Preterm Birth Genome Project (PGP) – validation of resources for preterm birth genome-wide studies

Craig E. Pennell1, Felipe Vadillo-Ortega2, David M. Olson3, Eun-Hee Ha4, Scott Williams5, Tim M. Frayling6, Siobhan Dolan7, Michael Katz8, Mario Merialdi9, Preterm Birth Genome Project (PGP) Consortium10 and Ramkumar Menon11,*

1School of Women’s and Infants’ Health, The University of Western Australia, Perth, Australia
2School of Medicine, Universidad Nacional Autonoma de Mexico, Mexico
3Department of Obstetrics and Gynecology, University of Alberta, Canada
4Ewha Women’s University Medical School, Department of Preventive Medicine, Seoul, Korea
5Center for Human Genetics Research, Division of Human Genomics, Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN, USA
6Genetics of Complex Traits, Peninsula Medical School, St. Luke’s Campus, Exeter, UK
7Department of Obstetrics and Gynecology, Albert Einstein College of Medicine, NY, USA
8Research and Global Programs, March of Dimes Foundation, White Plains, NY, USA
9Department of Reproductive Health and Research, World Health Organization, Geneva, Switzerland
10Complete list of collaborators at the end of the article
11Department of Obstetrics Gynecology, The University of Texas Medical Branch, Galveston, TX, USA

Abstract

We determined a series of quality control (QC) analyses to assess the usability of DNA collected and processed from different countries utilizing different DNA extraction techniques prior to genome-wide association studies (GWAS). The quality of DNA collected utilizing four different DNA extraction techniques and the impact of shipping DNA at different temperatures on array performance were evaluated. Fifteen maternal-fetal pairs were used from four countries. DNA was extracted using four approaches: whole blood, blood spots with whole genome amplification (WGA), saliva DNA was extracted using four approaches: whole blood, saliva and buccal swab. Samples were sent to a genotyping facility, either on dry ice or at room temperature and genotyped using Affymetrix SNP array 6.0. QC measured included extraction techniques, effect of shipping temperatures, accuracy and Mendelian concordance. Significantly fewer (50%) single nucleotide polymorphisms (SNPs) passed QC metrics for buccal swab DNA (P<0.0001) due to missing genotype data (P<0.0001). Whole blood or saliva DNA had the highest call rates (99.2 0.4% and 99.3 0.2%, respectively) and Mendelian concordance. Shipment temperature had no effect. DNA from blood or saliva had the highest call rate accuracy, and buccal swabs had the lowest. DNA extracted from blood, saliva and blood spots were found suitable for GWAS in our study.

Keywords: DNA; genetic analysis; prematurity; samples; single nucleotide polymorphisms (SNPs).

Introduction

Preterm birth (PTB), delivery at <37 weeks’ gestational age, is a complex disease resulting from multiple pathophysiologic pathways. This complexity is due to pathophysiologic, environmental and genetic heterogeneity [8]. Our lack of understanding of an individual’s risk (genetic, environmental, gene × gene and gene-environment interactions) has led to our failure to impact the rate of PTB because, at best, only generic “one size fits all” treatments have been applied. One relatively simple approach to address this issue is to consider genetic factors and how they interact with the environment in the pathophysiology of PTB in the design of individualized treatments.

Over the last decade, our understanding of the genetics of complex disease has increased substantially due in large part to the robustness of genome-wide association studies (GWAS) [7]. In contrast, genetic studies into PTB aimed at identifying individualized risk have had some success as they have focused on candidate gene studies in determining the risk of PTB [1, 3–5, 11, 13–17]. To overcome the shortcomings of candidate gene studies, GWAS have been proposed as an alternate approach to avoid bias in gene selection. The Preterm Birth Genome Project (PGP) is a consortium initiated by the Preterm Birth International Collaborative (PREBIC), March of Dimes (USA) and the World Health Organization (Geneva) to study genetic predisposition in PTB using GWAS [2]. Candidate gene associations have been reported widely in PTB, and PREBIC has recently summarized these data in a systematic review by Dolan et al. This report listed several positive and negative associations as well as data that were reproduced in multiple studies [5].

