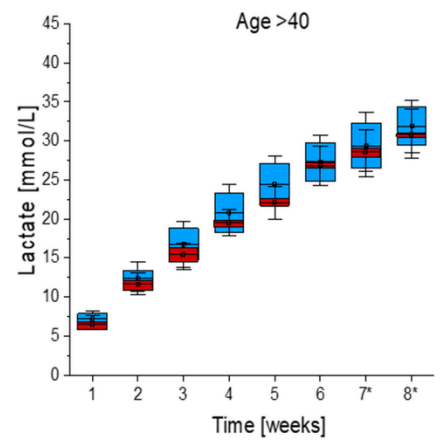
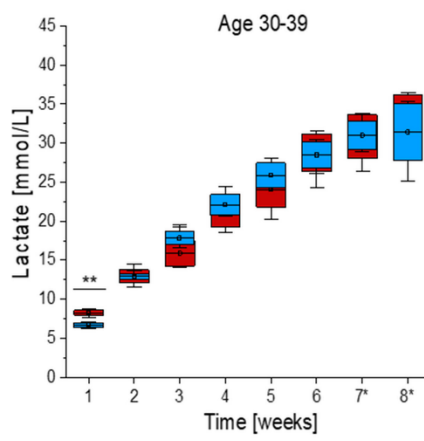
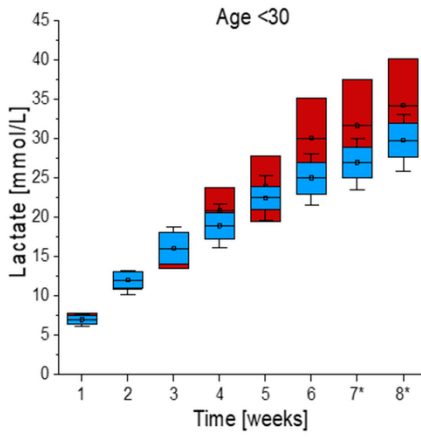
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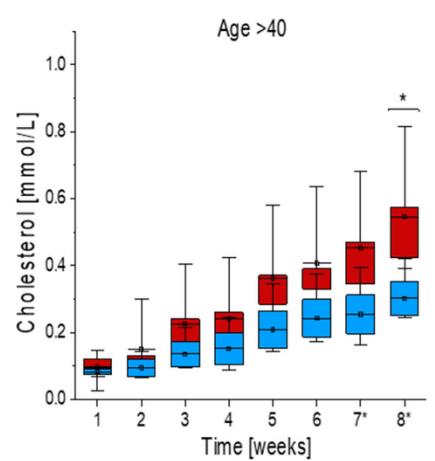
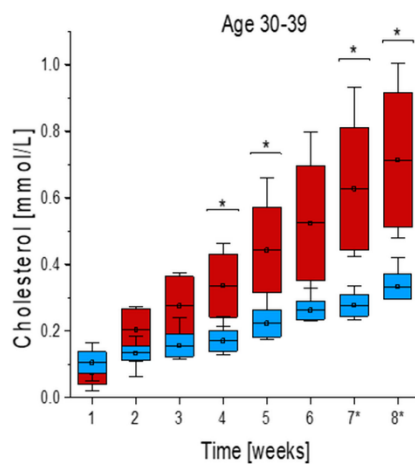
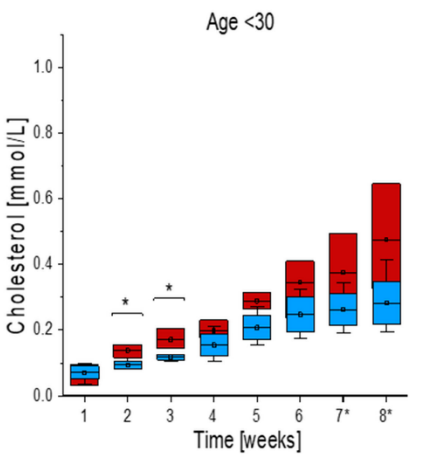
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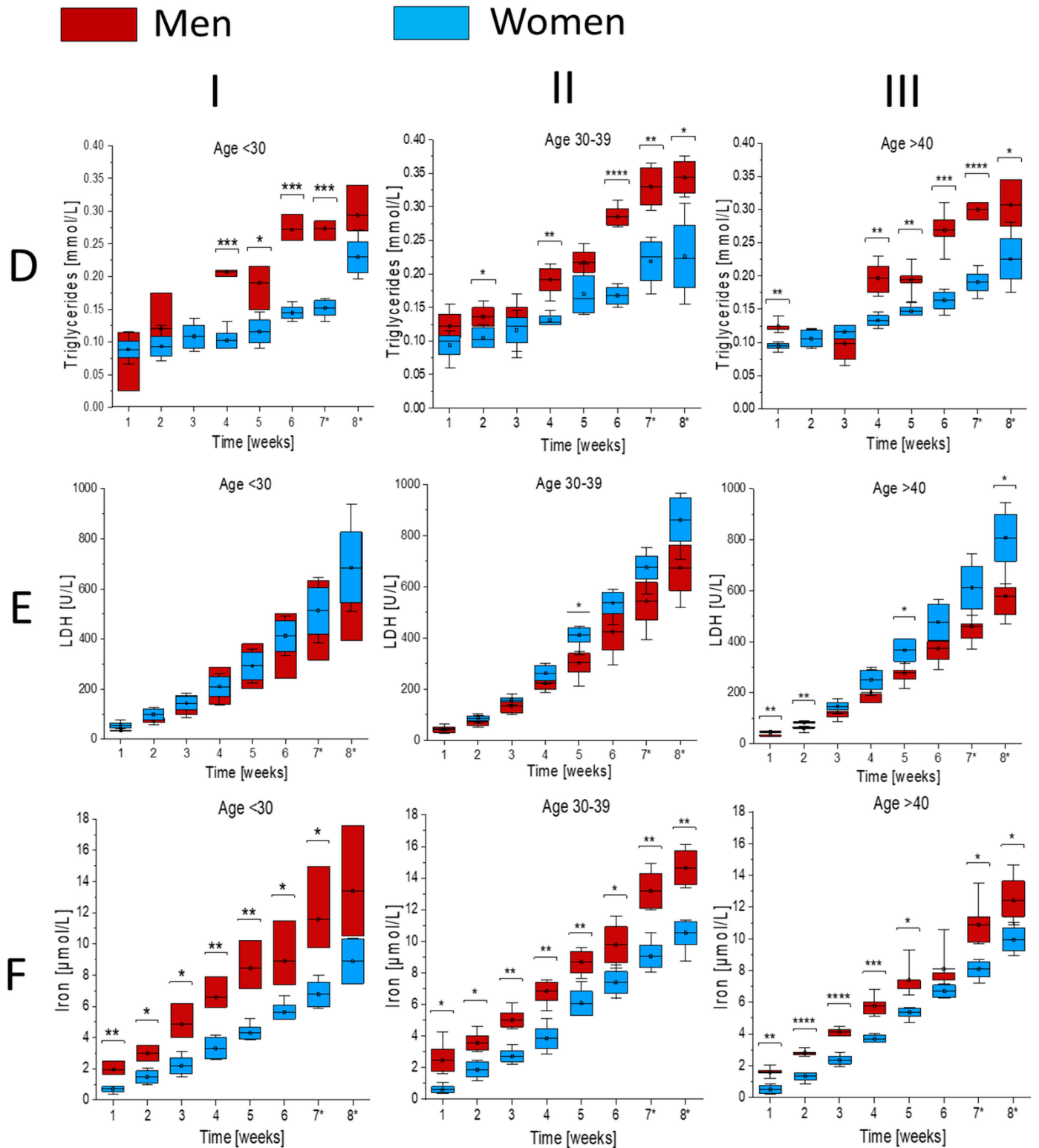


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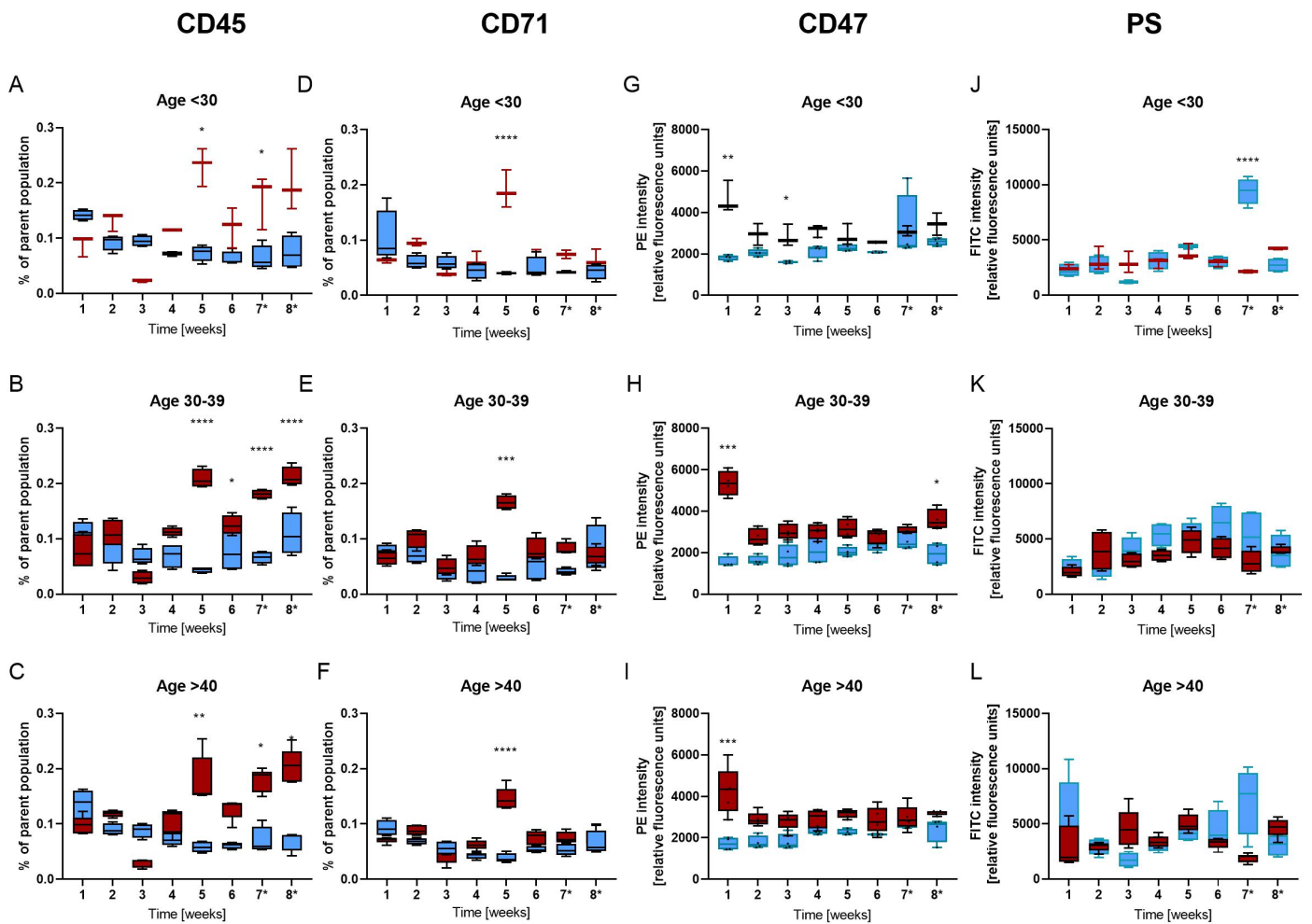


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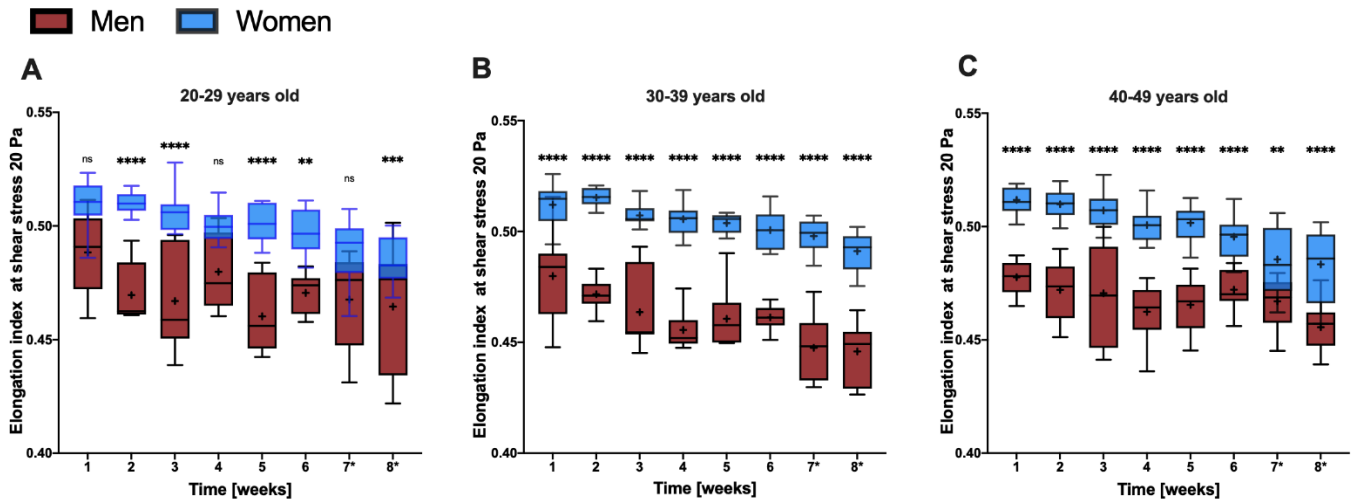




Supplementary Figure 1. Storage time-dependent changes in biochemical parameters: (A) Glucose, (B) Lactate, (C) Cholesterol, (D) Triglycerides, (E) LDH and (F) Iron in human pRBCs. The blood was withdrawn from both men (N=12) and