RESEARCH ARTICLE

Human erythrocyte surface fucose expression increases with age and hyperglycemia [version 1; peer review: 1 not approved]

Huan Cao¹, Ananyo Bagchi¹, Dimitrios Tampakis^{2,3}, Irina Laidvee¹, Maria Williams¹, Beverley Minter⁴, Sonja Wright⁵, Aristotelis Antonopoulos⁶, Stuart M. Haslam⁶, Robert N. Barker^{1*}, Mark A. Vickers^{1,7*}

¹School of Medicine, Medical Sciences and Nutrition, University of Aberdeen, Aberdeen, UK

²Centre for Biological Engineering, School of Mechanical, Electrical and Manufacturing Engineering, Loughborough University, Loughborough, UK

³Division of Cancer Studies, King's College London, London, UK

⁴Department of Archeology, University of Aberdeen, Aberdeen, UK

⁵Department of Haematology, Aberdeen Royal Infirmary, Aberdeen, UK

⁶Department of Life Sciences, Imperial College London, London, UK

⁷Scottish National Blood Transfusion Service, Aberdeen, UK

* Equal contributors

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Abstract

Background: Reactive oxygen species and other free radicals, together with glucose and its metabolites are believed to play important roles in the aging process. The carbohydrate components of glycosylated proteins are important in mediating cell-cell interactions and a role has been suggested for them in the aging process. Erythrocytes are critical cells in the human body, heavily glycosylated and relatively easily available and so are good candidates to yield insights into how patterns of glycosylation change with age and disease. It has been claimed, based on a periodic acid Schiff assay, that human aging is associated with a decline of erythrocyte surface sialic acids. Plant lectins allow for more specific assays for glycans, including determining the linkage of sialic acids and analysis of single cells by flow cytometry.

Methods: Plant lectins, including *Maackia amurensis* lectin II (MAL), binding to α -2,3 linked sialic acids and *Sambucus nigra* (SNA), α -2,6 sialic acids, were used in flow cytometry and western blot of erythrocyte surface membrane. N-glycomics mass spectrometry determines glycan structures. Donors varying in age and hyperglycemia, as indicated by HbA1c were analysed.

Results: Erythrocyte surface sialic acids have no significant associations with donor age. A combination of storage and cellular

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report

1. Nitin Bhardwaj, Gurukul Kangri University, Haridwar, India

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aging produces a specific loss of α -2,6 sialic acids. By contrast, erythrocyte surface terminal fucoses increase significantly with donor age. In order to determine which aspects of aging are important in determining this change, we investigated whether this novel human aging biomarker is associated with higher plasma glucose values, assessed by glycated hemoglobin (HbA1c) and reactive oxygen species (ROS) generation. Fucose levels were associated with HbA1c levels, but not ROS generation.

Conclusion: Our study identifies novel glycan-based biomarkers for human aging and disease. The simplicity of lectin-based assays provide an attractive cellular tool to study aging and disease processes.

Keywords

fucose, aging, hyperglycaemia, erythrocyte, lectins

Corresponding author: Huan Cao (h.cao@abdn.ac.uk)

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Abbreviations

AAL, *Aleuria aurantia* lectin; APF, α -fetoprotein; DSA-FACE, DNA sequencer-assisted fluorophore-assisted carbohydrate electrophoresis; gMFI, geometric mean fluorescence intensity; GPA, Glycophorin A; HbA1c, glycated haemoglobin; HCC, hepatocellular carcinoma; MAL, *Maackia amurensis* lectin II; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; MBL, mannose binding lectin; NANA, N-acetylneuraminic acid; PAS, periodic acid Schiff; ROS, reactive oxygen species; SNA, *Sambucus nigra*; U. euros, *Ulex europaeus* agglutinin I.

Introduction

The main function of erythrocytes is to carry oxygen from the lungs to the rest of the body and remove carbon dioxide as a waste product. Erythrocytes are highly glycosylated, with glycophorin as the main carrier¹, especially sialic acids. Sialic acids are negatively charged sugars and confer a bulk surface charge causing repulsion between cells, so preventing agglutination². They are also important signalling molecules as they are found at the periphery of cell surfaces³. Siglecs, sialic acid-binding immunoglobulin-type lectins, are surface receptors on immune cells that recognize sialic acids. For instance, erythrocyte sialoproteins inhibit neutrophil activation through interaction with Siglec-9⁴ and inhibit B cell activation through interaction with the lectin CD22 (unpublished reports, Charlotte S. Lennon, Huan Cao, Andrew M. Hall, Mark A. Vickers and Robert N. Barker).