Based on the minimal and optimal phenotype data sets outlined by Pennell et al., this consortium has accumulated
DNA samples from PTB studies across the globe, currently totaling more than 5000 PTB cases and 5000 term controls [11]. Unlike other consortia, the PGP is utilizing a single genotyping center for GWAS using DNA samples collected from multiple countries, samples and extraction methods. Although this approach has a number of advantages, including uniformity in genotyping, it requires validation in quality control (QC) across recruitment sites and methods to ensure that sample processing is of adequate quality to generate reliable data. Therefore, the PGP performed a series of QC analyses to assess the usability of DNA collected and processed from different countries utilizing different DNA extraction techniques, prior to GWAS. In this report, we describe the data from phase 1 (QC phase) of the five phases proposed by the PGP consortium (www.prebic.net). Further, we have evaluated the impact of shipping at different temperatures on down-stream array performance. These studies are intended to inform clinical investigators, key players in GWAS level studies of PTB, as to the best practices for sample processing at their respective centers.

Method

Fifteen maternal-fetal pairs were identified from four countries (Korea, Denmark, Mexico and Canada) that met the criteria for utilization for GWAS within the PGP consortium. The DNA was extracted in each country from different biological samples: 1) whole blood (Korea); 2) blood spots with whole genome amplification (Denmark); 3) saliva utilizing the salivate for DNA collection (Mexico); and 4) buccal swab (Canada). The 15 maternal samples (M1–M15) from each country were aliquoted twice, with one set of aliquots shipped to the centralized genotyping facility at University of Western Australia (Perth) on dry ice and the other sent at room temperature. The 15 fetal samples from each country were all sent on dry ice. All samples were shipped via expedited delivery and reached the genotyping facility within 3 days from the date of shipment.

Fifty arrays were performed on samples from each country: M1–M15 shipped on dry ice, M1–M15 shipped at room temperature, M1–M5 shipped on dry ice (replicates) and F1–F15 shipped on dry ice. All samples had OD260:280 ratios between 1.8 and 2.0. Genotyping was performed using the Affymetrix genome-wide human SNP array 6.0 following standard protocols.

Standard QC procedures were applied to data before analyses, including: minor allele frequency >0.01, maximum missingness in genotype calls <0.05 and Hardy-Weinberg equilibrium P>0.0001. A comparison of QC metrics was performed using χ²-analyses. Analyses for comparing extraction techniques were performed utilizing all samples shipped on dry ice (15 maternal samples, five maternal samples replicates and 15 fetal samples). Analysis investigating the effect of shipping temperature from the four countries compared 15 maternal samples shipped on dry ice with 15 paired maternal samples shipped at room temperature. Accuracy of genotyping calls was assessed using two techniques: 1) replication of M1–M5 samples shipped on dry ice from each country and 2) Mendelian concordance on maternal-fetal pairs shipped on dry ice. Call rates are presented as mean±standard deviation (SD). Comparisons of call rates between countries were performed using Kruskal-Wallis non-parametric analysis of variance. P values <0.05 were considered significant. DNA samples were collected from respective institutions under specific IRB approved protocols.

Results

The biological source of the DNA affected the number of SNPs passing QC metrics (Figure 1), with significantly fewer SNPs passing QC metrics when DNA was isolated from buccal swabs (P<0.0001; Canadian samples 506,607/906,600) compared to DNA from blood (Korean 638,981/906,600; Danish 694,584/906,600) or saliva (Mexican 757,863/906,600). The difference in QC metrics was primarily driven by a significant increase in missing genotype data in chips utilizing DNA derived from buccal swabs (P<0.0001). Using DNA extracted from buccal swabs, only 11 of the 35 arrays could be processed to completion due to poor DNA quality and samples failing Affymetrix QC metrics.

The highest average call rates were obtained from arrays where DNA was extracted from whole blood or from Salivette™ containers (whole blood 99.21±0.36 vs. Salivette™ 99.32±0.23%; P=0.314; Table 1). Significantly lower call rates were obtained from DNA obtained from blood spots with WGA (Danish, 98.89±0.40; P<0.0001) or DNA obtained from buccal swabs (Canada, 96.14±1.19; P<0.001). Taken together, these data suggest that DNA from buccal swabs was the worst performing of the four options evaluated in this study.

