Editorial

Mortality after red blood cell transfusions from previously pregnant donors: complexities in the interpretation of large data

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Red blood cell (RBC) transfusion is a common and potentially life-saving intervention. The requirement for RBC transfusions inherently reflects a degree of illness in the recipient, and healthy individuals do not generally require blood transfusions. In the recent past, restrictive blood transfusion policies have improved patient outcomes with the added benefit of driving down medical costs (1). Other policies and advances in cell preservation technology such as ABO and Rh matching, leukoreduction and limitations in the age of storage (to the age of RBC viability) of blood products have decreased recipient morbidity and mortality (1-3). However, the concept that RBC donor female gender and parity may influence recipient mortality has been a subject of much debate in the blood banking community and differing conclusions are reported in several large retrospective studies (4,5). Here we comment on the recent article by Caram-Deelder *et al.* (6) published in *JAMA* reporting increased mortality in male recipients of RBC units from female donors who had ever been pregnant compared with male recipients of RBC units from male donors.

In this retrospective cohort study of first ever RBC transfusion recipients, the authors aimed to "*quantify the association between RBC transfusion from female blood donors, with and without a history of pregnancy, and patient mortality in female and male transfusion recipients.*" They utilized three methodologies analyzing mortality results over a 10-year follow-up period in 42,132 recipients of RBC transfusions. The primary methodology, utilizing a "no-donor-mixture" cohort, was designed to prevent dilution effects by censoring or excluding recipient data when RBC products from more than one donor type were given. In these analyses, 31,118 recipients received 59,320 units from predominantly male (88%) donors. Never-pregnant female (NPF) and ever-pregnant female (EPF) RBC units comprised 6% each of the total donor pool. The second methodology utilized recipients who received only a single RBC unit (16,959 recipients). Finally, the third methodology utilized the full cohort, including recipients of multiple donor unit types. In the Netherlands, all transfusion information and mortality data are tracked within a national registry that includes age, gender, and dates of death but excludes information about ethnicity/race. All blood products were leukocyte-reduced by pre-storage filtration. Pregnancy status was obtained via self-reporting and due to an unknown pregnancy status, 44% of the donations from females were not used. All other transfused products were ignored in the primary analyses.

There were 3,969 deaths over a 10-year follow-up period within the "no-mixture" cohort. Cox proportional hazards models were utilized and categorical variables were fit into the model to correct for potential confounding. These potential confounders included institution, ABO-RhD blood group, age of the donor, and cumulative number of transfusions. Resulting hazard ratios (HRs) were calculated for each of the two exposure groups (i.e., ever-pregnant female donors and NPF donors) utilizing

the male donor group as the control. In the "no-mixture" cohort, mortality was found to be higher in male recipients of ever-pregnant female RBC donors compared to male RBC donors: HR 1.13 (95% CI, 1.01–1.26); P=0.03. This effect was observed within the full cohort analysis, though to a lesser degree: HR 1.08 (95% CI, 1.02–1.15); P=0.02. The single-transfusion analysis approached, but did not reach significance and displayed a much wider confidence interval: HR 1.23 (95% CI, 0.98–1.54); P=0.08. A donor effect was not observed for male recipients of NPF RBC donors in any of the three methodologies, nor were donor effects observed for female recipients of either NPF or EPF RBC donors. The authors comment that effect modification analysis for age demonstrated a higher risk of death in male recipients aged 18–50. As shown in the supplemental data (eTable7), a HR of 1.634 (95% CI, 1.023–2.610); P=0.04; was calculated in male recipients of ages 0–17 of ever-pregnant female donor RBCs. However, a non-statistically significant HR of 1.501 (95% CI, 0.979–2.303); P=0.063; was calculated in male recipients of ages 18–50. The number of male recipients over the age of 50 was far greater than those under the age of 50. These data suggest a biologic mechanism affected by both gender as well as age. A recent study reported an association between male gender and increased RBC susceptibility to hemolysis in a murine model (7). These data suggest a potential mechanism given that sex-hormone levels wane with advancing age. Lastly, increased arginase levels, known to inactivate the amino acid arginine, which is essential for lymphocyte function, and may be released from hemolyzed RBCs has been proposed as a potential mechanism in the development of transfusion-related immunomodulation (TRIM) (8). *In vivo* data from a murine model suggests that female mice express higher levels of arginase II in the kidney (9).

