SUPPLEMENTARY APPENDIX

Donor sex, age and ethnicity impact stored red blood cell antioxidant metabolism through mechanisms in part explained by glucose 6-phosphate dehydrogenase levels and activity

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SUPPLEMENTARY MATERIAL

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Supplementary INTRODUCTION – EXTENDED

Over the past decade the application of omics technologies[1] and particularly metabolomics[2] to the field of red blood cell (RBC) storage has exponentially expanded our understanding of the temporal sequence and mechanisms of the storage lesion. Indeed, while refrigerated storage in the blood bank is a logistic necessity to make ~110 million units available for transfusion every year worldwide, the process comes at a significant cost in terms of RBC structural[3–5] and biochemical homeostasis.[1] Some of these "storage lesions"[6] are inevitable, since refrigeration temperatures negatively impact the activity of key enzymes regulating red cell energy[7] and ion pump homeostasis[8,9]. Glycolysis is further inhibited by intracellular acidification as a function of lactate accumulation, a phenomenon that is observed during storage in all currently licensed storage additives, including SAGM [9–12], additive solutions 1 [13], 3 [14,15], 5 [16] and PAGGSM [17,18], and is in part counteracted by the adoption of next generation alkaline storage additives.[17,19–21] In small scale studies, the metabolic lesion has been reproducibly assessed to a quantifiable extent, which allowed the definition of metabolic markers of the so-called "metabolic age" of stored RBCs.[3,22,23] These markers are so robust that in prior double-blinded metabolomics studies we could accurately predict the storage age of >98.7% of 599 stored RBC samples.[24] Despite the consistency of these laboratory observations, it is still a matter of debate whether the (metabolic) storage lesion could represent an etiological contributor to (or a reliable predictor of) transfusion outcomes in the recipient.[25] Indeed, storage-induced impairments in the homeostasis of high energy phosphate compounds adenosine triphosphate (ATP) and 2,3-diphosphoglycerate (DPG) should negatively impact RBC capacity to bind and off-load oxygen upon transfusion.[26] Depletion of ATP and DPG could represent a concern in massively transfused patients, since the rate at which these compounds are replenished within the first 72h upon transfusion may not be sufficient to meet the oxygen metabolic demands in severely hypoxic recipients.[27,28] Studies in animal models and humans have shown that some small molecule metabolites could represent reliable correlates to Food and Drug Administration gold standards for stored blood quality, i.e. storage hemolysis[17,29] and post-transfusion recovery[30–32]. For example, metabolites like hypoxanthine, an ATP-breakdown and oxidation product, have been correlated to hemolysis and post-transfusion recoveries in mice and humans.[17,32,33] Similar correlations have been reported for lipid oxidation products.[30]

Despite the overwhelming evidence from in vitro studies, randomized clinical trials[34–37] have hitherto failed to capture any signal associated with poorer outcomes when comparing transfusion of the freshest available units versus the standard of practice. On the other hand, a recent analysis of a linked donor and recipient database indicated that transfusion of RBC units less than 35 days old was associated with a higher recipient hemoglobin increment as compared to transfusion of 35-42 day old RBC units.[38] The apparent inconsistencies among the studies on the age of blood in the literature could be reconciled by the appreciation of the fact that RBCs, like people, do not always age the same.[39] In other terms, the molecular age of blood may be a distinct parameter from the storage age calculated in days since the time of donation[40]. Biological variability in donors[41] and different RBC component processing strategies[42] may for example impact hemoglobin oxygen saturation across donors/components,[43] which in turn affects RBC susceptibility to oxidative stress during storage.[32,44,45] Small-scale laboratory studies corroborated the hypothesis that RBC antioxidant capacity[46] and storage-induced susceptibility to oxidative stress may indeed be donor-dependent[47]. This statement holds true when considering some categories of routinely accepted donors who are more susceptible to storage-induced oxidative stress owing to common enzymopathies. For example, deficiency of glucose 6-phosphate dehydrogenase (G6PD) activity affects ~400 million people worldwide, including ~10% of the African American donor population in some metropolitan areas.[48] These subjects are characterized by a decreased capacity to activate the pentose phosphate pathway (PPP) and thus to generate the NADPH necessary to reduce oxidized glutathione and NADPH-dependent antioxidant enzymes[48]. RBCs from G6PD deficient donors are characterized by altered energy and redox metabolism[49,50], a feature that has been preliminarily associated with poorer capacity to circulate upon transfusion to sickle cell recipients[51] and poorer post-transfusion recoveries in autologous volunteers.[52] As such, population screening in regions where the prevalence of G6PD deficiency is 3–5% or greater (in males) is recommend by the World Health Organization (WHO),[53] but no specific screening for G6PD activity is routinely in place for blood donors in the United States.