The temperature of DNA shipment did not alter the call rates between samples from any country (Table 2). These data were reassuring given the costs incurred with shipment of samples on dry ice.

Differences in call rate accuracy, as assessed by replication of genotyping, reveal higher rates of accuracy in arrays using DNA extracted from blood or saliva, whereas there was a significantly higher rate of inaccuracy in calls of buccal swab derived DNA (P=0.004, Table 3). Similarly, when assessed for Mendelian concordance, accuracy was significantly less with DNA extracted from buccal swabs (P=0.0009, Table 4) compared to DNA extracted from blood or saliva.

Figure 1 Comparison of QC metrics between DNA extraction techniques for GWAS.
Discussion

DNA extracted from blood, Salivette™ and blood spots with whole genome amplification were found to be adequate and similar in outcomes in our pilot study. Our data suggest that DNA extracted from buccal samples were not suitable for GWAS analysis for the following reasons: 1) Only 11/35 samples passed the Affymetrix QC checks during sample preparation for GWAS and 2) the samples that qualified for GWAS had significantly lower call rates, reducing the number of informative analyses that could be performed.

Our data suggest that DNA obtained from blood samples provides the best combination of cost, call rate, accuracy and reproducibility. Although blood spots are cheaper to collect, easier to store and performed equally well on the arrays compared to DNA from whole blood, the process of whole genome amplification required for these samples is expensive and is technically challenging. Similarly, Salivette™ was effective at providing good quality DNA for GWAS analysis. Although this approach is more expensive than blood sample collection due to the cost of the sample containers, it is a non-invasive option and ideal for certain populations.

Our data indicate that DNA samples do not need to be shipped on dry ice. In our study, the shipping time was similar for dry ice and room temperature by design; therefore, we cannot address the effect of prolonged shipping of samples at room temperature in GWAS. Given the stability of DNA, we do not anticipate that shipment time would be a major issue in most circumstances.

This study demonstrates that the source (whole blood, blood spots or saliva), extraction procedure and shipment temperature can have impact on the outcome of GWAS data in terms of accuracy and reproducibility. However, even the worst performing collection and processing method (buccal swabs) can yield a reasonably large amount of quality data if alternatives

Table 1 Comparison of extraction techniques.

<table>
<thead>
<tr>
<th>Country of origin</th>
<th>DNA extraction technique</th>
<th>Arrays processed to completion</th>
<th>SNPs passing QC metrics</th>
<th>Call rate</th>
<th>Call rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Korea</td>
<td>Blood</td>
<td>33/35</td>
<td>638981 (71%)</td>
<td>0</td>
<td>99.21±0.36</td>
</tr>
<tr>
<td>Mexico</td>
<td>Salivette</td>
<td>34/35</td>
<td>757863 (84%)</td>
<td>0</td>
<td>99.32±0.23</td>
</tr>
<tr>
<td>Denmark</td>
<td>Blood spot + WGA</td>
<td>35/35</td>
<td>694584 (77%)</td>
<td>2</td>
<td>98.89±0.40</td>
</tr>
<tr>
<td>Canada</td>
<td>Buccal swab</td>
<td>11/35</td>
<td>506607 (56%)</td>
<td>11</td>
<td>96.14±1.19</td>
</tr>
</tbody>
</table>

*Objective to complete M1–M15 (dry ice), M1–M5 replicate (dry ice), F1–F15 (dry ice).

† Using DNA extracted from buccal swabs, only 11 of the 35 arrays were processed to completion due to poor DNA quality and samples failing Affymetrix QC metrics.

Table 2 Comparison of shipping DNA on dry ice compared to room temperature.

