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ABSTRACT

Cell therapy using endothelial progenitors holds promise for vascular repair in ischemic retinopathies. Using a well-defined subpopulation of human cord blood-derived endothelial progenitors known as endothelial colony-forming cells (ECFCs), we have evaluated essential requirements for further development of this cell therapy targeting the ischemic retina, including dose response, delivery route, and toxicity. First, to evaluate therapeutic efficacy relating to cell dose, ECFCs were injected into the eye requires extensive preclinical testing. Our study provides supporting evidence to indicate that cell therapy could be a valid therapeutic option for eye disease such as age-related macular degeneration, Stargardt’s disease, and retinitis pigmentosa [1, 2]. Ischemic retinopathies such as diabetic retinopathy, retinopathy of prematurity, and retinal vein occlusion are major causes of visual impairment and it has recently been suggested that the common, underlying vascular insufficiency of these diseases could also be treated using vasoregenerative cell therapy [3]. Some difficulties associated with inducing therapeutic angiogenesis using proteins or gene therapy can be overcome with cell therapy [4]. Various different cell types have been shown to promote revascularization of the ischemic retina such as CD34+ cells [5, 6], mesenchymal stromal cells [7], bone marrow Lin- hematopoietic stem cells [8], and myeloid angiogenic cells [9]. However, it could be argued that in order to adequately regenerate damaged retinal vasculature, a bona fide endothelial progenitor is needed [10].

INTRODUCTION

Emerging evidence from preclinical investigations indicate that cell therapy could be a valid therapeutic option for eye disease such as age-related macular degeneration, Stargardt’s disease, and retinitis pigmentosa [1, 2]. Ischemic retinopathies such as diabetic retinopathy, retinopathy of prematurity, and retinal vein occlusion are major causes of visual impairment and it has recently been suggested that the common, underlying vascular insufficiency of these diseases could also be treated using vasoregenerative cell therapy [3].
Endothelial colony-forming cells (ECFCs) are a distinct subpopulation of endothelial progenitors [11], characterized by their high proliferative potential and vasculogenic capacity [12]. In preclinical studies, administration of ECFCs have demonstrated therapeutic efficacy by promoting vascular repair in ischemic tissues, including the myocardium [13], brain [14], hind limb [15], and kidney [16]. ECFC cell therapy has also been shown to have impressive efficacy in murine models of retinal disease [17–20], and there is a growing basis for using these cells in patients. Interestingly, there is debate about the mechanism of action for cell therapies. ECFCs exhibit an unequivocal endothelial phenotype, and therefore a cell replacement mechanism, whether as supportive to angiogenesis or de novo vasculogenesis has been reported [17]. In addition, a paracrine mechanism of action has also been described [21, 22]. Nevertheless, irrespective of the mechanism of action, there is consistent evidence for a therapeutic effect of ECFCs in revascularizing the ischemic retina [17, 19, 20, 23].

We have previously reported that intravitreal delivery of $1 \times 10^5$ ECFCs promotes vascular repair in a mouse model of ischemic retinopathy [17, 19, 23], although many obstacles remain to be addressed to optimize an ECFC cytotherapy strategy. In the present study, we sought to focus on some of these important bottlenecks for clinical translation such as the cell purity, immunophenotypic definition, the minimal therapeutic dose, alternative delivery systems, and cell toxicity when injected into a healthy adult eye.

**Materials and Methods**

**Cell Isolation and Characterization**

ECFCs were obtained from human umbilical cord blood with appropriate maternal consent and under full ethical approval in accordance with the Declaration of Helsinki. Cord blood samples are untraceable to donors. The mononuclear cell fraction was isolated by density gradient fractionation, resuspended in EGM-2 (Lonza Ltd.) supplemented with 20% fetal bovine serum (HyClone), and plated in flasks precoated with rat tail collagen type I (BD Biosciences) and seeded at a density of $1 \times 10^5$ cells/ml. ECFC colonies were isolated from single umbilical cord blood samples without pooling different samples together and without considering the sex of the donor cord blood. ECFCs were characterized using standard flow cytometry immunophenotyping protocols using antibodies against CD31, CD105, CD14, and CD45 (eBioscience) and an Acoustic Focusing Cytometer (Attune NxT, Life Technologies). Further characterization was done with immunocytochemistry and confocal microscopy using antibodies against vimentin, CD105, and CD31 (Dako); β-catenin (Cell Signaling Technology); and vWF (Abcam). In vitro three-dimensional (3D) tube formation assays were performed by resuspending $1 \times 10^5$ ECFCs in 50 μl Growth Factor-reduced Matrigel (BD Biosciences) and covered with previously described media. For all experiments, ECFCs at passage 9 were used. All cells used tested negative for mycoplasma by PCR (Sigma).