The phrase aging can be interpreted in two ways: “cell-aging”, referring to increasing lifetime or replication of cells, or “organismal-aging”, referring to the age of the body from which cells are derived. There are many biologically significant cell-aging specific changes described for erythrocytes, including loss of potassium ions, organic phosphates and water⁵, decrease in cell surface area and volume⁶ and decrease in the activity of Na⁺/K⁺ ATPase and Na⁺/H⁺ exchanger⁷. Old red blood cells (RBCs) can also undergo a form of programmed cell death in a process known as eryptosis, which is triggered by calcium action on Ca²⁺/K⁺ channels, scramblase, calpain and other proteases, and leads to cell clearance⁸. Long term blood storage causes oxidative damage to RBCs and leads to dysfunction in glucose metabolism that contributes to decreased metabolism of glutathione (GSH)⁹.

Erythrocytes are excellent “reporter cells”¹⁰ for organismal-aging as they are readily available. However, only limited organismal-aging changes have been described for erythrocytes. In mice, their antioxidant capacity decreases from six to 26 months of life¹¹. In humans, a strong negative association between donor age and erythrocyte surface sialic acids has been reported¹². Furthermore, increased sialic acids in the plasma of corresponding donors suggested a cleavage process, which was attributed to increased oxidative damage to erythrocytes in older donors. In cell-aging of fibroblasts, Itakura and colleagues reported a similar decline in sialic acids: α -2,3 sialylation, α -2,6 sialylation for both N- and O-glycans¹³. Fucose is a sugar that can be attached close to

the core or outer antennae of N-glycan chains. Enzymes that remove or add fucose have been studied with respect to aging in different cell types^{14,15}. However, fucose on erythrocytes has not been studied with reference to either “cell-aging” or “organismal-aging”.

Changes in carbohydrates have been described in association with several diseases such as cancer, Alzheimer’s disease and diabetes mellitus^{16–18}. Our understanding of glycosylation in cancer has improved as diagnostic biomarkers involving carbohydrate changes have been established¹⁹. For example, the core-fucosylated form of α -fetoprotein (AFP) in serum is associated with hepatocellular carcinoma (HCC) and can be used as a marker for diagnosis²⁰. Functionally, abnormal glycosylation in cancer can dysregulate cell signalling and communication, cell-matrix interactions, tumour cell dissociation and invasion¹⁹. For example, the over expression of one component of the N-glycosylation machinery, the β 1,6-N-acetylglucosaminyl transferase (GnT-V) enzyme, in glioma cells leads to focal adhesions and increased tumour cell invasion²¹. Protein glycosylation has been reported to be defective in Alzheimer’s disease²². Numerous connections between glycosylation and diabetes are starting to be recognized: for example, high levels of mannose binding lectin (MBL) in sera are associated with vascular complications¹⁷ and nephropathy¹⁸. In diabetes mellitus, in addition to N- and O-glycosylation, glycation, the non-enzymatic addition of sugars to proteins is particularly important^{23,24} and measurement of the glycated N-terminal amino acid of hemoglobin, HbA1c, is an important clinical diagnostic and prognostic marker of diabetes²⁵.

Cellular aging in other cell types such as mesenchymal stem cells have been shown to be associated with the loss of sialic acids¹³. Several studies have investigated the relationship between age of erythrocytes and cell surface sialic acids. Methods using periodic acid have been the commonest way of measuring total sialic acid content from erythrocyte membrane preparations, known as ghosts. It has been presumed, but not proven, that periodic acid Schiff (PAS) assay is specific for sialic acids. A more recently developed glycobiological technique known as DNA sequencer-assisted fluorophore-assisted carbohydrate electrophoresis (DSA-FACE) has been employed to study N-glycan profile changes during human aging and found age-dependent alterations in the ratio of two key structures of N-glycans beyond the age of 40 in humans²⁶. N- and O- glycome profiles of cell surfaces can be fully analysed by mass spectrometry²⁷ as well as state of art technology such as the solid phase chemoenzymatic method²⁸. Human and plant lectins are becoming increasingly popular for studies of cell surface changes, as they offer direct binding to specific terminal glycans^{29,30}. The use of lectins with flow cytometry to determine cell surface glycosylation expression has the advantage of accessing all cell surface glycans available for binding, thus mimicking cell-cell interactions, contrasting with conventional techniques such as PAS staining. In this study, we use plant lectins to evaluate carbohydrate expression on red blood cells during human organismal-aging.