<table>
<thead>
<tr>
<th>Country of origin</th>
<th>Pairs available for comparison</th>
<th>Dry ice call rate% (Mean±SD)</th>
<th>Room temperature call rate% (Mean±SD)</th>
<th>Temperature P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Korea</td>
<td>15/15</td>
<td>99.14±0.41</td>
<td>99.32±0.30</td>
<td>0.191</td>
</tr>
<tr>
<td>Mexico</td>
<td>15/15</td>
<td>99.32±0.22</td>
<td>99.35±0.16</td>
<td>0.945</td>
</tr>
<tr>
<td>Denmark</td>
<td>15/15</td>
<td>98.78±0.44</td>
<td>98.54±0.62</td>
<td>0.198</td>
</tr>
<tr>
<td>Canada</td>
<td>3/15</td>
<td>96.91±0.43</td>
<td>96.45±0.43</td>
<td>0.400</td>
</tr>
</tbody>
</table>

*Using DNA extracted from buccal swabs, only three of the 15 pairs of arrays were processed to completion due to poor DNA quality and samples failing Affymetrix QC metrics.

Table 3 Assessment of accuracy using replication of genotyping for samples shipped on dry ice.

<table>
<thead>
<tr>
<th>Country of origin</th>
<th>Pairs available for comparison</th>
<th>Call rate% (Mean±SD)</th>
<th>Inconsistency replication% (Mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Korea</td>
<td>5/5</td>
<td>99.21±0.36</td>
<td>0.26±0.22</td>
</tr>
<tr>
<td>Mexico</td>
<td>5/5</td>
<td>99.32±0.23</td>
<td>0.48±0.18</td>
</tr>
<tr>
<td>Denmark</td>
<td>5/5</td>
<td>98.89±0.40</td>
<td>0.47±0.55</td>
</tr>
<tr>
<td>Canada</td>
<td>4/5</td>
<td>96.14±1.19</td>
<td>4.37±2.27</td>
</tr>
</tbody>
</table>

*M1–M5 samples were genotyped on two separate arrays for genotyping call comparisons.

† Using DNA extracted from buccal swabs, only four of the five pairs of arrays were processed to completion due to poor DNA quality and samples failing Affymetrix QC metrics.

Table 4 Assessment of accuracy using Mendelian concordance.

<table>
<thead>
<tr>
<th>Country of origin</th>
<th>Maternal-fetal pairs available for comparison</th>
<th>DNA extraction technique</th>
<th>Mendelian concordance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Korea</td>
<td>15/15</td>
<td>Blood</td>
<td>99.95</td>
</tr>
<tr>
<td>Mexico</td>
<td>15/15</td>
<td>Salivette</td>
<td>99.96</td>
</tr>
<tr>
<td>Denmark</td>
<td>15/15</td>
<td>Blood spot+WGA</td>
<td>99.94</td>
</tr>
<tr>
<td>Canada</td>
<td>7/15</td>
<td>Buccal swab</td>
<td>99.30</td>
</tr>
</tbody>
</table>

*Paired comparison between M1–F1 to M15–F15 (all shipped dry ice).

Using DNA extracted from buccal swabs, only seven of the 15 pairs of arrays were processed to completion due to poor DNA quality and samples failing Affymetrix QC metrics.

‡ P=0.0009.
are not feasible. Therefore, although providing evidence for differences among methods, our data provide support for study designs that pool existing resources and utilize common genotyping facilities for GWAS studies of pregnancy outcomes. In conclusion, our results inform clinical investigators as to best practices in studies of the genetics of preterm birth and related fields while guiding clinical researchers still justifying the use of less than optimally collected samples. Studies, such as ours can serve future research, especially in obstetrics, which is not yet substantially invested in genetic analyses. By using those methods that we demonstrated yield the best results, researchers can design approaches to sample collection that are the most cost effective in the long-term. However, our results also provide evidence that even suboptimal methods can be used to minimize the need for new sample collection, thereby providing a cost effective strategy to perform GWAS studies on pregnancy outcomes utilizing existing resources.

Preterm birth is a complex disease [8, 6] and identification of risk factor(s) is of extreme importance for appropriate diagnosis and interventions.

Acknowledgments

The Preterm Birth Genome Project has been generously funded by the World Health Organization, the March of Dimes Foundation, the Alberta Health and Medical Research Foundation, Telethon (Western Australia), the Women and Infant Research Foundation and the Government of Mexico.