**Oxygen-Induced Retinopathy Mouse Model**

Animal procedures were performed under U.K. Home Office licence in compliance with the Animals (Scientific Procedures) Act. C57Bl/6J mouse pups and their nursing dams (Harlan Laboratories) were exposed to hyperoxia (75% O2) in an oxygen chamber (Pro-Ox 110, Chamber Controller, Biospherix, Redfield, NY) for 5 days from postnatal day P7 to P12 as previously described [24]. Mice were randomly assigned to experimental groups and all were sacrificed at P17 by intraperitoneal injection of sodium pentobarbital. A control group were sacrificed at P12 to confirm reproducible vascular loss ($n = 7$).

**Intravitreal Delivery of ECFCs: Dose-Escalation Study**

P13 mice were anaesthetized via intraperitoneal injection of xylazine (5 mg/kg, Bayer) and ketamine (100 mg/kg, Pfizer) and ECFCs were injected into the vitreous of the left eye at a dose of $1 \times 10^3$ ($n = 7$), $1 \times 10^4$ ($n = 5$) or $1 \times 10^5$ ($n = 7$) cells, resuspended in 1 μl, and using a 10 μl glass syringe with a 34G needle. The right eye received an equivalent 1 μl injection of vehicle (phenol red-free Dulbecco’s Modified Eagle Medium, DMEM). A subset of pups received a sham injection (empty 34G needle inserted and withdrawn) into the left eye and the right eye served as an un.injected control ($n = 3$). At P17, fluorescein angiographs were acquired using a confocal scanning laser ophthalmoscope (cSLO, Heidelberg Retina Angiograph 2, Heidelberg Engineering, Germany) prior to sacrifice, with the eyes enucleated for immunohistochemistry.

**Systemic Delivery of ECFCs and Microspheres**

In a separate cohort of mice, the left common carotid artery was exposed by blunt dissection under xylazine/ketamine anaesthesia at P13. 5,000 red-orange (580/620) fluorescent 15 μm diameter polystyrene microspheres (FluoSpheres, Invitrogen, U.K.) were resuspended in a volume of 5 μl and injected into the left common carotid artery using a 10 μl glass syringe with a 34G needle. Pups were sacrificed 2 hours post-injection (P13) or at P14, P15, P16, or P17 ($n = 1$ for each time point) and the eyes were enucleated for immunohistochemistry. For the cell treatment groups, ECFCs ($n = 7$) were injected into the left common carotid artery, at a density of $1 \times 10^5$ cells suspended in 5 μl of DMEM. Another group received an intracarotid injection of DMEM only ($n = 5$). To directly compare systemic and intravitreal ECFC delivery, an additional group of mice ($n = 8$) received an intravitreal injection of $1 \times 10^3$ ECFCs in 1 μl DMEM vehicle into the left eye, with the right eye used as a vehicle-injected control. At P17, all mice were sacrificed and the eyes were enucleated for immunohistochemistry.

**Immunohistochemistry**

Retinas were fixed in 4% paraformaldehyde for 1.5 hours, dissected, and incubated with a biotin-conjugated isoelectric B4 antibody (20 μg/ml, Sigma-Aldrich, U.K.) and labeled with streptavidin AlexaFluor 488 (1/500, Invitrogen, U.K.). Retinas were imaged at ×4 magnification using an epifluorescent microscope (Nikon Eclipse E 400, Nikon) and experimental groups blinded for quantification of avascular, neovascular, and normovascular areas by manual delineation, using Image J software.