Results

Neuraminidase sensitivity observed by lectin flow cytometry but not periodic acid Schiff assay

Neuraminidase is an enzyme that specifically removes terminal sialic acids from glycoproteins³¹. Using this activity, we compared the classical PAS assay with the more modern approach of lectin flow cytometry for sialic acids on the cell surfaces of erythrocytes. *Maackia amurensis* lectin II (MAL) is a lectin specific for α -2,3 linked sialic acids and *Sambucus nigra* agglutinin lectin (SNA) is specific for α -2,6 linked sialic acids. Both lectins demonstrate high levels of binding by flow cytometry to untreated (sialylated) erythrocyte cell surfaces, but binding is lost once the erythrocytes have been treated with neuraminidase (Figure 1a). However, while the PAS assay detected increasing amounts of sialic acids in the form of synthetic sialic acids (N-acetylneuraminic acid, NANA) and the glycoprotein fetuin, which contains high levels of sialic acid, the assay was not sensitive to neuraminidase treatment of whole erythrocytes (Figure 1b). These data indicate that the PAS assay measures compounds in addition to surface accessible sialic acid residues, for which plant lectins MAL and SNA are more specific.

Specific cell surface α -2,6 sialic acid loss in storage plus cell-aging but no change to sialic acids in organismal-aging

Using lectins MAL and SNA, we probed gravity Percoll fractionated stored erythrocytes from healthy donors to assess changes during cell-aging. It is well known that during cell-aging erythrocytes increase their density, become more rigid and fragile, and more deposition of C3 is observed. Our gravity based Percoll fractionation technique separates erythrocytes based on density into 5–7 fractions (Supplemental Figure 1a and 1b,

Extended data)³², with approximately 1% cells represented in the densest fractions (5, 6, and 7). We confirmed higher fragilities (Supplemental Figure 1d, Extended data)³² and more C3 deposition (Supplemental Figure 1c, Extended data)³² in the densest fractions. Although we failed to observe any differences in MAL binding to RBCs from any fraction (Figure 2a–d), we observed highly specific SNA binding loss in the densest fractions (Figure 2a–d). The data from Figure 2 are generated from erythrocytes stored for seven or more days prior to fractionation. Western blotting identifies the SNA binding loss as arising from bands around 70kDa and 100kDa, corresponding to dimeric glycophorin A (GPA) and Band 3, respectively (Supplemental Figure 2, Extended data)³². We also tried to reproduce these findings in murine erythrocytes, but found they lacked SNA binding (Supplemental Figure 3a, Extended data)³², suggesting poor lectin visibility of surface α -2,6 sialic acids. There were no changes in MAL or SNA binding when murine RBCs were fractionated by density (Supplemental Figure 3b and 3c, Extended data)³².

With respect to organismal-aging, we observed that the binding of MAL and SNA to unfractionated erythrocytes showed no significant association with age of donors ($n = 24$) (Figure 3a and 3b), in contrast to the claims of Mehdi *et al.*¹². Overall, these data demonstrate, while there is a specific loss of α -2,6 sialic acid under the combination of storage and cellular aging, there are no overall cellular or organismal-aging related sialic acid changes to erythrocytes in humans.

Fucose specific lectin binding increases with donor age

In contrast to sialic acid binding lectins, binding of those specific for fucose on red blood cells increased significantly with age of donor (Figure 4a and 4b). *Aleuria aurantia* lectin

Figure 1. **a**) Flow cytometry plot of MAL and SNA staining of untreated and neuraminidase treated erythrocytes. Red, fully stained erythrocytes; green, secondary only control. **b**) PAS assay on neuraminidase treated (+) and untreated (-) erythrocytes, N-acetylneuraminic acid, NANA and fetuin at different C3 concentrations. MAL, *Maackia amurensis* lectin II; SNA, *Sambucus nigra*; PAS, periodic acid Schiff; NANA, N-acetylneuraminic acid.

Figure 2. **a)** Flow cytometry plot of MAL and SNA staining of gravity Percoll fractionated erythrocytes from Donor 1 (10 days storage prior to fractionation). Green: secondary only control, red, F1 and F2 combined; blue, lower fractions as indicated. Red box indicates prominent changes in lower fractions. **b)** As **a)** but from donor 2 (22 days storage prior to fractionation). Denser fractions compared to F1. **c – d)** As **a)** but from donor 3 – 4 (seven and nine days storage prior to fractionation, respectively). MAL, *Maackia amurensis* lectin II; SNA, *Sambucus nigra*.

Figure 3. **a)** Plot of geometric mean fluorescence intensity (gMFI) obtained from MAL lectin binding assay as a function of donor age. $p = 0.2541$. **b)** Plot of gMFI obtained from SNA lectin binding assay as a function of donor age. $p = 0.7528$. Data are pooled from two experiments ($n = 24$): Spearman's rank. MAL, *Maackia amurensis* lectin II; SNA, *Sambucus nigra*.