In the past few years, large scale studies have been designed to focus on the impact of donor biology on storage quality and transfusion outcomes. Within the framework of the National Heart Lung and Blood Institute (NHLBI, NIH) Recipient Epidemiology and Donor Evaluation Study (REDS)-III RBC-Omics study, four blood centers across the United States enrolled ~13,800 healthy donor volunteers of different ages, sex and ethnicities. Preliminary analyses of the data

obtained from this cohort allowed us to conclude that (i) donor sex (and testosterone levels[54]), age and ethnicity impact the hemolytic propensity of stored RBCs[55,56]; (ii) stored RBC from multiple units donated by the same donors have a similar propensity to hemolyze following pro-oxidant or osmotic insults;[57] and (iii) the storage duration contributes to explain ~13% of the total metabolic heterogeneity of stored RBCs, a percentage similar to the impact noted for storage additives in a subgroup of recalled donors from the original RBC-Omics cohort.[24]

In the light of this background, the continued characterization of the impact of donor biology on storability and transfusion outcomes is a critical step towards the establishment of personalized transfusion medicine practices. In the present study we sought to expand the characterization of the metabolic mechanisms that contribute to explaining donor and storage-dependencies of hemolysis following oxidative insults. Leveraging the RBC-Omics recalled donor population (which enrolled 662 subjects with extremes in hemolytic propensity from a screened original cohort of ~13,800 consenting donors), we performed metabolomics analyses on 599 samples from 250 donors of different ages, sex, ethnicities. We thus correlated metabolic measurments to oxidative hemolysis and biological variables like donor sex, age and ethnicity, both as a function of or independently of storage duration.

Supplementary METHODS – EXTENDED

Sample processing and metabolite extraction: An isotopically labeled internal standard mixture including a mix of $^{13}C^{15}N$-labeled amino acid standards (2.5 μM) was prepared in methanol. A volume of 100μl of frozen RBC aliquots was mixed with water and the mixture of isotopically labeled internal standards (1:1:1, *v/v/v*). The samples were extracted with methanol (final concentration of 80% methanol). After incubation at −20°C for 1 hour, the supernatants were separated by centrifugation and stored at −80°C until analysis. Samples were vortexed[58] and insoluble material pelleted as described.[59,60]

Ultra-High-Pressure Liquid Chromatography-Mass Spectrometry metabolomics: Analyses were performed using a Vanquish UHPLC coupled online to a Q Exactive mass spectrometer (Thermo Fisher, Bremen, Germany). Samples were analyzed using a 3 minute isocratic condition[61] or a 5, 9 and 17 min gradient as described.[30,60] Solvents were supplemented with 0.1% formic acid for positive mode runs and 1 mM ammonium acetate for negative mode runs. MS acquisition, data analysis and elaboration was performed as described.[60,61] Additional analyses, including untargeted analyses and Fish score calculation via MS/MS, were calculated against the ChemSpider database with Compound Discoverer 2.0 (Thermo Fisher, Bremen, Germany). Graphs and statistical analyses (either t-test or repeated measures ANOVA) were prepared with GraphPad Prism 5.0 (GraphPad Software, Inc, La Jolla, CA).