**Intravitreal Delivery of ECFCs in Healthy, Adult Mice**

In order to examine possible toxicity of ECFC therapy, irrespective of therapeutic efficacy, $1 \times 10^5$ ECFCs resuspended in 1 μl of DMEM were injected into the vitreous of the left eye of healthy 12-week old male C57Bl/6J mice under xylazine/ketamine anaesthesia. The right eye received an equivalent injection of DMEM. Mice were sacrificed at 2 hours, 12 hours, 24 hours, 3 days or 7 days post-injection ($n = 5$ each group). Eyes were enucleated and processed for histology or Alu-PCR as previously described [25]. Whole retinas were dissected from the eye to detect the presence of human DNA in each retina. The number of ECFCs in the retina was quantified using a standard curve constructed from a serial dilution of cells to relate human DNA concentration to the number of ECFCs. For histology, eyes were fixed in 4% paraformaldehyde for 4–6 hours, paraffin processed, and cut into 5 μm thick
sections before H&E staining. Retinal sections were examined by light microscopy (Nikon Eclipse E 400, Nikon) to identify hematoxylin stained nuclei in the vitreous.

Statistical Analysis
All data analyses were performed blind to treatment group. Data presented as mean ± SD. Statistical analyses were undertaken using Prism 5 (GraphPad Software, San Diego, CA). One-way ANOVA was used to compare avascular, neovascular and normovascular areas between groups with Bonferroni’s post-test. Statistical significance was set at $p < .05$.

RESULTS

Characterization of ECFCs Purity, Potency, and Viability
ECFCs were isolated following protocols previously described [17, 26]. ECFCs appeared as cobblestone-shaped cell monolayers that stained positive for Vimentin and β-catenin (Fig. 1A). In addition, the endothelial nature of ECFCs was confirmed by positivity to prototypical endothelial markers CD31, CD105, and von Willebrand Factor (Fig. 1A). ECFCs immunophenotype was characterized as a surrogate for purity using four markers by flow cytometry. ECFCs were consistently negative for hematopoietic markers CD14 and CD45; however, they highly expressed endothelial markers CD105 and CD31 (Fig. 1B). This, combined with extensive, previously published evidence, demonstrates that ECFCs are a highly pure population of endothelial cells (>99%) with no or minimal hematopoietic cell contamination (<1%). In addition, ECFCs were capable of forming tubes within 72 hours in an in vitro 3D Matrigel model (Fig. 1C). For cell delivery, ECFCs must pass through microneedles; therefore the effect of such physical stress on $1 \times 10^5$ ECFCs resuspended in $1 \mu l$ going through 34G and 36G needles was evaluated by assessing cell viability. Three methodologies (trypan blue exclusion, CASY electrical current exclusion, and calcein staining) indicated that ECFCs...
viability was comparable among cells that passed through the needles and controls (Supporting Information Fig. S1).

Intravitreal Delivery of Low-Dose ECFCs Demonstrates Comparable Therapeutic Efficacy to Higher Cell Doses in the Murine Oxygen-Induced Retinopathy Model

Three different cell doses were tested by intravitreal injection into murine ischemic retinas, and the retinal vasculature was assessed by angiography and immunohistochemistry. Fluorescein angiograms showed that all ECFC doses reduced retinal avascular area when compared to sham and vehicle-injected eyes (Fig. 2A). In agreement with this, flat-mounted retinas stained with isoelectric B4 identified the retinal vasculature. Avascular areas are delineated in white. (C): Quantification of avascular area expressed as a percentage of total retina area. Figure shows individual data points with mean, interquartiles, and range. *, p < .05; ***, p < .001, ns: not significant. One-way ANOVA with Bonferroni’s post-test. Figure shows statistical significance compared to vehicle treated retinas. Abbreviation: ECFCs, endothelial colony-forming cells; P12, post-natal day 12.

Intravitreal delivery of low dose ECFCs demonstrates therapeutic efficacy comparable to higher cell doses in the murine oxygen induced retinopathy model. (A): Representative fluorescein angiographs acquired in post-natal day 17 (P17) mice demonstrating the retinal vasculature in vivo. Avascular areas are highlighted with an asterisk. Scale bars = 1 mm. (B): Representative flat-mounted retinas (magnification ×4) from P17 mice stained with isoelectric B4 (Alexa488) to identify the retinal vasculature. Avascular areas are delineated in white. (C): Quantification of avascular area expressed as percentage of total retina area. Figure shows individual data points with mean, interquartiles, and range. *, p < .05; ***, p < .001, ns: not significant. One-way ANOVA with Bonferroni’s post-test. Figure shows statistical significance compared to vehicle treated retinas. Abbreviation: ECFCs, endothelial colony-forming cells; P12, post-natal day 12.