(AAL) binds preferentially to fucose linked (α -1,6) to N-acetylglucosamine or fucose linked (α -1,3) to N-acetylglucosamine related structures³³. *Ulex europaeus* agglutinin I (*U. Euro*) binds to many glycoproteins and glycolipids containing α -linked fucose residues (Boyd, 1963). While *U. Euro* has a

preference for blood group O erythrocytes, AAL has no specificity for blood groups³³. For both lectins, the associations between binding and age of donor were similar, which indicates that the underlying basis for the increase in binding with age is an increase in terminal fucoses.

Figure 4. **a**) Plot of *Aleuria aurantia* (AAL) lectin binding by flow cytometry (normalised gMFI) in relation to donor age. Data are pooled from two experiments: $n = 24$, Spearman's rank. **b**) As (a) but with *U. Euros* lectin. **c**) MALDI-TOF mass spectra (m/z versus intensity) for glycomic analysis of N-glycans from membrane ghosts from healthy donor. All ions are $[M+Na]^+$. Structures above the brackets have not been unequivocally defined. "M", "m" and "vm" annotations above the structures corresponds to major, minor and very minor abundances. Annotation uses conventional symbols for carbohydrates in accordance with <http://www.functionalglycomics.org> guidelines: purple diamond, sialic acids; yellow circle, galactose; blue square, N-acetyl glucosamine; green circle, mannose; red triangle, fucose. **d**) MALDI-TOF mass spectra (m/z versus intensity) for glycomic analysis of O-glycans from membrane ghosts from healthy donor. All ions are $[M+Na]^+$. Structures outside the brackets have not been unequivocally defined. gMFI, geometric mean fluorescence intensity; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; A. Aur, *Aleuria aurantia*; U. Euros, *Ulex europaeus* agglutinin I.

Mass spectrometry based glycomic analysis identifies sialic acid and fucose containing structures on erythrocytes

Erythrocyte membrane ghosts were subjected to N-linked (Figure 4c) and O-linked (Figure 4d) glycomic analysis by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). Fucose bearing structures were identified only on N-glycans, while sialylated structures were found as multiple complex N-glycans and three dominant O-glycans. While the N-linked glycomic analysis here does not distinguish between different linkages for fucose attachment, an abundance of complex and hybrid structures were shown to contain fucose. Further experiments combining lectin binding and glycomic analyses could help to identify the age specific glycan structure.

Binding of fucose-specific lectin AAL is associated with hemoglobin HbA1c levels but not cellular ROS generation

We investigated the underlying basis for the association between erythrocyte fucose expression and age by assessing whether it might be secondary to two aspects that have been linked with

aging, glycation and oxidative stress^{34–37}. Glycated hemoglobin (HbA1c) is a well-established clinical marker of long-term glycemic control³⁸, so we collected samples from patients with known HbA1c levels irrespective of age. Because of the age effect on fucose expression (Figure 3), we restricted our analysis to >45 years of age. We observed a positive association between HbA1c and AAL lectin binding levels (Figure 5a), which is the first erythrocyte surface marker associated with HbA1c changes.

Oxidative stress is associated with diabetes^{39,40} but no study has investigated the association between HbA1c levels and erythrocyte oxidation status. Here we show that the rate of internal reactive oxygen species (ROS) production is significantly elevated in red blood cells from patients with high HbA1c compared to a control HbA1c group (Figure 5b, c). Furthermore, there is a trend of positive association between HbA1c and ROS across both groups for patients of age 45 or above (Figure 5c). Yet, the correlation between ROS production and the binding of the fucose specific lectin AAL (Figure 5d) does not appear significant. These results indicate that the relationship between HbA1c and AAL binding levels is likely to be independent of erythrocyte ROS generation capacity.

Figure 5. Analysis of HbA1c levels in patients of age 45 or over. **a)** Comparison between HbA1c and AAL binding (normalised gMFI). Spearman's rank. **b)** Rate of ROS formation in erythrocytes compared between control and high HbA1c groups. **c)** HbA1c correlation with ROS. Spearman's rank. **d)** AAL correlation with ROS. Spearman's rank. AAL, *Aleuria aurantia* lectin; gMFI, geometric mean fluorescence intensity; ROS, reactive oxygen species.

Discussion

Periodic acid Schiff assay versus lectin staining

Traditionally, PAS assays have been used to stain for polysaccharides such as glycolipids and glycoproteins. They have also been used to measure sialic acids on red blood cells in a number of publications, despite no demonstration of specificity for the sialic acid sugar itself. We show here for the first time that the PAS assay does not specifically recognise sialic acids on the surface of erythrocytes by comparing staining of neuraminidase treated and untreated red blood cells, for which lectins such as MAL and SNA do demonstrate specific binding.