women (N=12) aged (I) <30, (II) 30-39 and (III) >40. Data distribution is presented as box plots (mean, Q1, Q3, interquartile range, min-max whiskers). Q1, Q3 indicate 25th and 75th percentiles, respectively. Data normality was assessed using Shapiro-Wilk test. The significance of the differences between the means was evaluated either by one-way ANOVA with Tukey's post-hoc test or by Kruskal-Wallis test if appropriate; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ indicates significant difference between men and women in each week. * weeks 7 and 8 are additional measurements exceeding expiration date.



Supplementary Figure 2. Storage time-dependent changes in CD45 (A-C), CD71 (D-F), CD47 (G-I) and phosphatidylserine-PS (J-L) expression in human pRBCs. The blood was withdrawn from men (N=12) and women (N=12) aged (A,D,G,J) <30, (B,E,H,K) 30-39 and (C,F,I,L) >40. Fluorescence was measured weekly with use of flow cytometry throughout 8 weeks of storage, * weeks 7 and 8 are additional measurements exceeding expiration date. Data are presented as a percent of parent population. Data distribution is presented as box plots (median, Q1, Q3, interquartile range, min-max whiskers). Q1, Q3 indicate 25th and 75th percentiles, respectively. Data normality was assessed using Shapiro-Wilk test. The significance of the differences between the medians was evaluated either by one-way ANOVA with Sidak's post-hoc test or by Kruskal-Wallis test with Dunn's post-hoc test if appropriate; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, indicates significant difference between men and women in each week.



Supplementary Figure 3. Storage time-dependent changes in pRBCs deformability in men and women according to their age: A) 20-29 years old B) 30-39 years old C) 40-49 years old. Elongation index was measured weekly with use of ektacytometry throughout 8 weeks of storage, * weeks 7 and 8 are additional measurements exceeding expiration date. Data distribution is presented as box plots (median, Q1, Q3, interquartile range, min-max whiskers). Q1, Q3 indicate 25th and 75th percentiles, respectively. Normality was assessed using Shapiro-Wilk test. The significance of the differences between the medians was evaluated by Kruskal-Wallis test with the Dunn's post-hoc test if appropriate; ****p<0.0001 indicates significant difference between men and women in each week.

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