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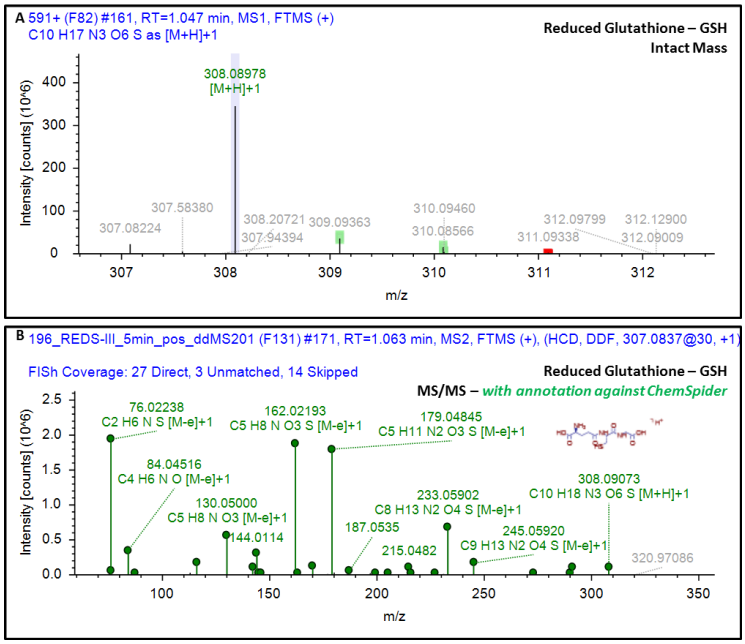
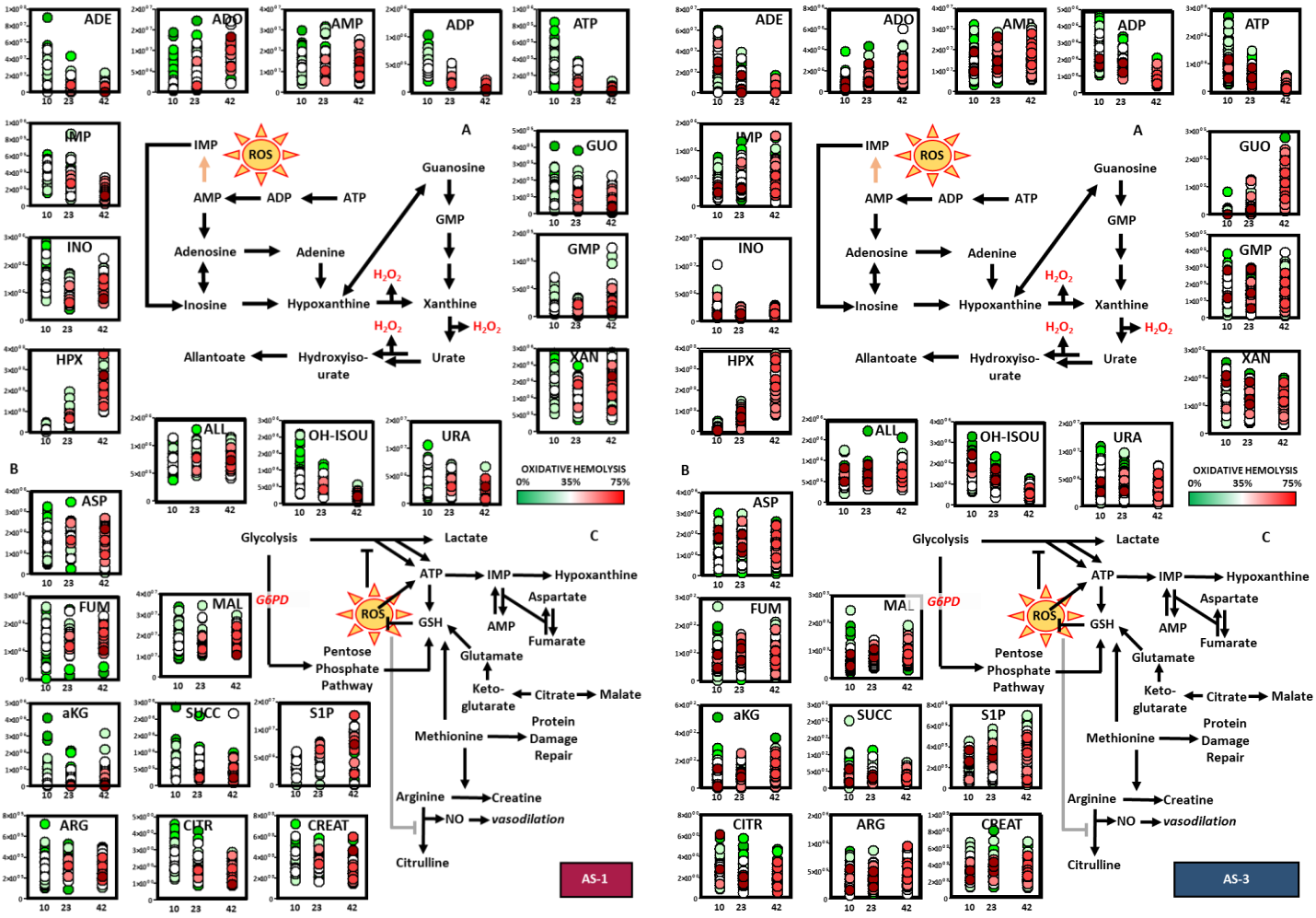
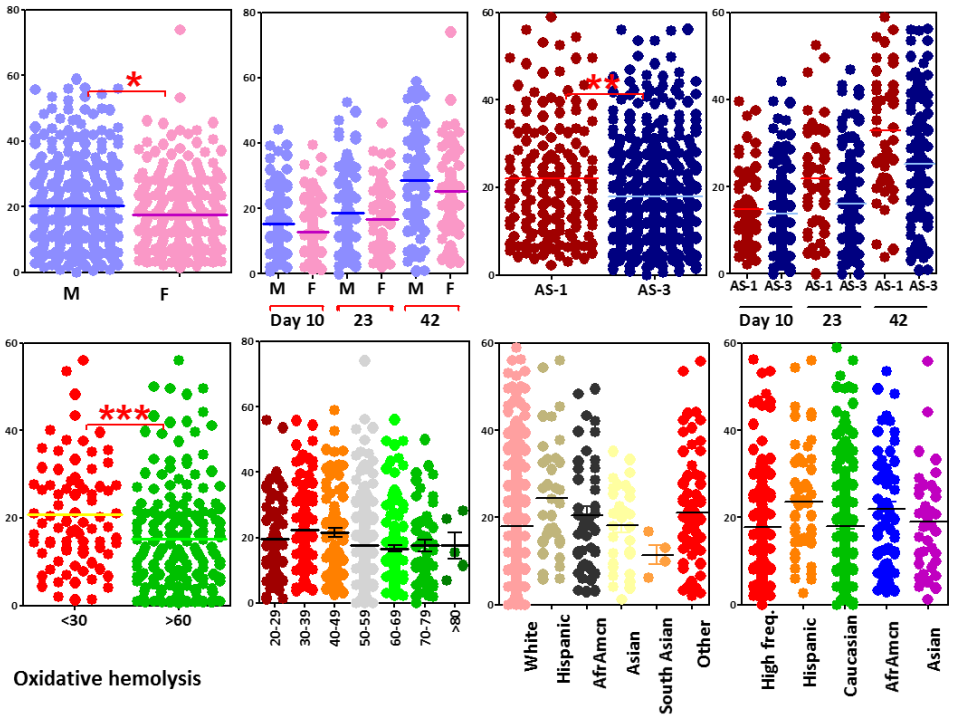
Supplementary Figures