Consortium contributors

Australia (Genotyping facility and array quality control analyses; study design, manuscript preparation)
Craig E Pennell1, Jennifer Henderson1, Q Wei Ang1, Blagica Penova-Veselinovic1, Melanie Slater1
Mexico (sample collection, DNA extraction)
Felipe Vadillo-Ortego7
Canada (sample collection, DNA extraction)
David M Olson3, Inge Christiaens3
Korea (sample collection, DNA extraction)
Eun-Hee Ha4
Denmark (sample collection, DNA extraction)
Jørg Olson5, Mads Melby, Mads Hollegaard, David Hougard6
United States (study design, manuscript preparation)
Ramkumar Menon7, Scott Williams8, Michael Katz9, Siobhan Dolan10
United Kingdom (statistical analyses)
Tim Frayling11, Andrew Wood12, Caroline Relton13
Switzerland (study design, manuscript preparation)
Mario Merialdi14, Ana Pilar Betran14
1School of Women’s and Infants’ Health, The University of Western Australia, Perth, Australia
2School of Medicine, Universidad Nacional Autonoma de Mexico, Mexico
3Department of Obstetrics and Gynecology, University of Alberta, Canada
4Ewha Womans University Medical School, Department of Preventive Medicine, Seoul, Korea
5Department of Epidemiology, School of Public Health, University of California Los Angeles, Los Angeles, CA
6Statens Serum Institut, Dept. Clinical Biochemistry and Immunology, Section of Neonatal Screening and Hormones, Copenhagen, Denmark
7Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, GA
8Center for Human Genetics Research, Division of Human Genomics, Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN
9Research and Global Programs, March of Dimes Foundation, White Plains, NY
10Department of Obstetrics and Gynecology, Albert Einstein College of Medicine, NY, NY
11Genetics of Complex Traits, Peninsula Medical School, St. Luke’s Campus, Exeter, UK
12Sir James Spence Institute, Newcastle University, Royal Victoria Infirmary, Newcastle upon Tyne, UK
13Institute of Genetic Medicine, Newcastle University, UK
14Department of Reproductive Health and Research, World Health Organization, Geneva, Switzerland

Preterm Birth Genome Project (PGP) Consortium

Management Committee: George Davey-Smith1, Eunyoung, Ha2, Ramkumar Menon7, Jeffrey C. Murray3, Craig Pennell5, Poul Thorsen6, Felipe Vadillo-Ortego7, Mario Merialdi (Chair)8
Steering Committee: Calvin Hobel9, Michael Katz (Director)10, Ramkumar Menon11, Mario Merialdi12, Bonnie Petrukas13, Scott M. Williams14, Marshall Lindheimer (Chair)15
Scientific Committee: Scott M. Williams12, Sample Selection: Stephen Fortunato16, Eunyoung, Ha2, Bo o Jacobsson18, Craig Pennell7, Felipe Vadillo-Ortego7, Steven S. Witkin19, Siobhan M. Dolan (Chair)17, Genotyping: Jeffrey C. Murray3, Carole Ober20, Kenneth Ward21, Scott M. Williams12, Timothy Frayling (Chair)20
Database Management/Data Analyses: Martha Karen Campbell21, Michael Kramer22, Jason H. Moore23, Nils-Halvdan Morken24, Caroline Relton (Chair)25
Prospective collection and replication: Mehmet R. Genc26, Errol R. Norwitz27, Poul Thorsen28, David Olson (Chair)29
Institutional Review Board/Informed Consent: Eunyoung, Ha2, Jeffrey C. Murray3, David Olson30, Felipe Vadillo-Ortego31, S. Vanderpoe1 (Chair)30
PGP Manuscript Committee: Siobhan M. Dolan32, Stephen Lye33, Ramkumar Menon34, Caroline Relton35, Calvin Hobel (Chair)36
Affiliations for participants: Medical Research Council Centre for Causal Analyses in Translational Epidemiology, Department of Social Medicine, University of Bristol, Bristol, BS8 2BN.
2Department of Biochemistry, Kyung Hee Medical Center, Kyung Hee University, Hoigj-dong, Dongdaemun-gu, Seoul 130-701, Republic of Korea.
3The Perinatal Research Center, Centennial Women’s Hospital, 2300 Patterson St., Nashville, TN 37203, USA.
4University of Iowa Department of Pediatrics, 2633 Carver Pavilion, UI Hospitals and Clinics, 200 Hawkins Drive, Iowa City, IA 52242, USA.
5Department of Epidemiology, Rollins School of Public Health, Emory University, 1518 Clifton Road, Atlanta, GA 30322, USA.
6Instituto Nacional de Perinatologia, Montes Urales 800, Lomas 11000, Mexico city.
7Instituto de Infecciosologia, Universidade do Estado do Rio de Janeiro, RJ, Brazil
8Center for Human Genetics Research, Division of Human Genomics, Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN
9Research and Global Programs, March of Dimes Foundation, White Plains, NY
10Department of Obstetrics and Gynecology, Albert Einstein College of Medicine, NY, NY
11Genetics of Complex Traits, Peninsula Medical School, St. Luke’s Campus, Exeter, UK
12Sir James Spence Institute, Newcastle University, Royal Victoria Infirmary, Newcastle upon Tyne, UK
13Institute of Genetic Medicine, Newcastle University, UK
14Department of Reproductive Health and Research, World Health Organization, Geneva, Switzerland