Systemic Cell Delivery, via the Common Carotid Artery, Provides an Alternative Administration Route for ECFC-Based Therapy

We have previously published that ECFCs cross the retinal inner limiting membrane (ILM) in neonatal mouse eyes following intravitreal injection [17], but since this route can sometimes be associated with complications [27] and may not be optimal for ECFC engraftment, we decided to use the common carotid artery as an alternative delivery route. Fluorescent microspheres were delivered into the left common carotid artery as a proof-of-principle study to demonstrate that beads delivered into the common carotid artery reach the retinal vasculature. Retinas examined 2 hours postinjection showed evidence of fluorescent microspheres within the vasculature (Fig. 3A). In addition, microspheres were only detected in the left retina, ipsilateral to the injection site, and were not detected in the contralateral right retina (Fig. 3A). Interestingly, fluorescent microspheres were not detected in the retinal vasculature in eyes sampled 24 hours postinjection, which indicated these microspheres reached the retinal vasculature but did not migrate into the retinal tissue. We then evaluated the potential for intracarotid delivery of 1 × 10^5 ECFCs in the OIR model. Flat-mounted retinal tissues showed that avascular areas in ECFC-injected animals were significantly reduced in ipsilateral retinas indicate that ECFC minimal therapeutic dose can be scaled down to 1 × 10^3 ECFCs per microliter, per eye, without losing efficacy.
Figure 3. Systemic administration, via the common carotid artery, can be successfully used as an alternative route of delivery for ECFC-cell based therapy. (A): High magnification photomicrographs of isoelectin B4 (Alexa488) stained mouse retina flatmounts following intracarotid injection of orange-red fluorescent microspheres. Left image shows retinal vasculature of the contralateral right eye on P13, where there is no evidence of microspheres 2 hours following systemic administration via the left common carotid artery (scale bar = 300 μm). Middle image shows microspheres in retinal vasculature of the ipsilateral left eye on P13, 2 hours after injection (scale bar = 300 μm). Image on right shows microspheres at higher magnification (scale bar = 100 μm). (B): Representative photomicrographs of isoelectin B4 (Alexa488) stained retina flatmounts of both ipsilateral and contralateral eyes from P17 oxygen induced retinopathy mice following injection of either vehicle or 1 × 10^5 ECFCs into the left common carotid artery (magnification ×4). Avascular areas are outlined in white. (C): Quantification of avascular areas on retina flatmounts of vehicle and ECFC treated mice. Data presented as individual data points with mean, interquartiles, and range. **, p < .01, One-way ANOVA with Bonferroni’s post-test. Figure shows statistical significance in ipsilateral left eyes compared to contralateral right eyes in ECFC treated mice. (D): Quantitative comparison of the therapeutic efficacy of intracarotid and intravitreal ECFC delivery routes. Ipsilateral retinas (left eye) from intracarotid ECFC treated mice were compared to retinas where ECFCs were delivered directly into the vitreous at the same cell dose (1 × 10^5 ECFCs). All retinas were examined at P17. Data presented as individual data points with mean, interquartiles, and range. Two sample t tests for intracarotid versus intravitreal, p > .05, ns: not significant. Abbreviations: ECFC, endothelial colony-forming cell; P, postnatal day.
but not in the contralateral retinas (right eye) (Fig. 3B, 3C). In addition, there was no significant difference in neovascularization among the experimental groups (Supporting Information Fig. S3A); however, only ipsilateral ECFC retinas demonstrated a significant increase in normovascular areas (Supporting Information Fig. S3B). Furthermore, we directly compared the therapeutic efficacy of ECFCs delivered systemically, with the same dose of ECFCs ($1 \times 10^5$) administered locally by injection directly into the vitreous. The vasoreparative effects of both administration groups were comparable, with no significant difference in avascular, neovascular, and normovascular areas, demonstrating that both delivery routes have similar therapeutic benefit (Fig. 3D). ECFCs labeled with fluorescent Qdots (ThermoFisher) were identified incorporating into host retina irrespective of delivery route; however, there were fewer ECFCs in the systemic delivery than in the local intravitreal delivery (Supporting Information Fig. S4). Taken together, these data demonstrate that intracarotid cell delivery is a viable alternative to the intravitreal route for ECFCs.