Lack of age dependent sialic acid changes

In contrast to previous reports^{12,41} of strong negative associations of sialic acids on the surface of erythrocytes with age of donor, we saw no significant associations between age and either α -2,3 or α -2,6 linked sialic acids. Lutz and Fehr⁴² also reported, using PAS staining, a loss of sialic acids attached to glycoproteins on aging erythrocytes. Such conclusions based on determining sialic acid changes through PAS staining may warrant re-examination. Nevertheless, upon storage and further fractionation based on density, we do observe a specific loss of α -2,6 linked sialic acids, which appears to be associated with dimeric GPA and Band 3. This specific glycan loss is only observed under both storage and fractionation, while α -2,3 linked sialic acids are consistently unchanged. Overall, our report indicates the stability of surface sialic acids during different types of aging of erythrocytes.

Fucose related changes

Unexpectedly, we showed a positive association of fucose expression on erythrocytes with age of donor. This has not been described before, although there are reports of decreases in plasma levels of N-glycans containing core fucose with aging⁴³. Perhaps both increased RBC surface fucose and reduced plasma fucose are explained by lower rates of enzymatic removal of RBC surface fucoses with age. However, since the lectins used in this study bind only to terminal fucose, we are not able to define the exact structure that is changing with age. N-glycomics mass spectrometry analysis provides an overview of possible fucose terminating structures, but further work would be needed to determine the structural changes with human organismal-aging.

Glycans as potential markers of aging

Changes in glycosylation are caused by diabetes, which is more prevalent amongst older populations. Glycation affects proteins in two ways. Early glycation simply adds carbohydrates, non-enzymatically, to amino groups carried on lysine or arginine residues, as well as N terminal amino acids, such as HbA1c modification. Advanced glycation, which is associated with oxidation, causes much more significant structural damage by creating cross-links between and within proteins through formation of advanced glycation end products (AGEs)^{44,45}. Our data show fucose expression on RBCs associated with the diabetes indicator, HbA1c, which is itself a product of glycation. Yet, fucose expression did not appear significantly related to oxidation capacity within RBC. These observations indicate that fucose levels are sensitive to the early phases of glycation.

Conclusions

Our study shows that by using more specific reagents for sialic acids, cell- and organismal-aging do not produce significant changes to the expression of α -2,3 and α -2,6 sialic acids on erythrocytes. A combination of storage and cell-aging can cause specific α -2,6 sialic acid loss, as shown by density fractionation. Surprisingly, human organismal-aging of erythrocytes appears to be associated with increased terminal fucose expression, which is also related to the early onset of diabetes, yet independent of erythrocyte oxidation capacity. These results illustrate the potential of using simple lectins to study the complex processes of ageing and disease.

Methods

Ethical statement

Anonymised blood samples were retrieved from residual clinical samples obtained after clinical testing from the NHS Grampian Biorepository (15/WM/0430). The Grampian Biorepository is accredited by Health Improvement Scotland, which provides a regulatory framework for the removal, storage and use of relevant biological material (tissue, cells and body fluids) for research into disease, or the functioning of the human body (<https://www.biorepository.nhsgrampian.org>). Independent ethical approval and consent is not required for samples obtained through the Biorepository.

Blood donors

Blood samples were retrieved from residual clinical samples obtained after clinical testing from the NHS Grampian Biorepository (15/WM/0430). For lectin-age studies (Figure 2 and Figure 3), blood samples were collected irrespective of age, gender or HbA1c levels. For lectin-hyperglycaemia and oxidation studies (Figure 4), blood samples were also collected irrespective of age or gender, selected by HbA1c levels in the normal (< 40 mmol/mol) and raised (>70 mmol/mol) ranges were collected at a 1:1 ratio.

Blood cell separation

Erythrocytes were separated from white blood cells in donor blood using Lymphoprep (Stemcell Technologies Inc.). Briefly, whole blood was diluted 1:1, layered over Lymphoprep and centrifuged at 400g for 35 minutes. The buffy coat was removed, while erythrocytes were isolated for analysis.