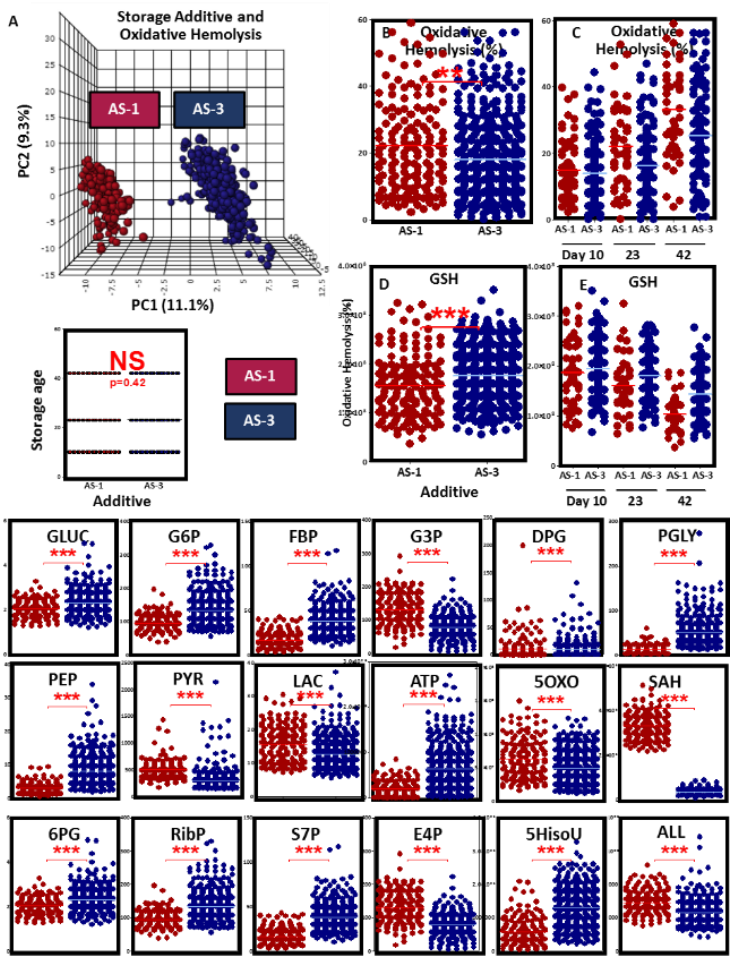
Supplementary Figure 1 – Representative MS (top) and annotated MS/MS spectrum (bottom) for reduced glutathione (GSH), as quantified through high-throughput, high-resolution UHPLC-MS.