However, there may be a more obvious answer for the increased mortality observed in the young male recipients driving the statistical significance that was observed in the total male recipient group. The risk of morbidity is highly dependent on the underlying type and degree of illness and trauma patients in particular, have very high mortality rates and associations with unique biological mechanisms. Furthermore, trauma patients are predominantly young and male (17–50 years of age). Post-injury hyperfibrinolysis and fibrinolysis shutdown are both associated with mortality in a U-shaped distribution (10). In addition to being young and male, the patients who die tend to have higher injury scale scores, are the recipients of more blood products and have higher rates of massive transfusion (7). In a recent prospective observational study of mortality risk factors in trauma patients, in which 88% of the trauma patients were young and male, persistent fibrinolysis shutdown and hyperfibrinolysis were both highly associated, independent risk factors for late mortality (11). Contrary to our general understanding that RBCs may confer a prothrombotic phenotype, the likelihood of developing persistent fibrinolysis shutdown actually decreased with each unit of packed RBCs transfused (11). In addition, the disease type and severity should be carefully controlled for in any large study of risk factor association with mortality.

The evidence linking female-derived plasma and poor patient outcomes is far more robust and various gender dependent differences have been identified in donated plasma (3,12). Infusion of female donor-derived anti-HLA or anti-neutrophil antibodies has been well documented to play roles in the etiology of TRALI (3,12). Furthermore, male-only donor-derived plasma programs have significantly reduced the incidence of TRALI (12). Again, though the association is evident, the biologic mechanisms are not completely understood (12). HLA-derived antibodies cause TRALI as part of the two-hit mechanism in animal models and these HLA-antibodies have been identified from female donor plasma correlating with clinically proven TRALI (3). Furthermore, proteomic analyses of human plasma showed differences between male and nulliparous female donors. Female plasma contained higher factor V (FV), pregnancy zone protein, α1-antitrypsin, β2-microglobulin and Complement Factors H and C4B, whereas male plasma contained higher Fc-binding protein, protein Z-dependent protease inhibitor, phosphatidylinositol glycan-specific phospholipase, protein S-100 and transgelin (13). FV is critical to clot formation and elevated circulating concentrations of FV have been associated with VTE. Amino acid differences and metabolites also appear to have different distributions between men and women with creatine levels detected at higher levels in females; and phenylalanine, glutamine, proline, histidine, glutamate, tyrosine, valine, propylene glycol, leucine, isoleucine, creatinine and acetone higher in males (14). In the Caram-Deelder manuscript, the authors state that although RBC products undergo leukoreduction, the quality control standards may allow for up to 5 million leukocytes in a small percentage of products, which implies that variable plasma volumes, and consequently variable metabolites, proteins or amino acids, are contained in individual RBC units as well. Inherent biologic differences in RBCs affecting their survival have been reported with

higher hemolysis after prolonged storage in leukocyte-reduced RBC concentrate derived from male donors (7). Furthermore, ethnic background was associated with resistance to osmotic hemolysis as well as extreme (>1%) levels of storage hemolysis, exceeding The US Food and Drug Administration regulations (15).

The authors assert that an association of pregnancy and recipient mortality indicates an immunologic mechanism. The median follow-up time was 380 days, implying that inherent RBC characteristics, proteomics or plasma metabolites are not the most likely explanation for these findings. Even antibody (Ab)-mediated processes would be expected to occur rapidly; TRALI, which is often Ab-mediated, occurs by definition within 6 hours of having received a transfusion (3,12). The relatively late mortality reported in this study suggests other mechanisms; potentially immunologic modulation or even epigenetic modification. It is puzzling that results were not similar for both male and female recipients, though uneven sample distribution may be in part to blame. It is important to consider some gender-specific immunologic mechanisms. Similarly, conflicting results have been observed in the hematopoietic stem cell transplant population. In hematopoietic stem cell transplantation, male recipients of female stem cells have increased risks for poorer outcomes and increased graft-versus-host disease (16). A recent retrospective analysis by Friedrich *et al.* (17) showed that donor gender had no impact on overall outcomes in young patients receiving a matched sibling HSCT. They did, however, see a slight increase in chronic graft versus host disease associated with female donors over the age of 12 years. In addition, antibodies directed against holandric (minor histocompatibility) antigens, encoded by the Y-chromosome (HY-Abs), have been detected in recipients and appear to correlate with the development of chronic graft-versus-host disease (18). The kinetics of alloantibody persistence have not been fully characterized, but evidence suggests that pregnancy, immunosuppression and trauma may allow for prolonged detection of anti-HLA antibodies in the plasma (2).