Lectin staining of erythrocytes and flow cytometry analysis

Biotinylated lectins (Vector Laboratories), *Maackia amurensis* Lectin II (MAL-II), *Sambucus nigra* Agglutinin (SNA), *Aleuria aurantia* (AAL) and *Ulex europaeus* (U.Euros) were used in conjunction with streptavidin PE-Cy7 or phycoerythrin (PE) for flow cytometric analysis. The lectins were titrated to minimise agglutination, as indicated by abnormally high forward versus side scatter (FCS) and high PE-Cy7 or PE intensity. Optimal flow cytometry concentrations for the lectins were MAL-II (Cat#, B-1265-1, Vector): 67 ng/ml; SNA (Cat#, B-1305-2, Vector): 57ng/ml; AAL (Cat# B-1395-1, Vector): 200ng/ml; and U.Euros (Cat# B-1065-2, Vector): 100ng/ml. Biotinylated lectins were applied, followed by streptavidin to purified erythrocytes, post-blocking with Carbo-Free-Block (Cat# SP-5040-125, Vector)

at room temperature for 30 minutes each before subjecting to flow cytometry⁴⁶. Data were acquired on a FACSCalibur (BD) and analysed using FlowJo v10.0 (Treestar) software. WinMDI can be used as a free alternative to FlowJo for analysis of the flow cytometry data. Normalized geometric mean fluorescence (gMFI) were calculated by subtracting the gMFI of streptavidin-only paired controls.

Neuraminidase treatment of erythrocytes

Purified erythrocytes were washed and treated with neuraminidase (*Vibrio cholerae*, Sigma) according to manufacturer's instructions for 30 minutes at 37°C in DMEM. Cells were washed with cold (4°C) PBS thoroughly to remove contaminating enzyme or free sialic acids.

Periodic acid Schiff assay

Neuraminidase treated erythrocytes and control erythrocytes, N-acetylneuraminic acid (NANA) and fetuin, were subjected to a PAS assay adapted from Mehdi and colleagues¹². Samples were oxidized using 0.04 M periodic acid (4°C, 30 minutes, 1:5 ratio of acid to sample). Two volumes of resorcinol working solution (5 ml of 6.0% resorcinol solution, 0.125 ml of 0.1 M copper sulphate solution and 19.875 ml of distilled water, brought to a final volume of 50 ml with 10 M HCl) was added, heated at 98°C for 5 minutes and cooled in an ice bath for 2 minutes. 1.8 volumes of n-butanol was added, mixed vigorously and the mixture incubated for 3 minutes at 37°C. Absorbance at 625nm was measured using a multiscan plate reader (Labsystems, Basingstoke, UK), against a reagent blank.

Gravity Percoll fractionation of erythrocytes

Erythrocytes were washed in PBS then SAH buffer (2.63 g/L BSA (Biosera), 132mM NaCl, 4.6mM KCl, 10mM HEPES, pH 7.1). Different percentages of Percoll/SAH mixtures were created by making a master mix of 97.5% density Percoll with the same salt and HEPES concentration as SAH, then diluting with SAH. The following fraction numbers indicate percentages of Percoll: Fraction 1, <18%; Fraction 2, 18–30%; Fraction 3, 30–50%; Fraction 4, 50–65%; Fraction 5, 65–80%; Fraction 6, 80–90%; Fraction 7, 90–97.5%. For layering the fractions by gravity, the highest desired density of Percoll/SAH is added to an empty 15ml Falcon™ tube and 1 ml of erythrocyte/SAH mixture gently layered on top. Consecutive layers of lower density Percoll is applied directly on top of the erythrocyte/SAH mixture. No centrifugation is required. Fractions of erythrocytes rise to the interphases of various Percoll layers applied. Fractionated erythrocytes are extracted from each layer into fresh tubes and washed in PBS well before analysis.

Erythrocyte fragility assay

Washed erythrocyte samples were tested for fragility based on the protocol provided in Blasi and colleagues⁴⁷. Briefly, erythrocytes from different fractions were washed in PBS and subjected increasing strength of hypotonic solutions, created by diluting PBS with deionized H₂O. The degree of haemolysis after 15 minutes of incubation at room temperature was determined by

measuring absorbance at 540nm, using a multiscan plate reader (Labsystems, Basingstoke, UK).

Erythrocyte C3 deposition assay

A sensitive, quantitative indirect enzyme-linked antiglobulin test (IELAT) was employed to test for C3 deposition on erythrocytes⁴⁸. Briefly, erythrocytes were applied to a 96-well round bottom microtitre plate (Nunc, Roskilde, Denmark) and blocked in PBS containing 0.2% BSA. Mouse anti-C3 antibody (Cat# Ab11862, Abcam, UK) was added. Cells were stained for one hour at 37°C and washed three times in PBS/BSA before fixing for 30 minutes in 0.15% glutaraldehyde (Cat# G5882-50ML, Merck). Fixed RBCs were transferred to fresh, pre-blocked, 96-well plates and washed before incubation with goat-anti-mouse IgG antibody (AP124, Merck) for one hour at 37°C. Cells were washed and incubated with rabbit anti-goat IgG alkaline phosphatase (Cat# AP106A, Merck) for one hour at 37°C. Washed cells were incubated with phosphatase substrate solution (Cat# 487664, Merck) for one hour at 37°C. 50µl of the supernatant was measured in flat bottom microtitre plates (Nunc, Roskilde, Denmark) and the absorbance measured at 405nm, using a multiscan plate reader (Labsystems, Basingstoke, UK).