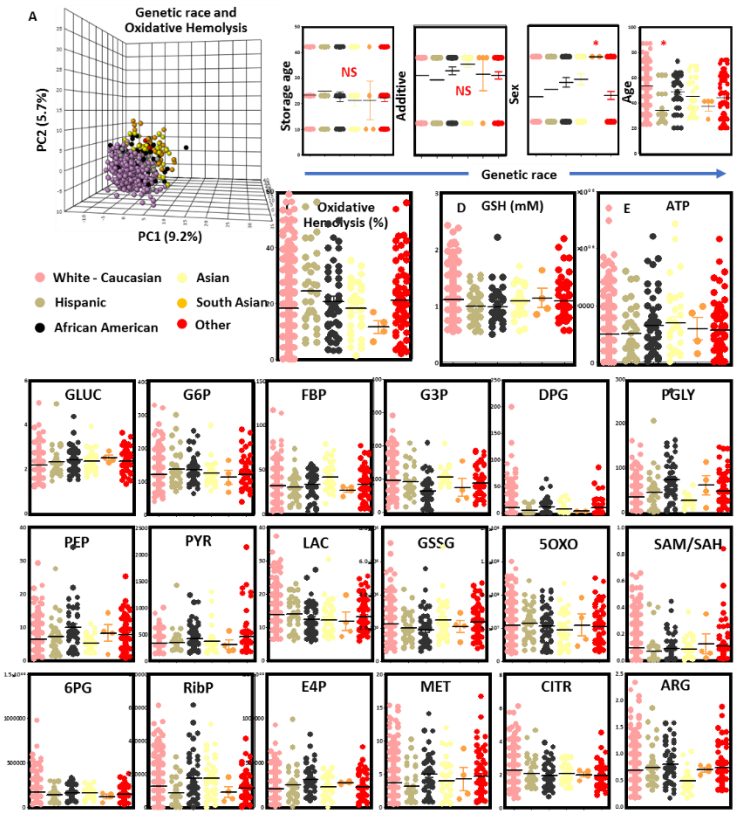
Supplementary Figure 2 – Metabolic changes in purine metabolism and oxidation, carboxylic acid and arginine metabolism, as a function of storage additives (AS-1 – **left**; AS-3 – **right**). On the x axis of each graph, storage day 10, 23 and 42 are represented. Each dot represents an independent measurement and colors are proportional to the oxidative hemolysis measurement for the same sample (green to red = low to high oxidative hemolysis).