**Human ECFCs Show No Adverse Effects Following Intravitreal Injection into Healthy Adult Mice**

To investigate potential adverse effects of ECFCs delivered into a healthy eye, including immunogenicity, tumorigenicity, and toxicity, we delivered $1 \times 10^5$ ECFCs intravitreally into healthy adult mice. H&E sections showed that up to 12 hours following injection, ECFCs form an aggregate of cells clearly observed in the vitreous (Fig. 4A). From 24 hours to 7 days postinjection, the number of ECFCs in the vitreous progressively declined until only a few cells could be visualized. From 24 hours onward, most of ECFCs in the vitreous exhibit a pyknotic nucleus suggesting cell death. This
is likely to be apoptosis because there was no evidence of a local inflammatory response. Alu-PCR was used to quantify the number of human ECFCs present in the host retina. This methodology was validated in vitro with data demonstrating we can consistently correlate amount of DNA from a defined amount of cells with Ct values significantly lower than water controls (Fig. 4B). Heatmap for Alu-PCR values peaked at 12 hours postinjection and gradually declined at 24 hours postinjection. From day 3 onward, no human DNA could be detected (Fig. 4C). Histological evaluation at different time points and up to 7 days after cell delivery showed that there was no immune cell infiltration, no tissue edema, no tumor formation, and no retinal detachment in ECFC-injected retinas. Importantly, retinal tissue integrity was preserved and histology appeared normal (Fig. 4D). These results are evidence that human ECFCs did not induce an inflammatory response when injected into healthy mouse retina, and that ECFCs did not persist in healthy retina beyond 24 hours.

**Discussion**

This study provides preclinical evidence to facilitate the translation of an ECFC-based cell therapy for the ischemic retina. Multiple critical steps in the development of cell therapy products have been described in international guidelines [28]. Here, we focused on cell purity, escalation of cell dose related to efficacy, delivery route, and immunogenicity. Our results are supportive for further development of human cord blood-derived ECFCs as a cell product, but at the same time, highlight that the developmental pathway for cell therapies is challenging. Among the next essential steps are the production of ECFCs in compliance with good manufacturing practice, evaluation of biodistribution, assessment of ECFC tumorigenicity, and the development of potency assays.

We showed evidence that ECFCs are not lysed when passed through 34G and 36G microneedles with internal diameters of 85 μm and 35 μm, respectively. The average diameter of early passage ECFCs in cell suspension is 12–18 μm. This highlights that ECFCs can be delivered with 36G microneedles while remaining intact.

We have demonstrated that local administration of 1 × 10^3 ECFCs promotes vascular repair of the murine ischemic retina with a therapeutic efficacy comparable to higher ECFC doses, that is, 1 × 10^5 ECFCs as previously reported [17]. These data will assist for mouse-to-human cell dosage conversion using advanced allometric scaling and modeling. This finding further confirms feasibility of manufacture of adequate cell numbers, since in mice, the minimal therapeutic dose for an ischemic eye is approximately 500-fold lower than the cell number required for an ischemic limb [29]. This also has important implications for the cell product cost of manufacture and suggests that blood from a single umbilical cord will be able to generate enough ECFCs to treat tens of patients. Therefore, ECFCs could be banked as a frozen product for allogeneic cell therapies and cell cryopreservation technology for endothelial cells is progressing rapidly [30, 31], which is applicable to ECFCs. In addition, ECFCs may be primed prior to use by coculturing with mesenchymal stem/stromal cells to further improve vasculogenic potential and engraftment capacity [32]. Our data also show that fluorescein retinal angiography is an effective tool to visualize areas of vascular loss in the OIR mouse model, and the images acquired in vivo are comparable to avascular areas observed on postmortem retinas. This has translational value because retinal angiography performed in the clinical setting is used as a primary read-out in clinical trials. Based on our preclinical data, we recommend that optical coherence tomography angiography and fluorescein retinal angiography are considered as potential endpoints, if an ECFC cell therapy for ischemic retinopathies moves into clinical trials.