RBC ghost preparation

Washed RBC were subjected to ice cold hypotonic lysis in 20 mM Tris, pH 7.6 and protease inhibitor (Cat# 05056489001, Roche)³⁰. Lysates were washed three times in hypotonic lysis buffer (37000 g, 4°C, 30 minutes) before resuspension in minimal hypotonic lysis buffer. Protein concentrations were determined by protein BCA assay (Cat# 23227, Pierce).

Lectin western blot

Ghost preparations were mixed in equal volumes with SDS sample buffer containing 8M urea³⁰ and heated at 100°C for 10 minutes. Ghost protein samples were fractionated by gel electrophoresis using NuPage 4–12% Bis-Tris gel (Invitrogen, Cat# NP0321BOX) and transferred by western blotting (30V, 1 hour) to polyvinylidene fluoride membrane (P 0.45 µm; Cat# 10600023 Amersham Hybond, GE Healthcare). Blots were probed with biotinylated MAL (0.2µg/ml) or SNA (2µg/ml) lectin (Vector Laboratories) and Streptavidin HRP (1:2500 dilution, Cat# 3999S, Cell Signalling) in calcium binding buffer (10mM HEPES, 150mM NaCl₂, 2.5mM CaCl₂, pH 7.4) containing 1x Carbo-Free Blocking Solution (Vector Laboratories, Cat# SP-5040) and protease inhibitor cocktail (Cat# 11836145001, Roche) before development in Amersham ECL Select substrate (Cat# RPN2235, GE Healthcare). 0.1% Tween-20 was added in probing and washing steps. Loading of wells was normalized by protein concentration (~6 µg per sample).

ROS production

Rate of ROS production was determined by loading purified oxidized or untreated RBC with oxidation sensitive dye CM-H2DCFDA (10 µM; Cat# C6827, Molecular Probes) in PBS and incubating for 60 minutes in the dark at 37°C. RBCs were

then washed three times, resuspended in DMEM (Thermo Fisher) and fluorescence determined immediately by spectrofluorimeter (Fluostar Optima; BMG Labtech), with excitation of 485 nm and emission 530 nm. The rate of ROS formation was calculated for the linear portion of the fluorescence/time curve generated over six hours, which typically lasted for three hours.

Glycomic analysis

Structural analysis was performed as described previously⁴⁹. Briefly, ghost membranes were subjected to sonication in the presence of CHAPS detergent, reduced in 4 M guanidine-HCl (Pierce), carboxymethylated, and digested with trypsin. The digested glycoproteins were then purified by Oasis Plus HLB Sep-Pak (Cat# 186000132, Waters Corp.). N-glycans were released by peptide N-glycosidase F (E. C. 3.5.1.52; Roche Applied Science) digestion, while O-glycans were released with reductive elimination (55 mg of KBH_4/mL in 0.1 M KOH). Released N- and O-glycans were permethylated using the sodium hydroxide procedure and purified by C_{18} -Sep-Pak.

MALDI-TOF MS and MALDI-TOF/TOF MS/MS were performed for the analysis of permethylated glycans. MS data were acquired using either a 4800 MALDI-TOF/TOF (Applied Biosystems) mass spectrometer. MS/MS data were acquired using a 4800 MALDI-TOF/TOF mass spectrometer. Permethylated samples were dissolved in 10 μL of methanol, and 1 μL of dissolved sample was premixed with 1 μL of matrix (10 mg/ml 3,4-diaminobenzophenone in 75% (v/v) aqueous MeCN), spotted onto a target plate, and dried under vacuum. For the MS/MS studies, the collision energy was set to 1 kV, and argon was used as collision gas. The 4700 Calibration standard kit, Calmix (Applied Biosystems), was used as the external calibrant for the MS mode, and [Glu1] fibrinopeptide B human (Sigma) was used as an external calibrant for the MS/MS mode.

The MS and MS/MS data were processed using Data Explorer 4.9 Software (Applied Biosystems). The processed spectra were subjected to manual assignment and annotation with the aid of a glycominformatics tool, GlycoWorkBench⁵⁰. The proposed assignments for the selected peaks were based on ^{12}C isotopic composition together with knowledge of the biosynthetic pathways. The proposed structures were then confirmed by data obtained from MS/MS experiments.