Despite the assertion that donors are randomly allocated, implying inherent randomization, Edgren *et al.* (5) in their recent *JAMA* publication suggest that residual confounding may still exist. Using similar approaches to that of the Canadian study by Chassé *et al.* (4), the group from Denmark reported negative associations with patient survival and donor gender or age after rigorously controlling for confounding factors. Although the initial analysis revealed associations between mortality with extremes in donor age and female gender, these associations were nullified after accounting for non-linearity in the data (5). Underlying disease severity in the patient, specified by the number of transfusions rather than donor characteristics, appeared to be most important in patient survival. Furthermore, the authors speculate that residual confounding, likely patient underlying disease severity, due to imperfect measurement of the confounding variable leading to its incomplete removal, may account for the positive results observed in other studies. These results were contrary to those observed by Chassé *et al.* (4). As per eTable 10, the deleterious effects of EPF RBCs remained, again in the younger males, after controlling for the cumulative number of plasma and platelet units received. However, these data may be non-linear as well. As eloquently stated in the commentary by Roubinian *et al.* (19), these disparate results suggest that statistical approaches vary in their ability to control for confounding, and large retrospective, observational studies deserve careful consideration in the application of statistical methods and study design.

Mortality and morbidity are highly dependent on the underlying type and degree of illness and it is imperative to consider the potential impact of missing data on the statistical analyses and the potential for added bias. Pregnancy status was unknown in 44% of female donors. It is reasonable to assume, but impossible to prove, that the missing data were random. One would expect that when analyzed separately, the missing data should reflect an intermediate level HR. Per eTable 8, utilizing the full cohort, this was not observed in all age categories when stratifying for recipient age. When considering absolute numbers of blood donors, a large discrepancy exists given missing data. Although similar in numbers, the reference sample (recipients who received male donor-derived RBCs), were almost always somewhat less than the numbers of recipients of female donor-derived RBCs, which is surprising given that there were far more male donor data available compared to the female donor data. It would even be reasonable to include multiple male (control) donor-derived recipients for every female donor-derived recipient, potentially increasing statistical power. Furthermore, although number of units per recipient was included as a potential confounder in the analyses, no report of the relative distributions per donor-type was listed. Sicker patients often require more units and because female donor-derived blood units contain less hemoglobin, these recipients are at risk of requiring additional units.

Therefore, these recipients were more likely to receive blood from more than one donor type and consequently, were more likely to be excluded from the "no-mixture" analyses.

To further highlight the complexities of analyzing large retrospective data sets, several studies interrogating mortality risk factors in cardiac surgical patients reveal conflicting conclusions in regard to RBC transfusion (20-23). In fact, Welsby *et al.* (20) reported less pulmonary dysfunction and fewer poor outcomes in the recipients of female-derived plasma. One may postulate that the cardiac surgical patient population reflects more homogeneity in terms of underlying illness and inflammation and may provide enhanced sensitivity in detecting small associations of donor sex with recipient mortality. Desmarets *et al.* (22) found that donor sex was not correlated with morbidity, and not surprisingly, the type of surgery and number of transfused RBC units were both strongly associated with death. In fact, a trend toward increased mortality associated with male donor-derived blood was observed, though this did not reach statistical significance. Lastly, similar to the Edgren paper (5), Holzmann *et al.* (21) reported significant nonlinear confounding that when appropriately controlled for, mitigated any effects of donor gender.

As the authors state, these results are tentative and should be corroborated by replicative studies utilizing strict statistical techniques to control for confounding variables. At this time, it is premature to suggest changes to our current blood banking practices. However, should these results be confirmed, RBC banking approaches will need to undergo major modifications. Modifications could potentially include the exclusion of ever-pregnant females, the practice of gender specific transfusion or more intensive forms of leukoreduction and pre-storage RBC separation from plasma. Exclusion of ever-pregnant females from the RBC donor pool could lead to a dramatic underproduction of this important blood product. Although very compelling, the results of this study must be confirmed with further basic, translational and clinical studies to determine both causality and to elucidate the underlying biologic mechanism(s) responsible for mortality in either gender mismatched or female to male RBC transfusion.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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