The current study demonstrates, for the first time, that ECFCs promote vascular repair when administered systemically, via the common carotid artery. Although intravitreal injections can enable a localized and concentrated cell delivery, the carotid artery delivery route may have advantages since it overcomes the need to cross the ILM, although ECFCs appear to readily cross this barrier in murine disease models [17, 19]. In addition, the intracarotid delivery route also eliminates possible complications associated with intravitreal injections, such as endophthalmitis, retinal detachment, increased intraocular pressure, and ocular hemorrhage [27]. Previously, intracarotid injections have been used to deliver cell therapy in rat stroke models [33, 34] and permits targeting of the ischemic retina while avoiding clearance via the venous system, where typically 70% of cells are found in the liver and spleen following intravenous administration [35]. Interestingly, ECFCs only promoted vascular repair of the retina ipsilateral to the intracarotid injection site and had no therapeutic effect on the contralateral retina. This may result from ECFCs homing toward the ischemic retina which is closest in proximity to the injection site. The possibility of targeting only one retina is also of clinical relevance, since ischemic eye diseases such as retinal vein occlusion present usually as unilateral disease, although recurrence in fellow eye is observed in up to 5% cases within 3 years of initial diagnosis [36].

The mechanism of action for ECFCs in vascular repair is likely to be a combination of both direct cell replacement and paracrine effects. There is previous convincing evidence for these [17, 21], and our data, while demonstrating efficacy, cannot establish a sole major mechanism of action. Our results demonstrate ECFC engraftment into mouse ischemic retinal vasculature, albeit at low levels. This might relate to limitations of the experimental model used, such as the fact that we are injecting human cells into immunocompetent mice. Additionally, in the OIR model, a long-lasting engraftment cannot be expected nor studied because endogenous mouse cells will spontaneously repair the ischemic retinal vasculature within 14–21 days [24]. Therefore, there is need for further studies using models that allow assessment of engraftment and toxicity after 6–12 months of cell delivery. The ultimate proof for cell engraftment and immunogenicity will come from a first-in-human trial, which for allogeneic therapies from cord blood-derived ECFCs will require some level of immunosuppression or alternatively an HLA-matched cell transplant.

Since the OIR model uses neonatal mice whose retinas are not fully developed, we sought to evaluate any potential detrimental tissue responses to intravitreal injection of human ECFCs in healthy adult immunocompetent C57Bl/6j mice. We show evidence to indicate that ECFCs administered in the adult mouse eye did not trigger inflammation or neovascular formation, up to 7 days following intravitreal ECFC delivery. Progressively declining numbers of ECFCs were visible in the vitreous and importantly, ECFCs did not penetrate into the healthy retina presumably since there was no hypoxic stimulus for their chemotaxis. Indeed, vitreous localized ECFCs eventually underwent cell death without an inflammatory response in the retinal tissue. This adds to the safety profile of ECFCs as a cell therapy product, which was also reported.
by systemic tail vein delivery without formation of tumors among nine organs after 7 months follow-up [37].

This preclinical study provides encouraging results that contribute to accumulating evidence for the validity of ECFCs as a cell therapy product for ischemic eye disease. Nevertheless, further studies are warranted to fulfill preclinical benchmarks required to prove safety and efficacy. This is extremely important to avoid preventable adverse effects such as the severe bilateral visual loss recently reported in three patients receiving intravitreal autologous “stem cell” injections [38]. This again highlights the importance of studies at the preclinical stage, which are essential but usually overlooked due to the “eagerness” and various pressures to translate cell therapies into patients. We support the “fast-track” development of novel cell therapies but always within national and international regulatory frameworks [28, 39].

## CONCLUSION

ECFCs are a well-defined and highly pure cell population with potential to be used as a cell therapy product for ischemic retinopathies. The ischemic retina is an attractive target because of easy access for both delivery and evaluation through angiography. In the mouse OIR model, we demonstrated that ECFC cell therapy can be successfully administered in a low dose, by local intravitreal injection or by systemic intracarotid delivery, with both promoting significant vascular repair of the ischemic retina.

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## AUTHOR CONTRIBUTIONS

E.R.: collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; J.G.-F. and C.O.: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript; L.-D.A. and S.E.J.C.: collection and/or assembly of data, final approval of manuscript; A.W.S.: conception and design, final approval of manuscript; R.J.M.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.

## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.