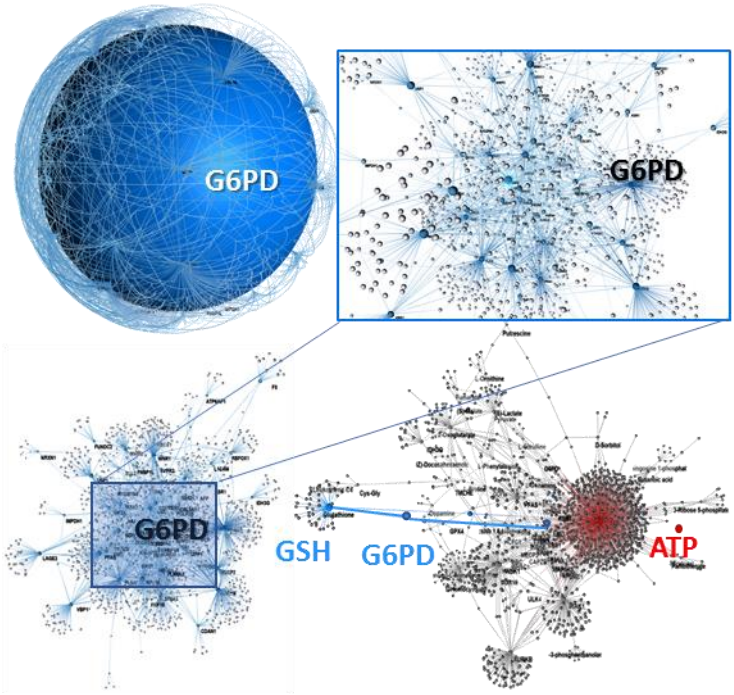
Supplementary Figure 3 – Overview of oxidative hemolysis as a function of gender, storage additive, age, ethnicity and storage day. In the top row, the first two panels show oxidative hemolysis measurements in male (M) vs female (F) donors at any given storage day (leftmost panel) or at storage day 10, 23 and 42 (second panel). In the third and fourth panel, a similar breakdown is shown for oxidative hemolysis as a function of storage additive (AS-1 vs AS-3) at any given storage day (third panel, top row) or at storage day 10, 23 and 42 (fourth panel, top row). In the bottom row, oxidative hemolysis measurements are shown for donors younger than 30 or older than 60 (first panel), for donors of different ages broken down by decade (second panel), for different ethnicities (third panel), for ethnic group and donation frequency (last panel).



Supplementary Figure 4 – Oxidative hemolysis as a function of storage additive (AS-1 vs AS-3 = red vs blue) (**A**). Oxidative hemolysis and reduced glutathione (GSH) are higher and lower, respectively in RBCs stored in AS-3 at any given storage day (**B-D**). Similarly, RBCs stored in AS-1 have altered glycolysis, lower activation of the pentose phosphate pathway, altered methionine and citrulline metabolism (samples are plotted with no distinction of storage days).



Supplementary Figure 5 – Oxidative hemolysis as a function of donor ethnicity (**A**). Oxidative hemolysis and reduced glutathione (GSH) are higher and lower, respectively in RBCs from Hispanic and African American donors (**B-D**). Bottom panels provide an overview of glycolysis, pentose phosphate pathway, glutathione and methionine homeostasis as a function of donor ethnicity (samples are plotted with no distinction of storage days).



Supplementary Figure 6 – OmicsNet elaboration of the gene-centric (top) and metabolite-centric (bottom) network view of the merged top 100 genes and metabolites correlated to oxidative hemolysis from the REDS-III Omics study confirms a central role for G6PD and GSH- or NADPH-dependent branching pathways (e.g, GPX4 and ALDH1) in mediating energy and redox homeostasis.

SUPPLEMENTARY TABLE 1 – *please refer to the XLS appendix*

Untargeted Metabolomics data were correlated to oxidative hemolysis (Spearman's correlation). The top 250 positive (red) and negative correlates (blue) from this analysis are reported in Supplementary table 1