Data availability

Underlying data

Open Science Framework: Human erythrocyte surface fucose expression increases with age and hyperglycemia. <https://doi.org/10.17605/OSF.IO/XF8AM>³².

This project contains the following underlying data:

- Figure 1a, Figure 2 Donor 1, Figure 2 Donor 2, Figure 2 Donor 3, Figure 2 Donor 4, Figure 3a 3b, Figure 4a 4b (Folders containing raw cytometry data for each figure. Flow cytometry files are stored as .FCS. Each experimental folder contains within it a 'Read me' file that details what each flow cytometry file refers to.)

- Figure 4c (Folder containing raw mass spectrometry data in XLSX format)
- Figure 5b (Folder containing raw ROS production data in XLSX format)
- Fucose_Source_data.xlsx (Raw data underlying Figure 1b, Figure 3a and b, Figure 4, Figure 5a and Supplemental Figure 1c)

Extended data

Open Science Framework: Human erythrocyte surface fucose expression increases with age and hyperglycemia. <https://doi.org/10.17605/OSF.IO/XF8AM>³².

This project contains the following extended data in the file 'Supplemental Figures.docx':

- Supplemental Figure 1.tif (Gravity Percoll fractionation technique for erythrocytes based on age. a) Photograph of an example of gravity Percoll fractionated erythrocytes from a single healthy donor. b) Cell counts for each fraction from a). '1+' and '2+' denotes the fraction and the intermediate Percoll section below this fraction. There are minor spill-over populations of cells in the intermediate Percoll sections between Fractions 1 to 3. c) C3 binding assessed by IELAT for a single donor from fraction 1 to 6. d) Osmotic pressure induced RBC lysis assay for different fractions separated by gravity Percoll method. Decreasing salt concentration induces increasing lysis of red blood cells, and release of hemoglobin, measured at 540nm. Underlying data for this figure can be found in the file 'Source Data for extended data.xlsx')
- Supplemental Figure 2.tif (Western blot performed with lectins MAL (a) and SNA (b) on fractionated samples corresponding to the flow cytometry displayed in Figure 1a (donor 1). Molecular weights in kDa are indicated to the left of each image. Underlying data for this figure can be found in the file 'Western Blot originals Supplemental 2a 2b.pptx')
- Supplemental Figure 3.tif (Murine RBCs lack surface SNA binding. a) Unfractionated C57/BL6 erythrocytes, stored in DMEM for 3 days, are probed with MAL and SNA in flow cytometry. Blue: secondary only control. Red: lectin staining. b) Photograph of murine (C57/BL6) and human erythrocytes are fractionated using the gravity Percoll fractionation assay. c) MAL and SNA lectin binding to erythrocytes isolated from Fractions 2 to 5 (blue) compared to binding to Fraction 1 (red). Secondary only control is shown in green.)

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](https://creativecommons.org/licenses/by/4.0/) (CC-BY 4.0).

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Nitin Bhardwaj

Department of Zoology and Environmental Science, Gurukul Kangri University, Haridwar, Uttarakhand, India

1. The introduction section should be more focused on the various changes associated with the erythrocytes maturation. The section describing changes in carbohydrates content in tumour should be omitted.
2. The method of percoll fractionation never separates young and old erythrocytes. There is always reticulocytes and young erythrocytes contamination in heavier fraction.
3. It is really surprising the increment in the fucose content on the erythrocytes. As the erythrocytes matures, there is decline of several molecules occurs. Since erythrocytes don't have any synthesizing machine, how this increment has been happened. What is the physiological significance of this increment? Why no such type of changes were seen in mouse model?
4. In case of donor 4, The MAL binding is increasing in lower fraction as shown by flow cytometric histogram.
5. Donor 3 Fraction 5 is not showing any differences in SNA binding loss with the increase age.
6. Why the authors have selected 7 or 9 days, 10, and 22 days? Is there any changes in SNA binding loss with the increase in storage?
7. How many events were counted in flow cytometric experiments. Show the PE isotype histograms in supporting files.
8. Western blot figures are not clear.
9. As author described, mass spectrometric data have no correlation with the age, there is no need to mention in present manuscript.

10. Show the flow cytometric histogram of Fig. 5a.

Few grammatical mistakes like

- Donors varying in age and hyperglycemia should be donors varying in age and having hyperglycemia
- Either arabic or numeral..like six to 26 should be 6 to 26. (Introduction para 3 line 4).
- Use either Erythrocytes or RBC throughout the manuscript.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

No

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Hematology, Immunology, Toxicology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.