1Department of Epidemiology, School of Public Health, University of California Los Angeles, Los Angeles, CA
2Statens Serum Institut, Dept. Clinical Biochemistry and Immunology, Section of Neonatal Screening and Hormones, Copenhagen, Denmark
3Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, GA
4Center for Human Genetics Research, Division of Human Genomics, Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN
5Research and Global Programs, March of Dimes Foundation, White Plains, NY
6Department of Obstetrics and Gynecology, Albert Einstein College of Medicine, NY, NY
7Genetics of Complex Traits, Peninsula Medical School, St. Luke’s Campus, Exeter, UK
8Sir James Spence Institute, Newcastle University, Royal Victoria Infirmary, Newcastle upon Tyne, UK
9Institute of Genetic Medicine, Newcastle University, UK
10Department of Reproductive Health and Research, World Health Organization, Geneva, Switzerland
Pediatric Institute, 100 Albany Street, Suite 130, New Brunswick, NJ 08901, USA. 12Vanderbilt University, 519 Light Hall, Nashville, TN 37232, USA. 13Department of Obstetrics and Gynecology, The University of Chicago, 841 S Maryland Ave # MC4076, Chicago, IL 60637, USA. 14Maternal Fetal Group, 210 23rd Ave N, Nashville, TN 37203, USA. 15Department of Obstetrics and Gynecology, Perinatal Centre, Goteborg, Sweden. 16Division of Immunology and Infectious Diseases, Department of Obstetrics and Gynecology, Weill Medical College of Cornell University, New York, NY 10065, USA. 17Department of Obstetrics and Gynecology and Women’s Health, Albert Einstein College of Medicine/Montefiore Medical Center, Belfer 501, 1300 Morris Park Avenue, Bronx, NY 10461, USA. 18Department of Human Genetics, The University of Chicago, Illinois 60636, USA. 19Lucina Foundation, 2749 Parleys Way, Salt Lake City, UT 84109, USA. 20PCMD Magdalen Road, Exeter, UK.

21Department of Epidemiology and Biostatistics, Kresge Building, Room 201, Schulich School of Medicine and Dentistry, The University of Western Ontario London, N6A 5C1, Canada. 22Departments of Pediatrics and of Epidemiology and Biostatistics, McGill University Faculty of Medicine, Montreal, Quebec, Canada. 23Section of Biostatistics and Epidemiology, Dartmouth Medical School, One Medical Center Drive, HB7937, Lebanon, NH 03756. 24Haukeland University Hospital, Jonas Lies vei, 5021 Bergen, Norway. 25Sir James Spence Institute, Newcastle University, Royal Victoria Infirmary, Newcastle upon Tyne, NE1 4LP. 26Brigham and Women’s Hospital, Department of Obstetrics and Gynecology, 75 Francis Street, Boston, MA 02115, USA. 27Yale University School of Medicine, New Haven, CT 06443, USA. 28Perinatal Research Centre, University of Alberta, 220 HMRC, Edmonton, Canada. 29S. Vanderpoel, World Health Organization, Geneva. 30Samuel Lunenfeld Research Institute, 25 Orde St., Room 6-1004-1, Toronto, Canada.

References


The authors stated that there are no conflicts of interest regarding the publication of this article.