

Platelet Rich Plasma Augments Adipose-Derived Stem Cell Growth and Differentiation

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Abstract

Background: Adipose-derived stem cells (ASCs) are a powerful tool for cosmetic surgery and regenerative medicine. The use of autologous platelet rich plasma (PRP), particularly in combination with ASC-based therapy, has significantly expanded in recent years. Unfortunately, the mechanisms and optimal dosing responsible for the beneficial effects of PRP remain poorly understood. Here we investigate the effect of PRP on ASC growth and differentiation.

Objectives: To assess the impact of different PRP feeding and cryopreservation protocols on ASC isolation, expansion, and differentiation.

Methods: Human PRP was isolated using the Magellan System (Arteriocyte). Fresh PRP (fPRP), flash frozen PRP (ffPRP), and cryopreserved PRP (cPRP) were added to human ASCs isolated from healthy patients. A panel of PRP supplementation protocols was analyzed for ASC adherence, proliferation, and osteogenesis.

Results: The fresh and cryopreserved PRP groups demonstrated reduced cell adherence compared to control (non-PRP) groups ($P < 0.001$), while the flash frozen PRP groups showed cell adherence equivalent to or better than controls. After 7 days of growth, ASC populations for fPRP and ffPRP Single Administration protocols were significantly higher than other feeding protocols and controls. This benefit was lost in cPRP groups. Optimized ffPRP protocols showed potential for spontaneous osteogenesis.

Conclusions: Addition of ffPRP improves initial ASC adherence while a single administration of either fresh or flash frozen PRP without additional cell manipulation significantly augments subsequent ASC proliferation. The potential for spontaneous osteogenic differentiation upon PRP exposure invokes the need for additional molecular studies of PRP activity prior to further expansion to clinical applications.

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The use of autologous platelet rich plasma (PRP) for cosmetic surgery and regenerative medicine applications has significantly expanded in recent years. Platelets are critical to wound healing as they initiate cell migration, proliferation, and differentiation in response to tissue injury or disease.¹ Upon exposure to fibrin and other stimuli, platelets become “activated” by undergoing a conformational change and releasing granules containing growth factors and other regulatory proteins that stimulate cell growth and repair.^{2,3} PRP has been advocated for an ever increasing array of clinical applications including aesthetic rejuvenation, healing of traumatic, chronic, autoimmune wounds,⁴⁻⁹ and augmentation of fat grafts in autologous fat grafting.¹⁰⁻¹² The mechanisms responsible

for the beneficial effects of PRP on tissue rejuvenation and the optimal PRP dosing protocols, however, remain poorly understood.

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Adipose-derived stem cells (ASCs) are a powerful therapeutic and research tool in cosmetic surgery and regenerative medicine due to their robust nature and ease of isolation from subcutaneous fat.¹³ Significantly, ASCs have also been demonstrated to be therapeutic in an increasing number of regenerative medicine applications including the treatment of myocardial ischemia,¹⁴ inflammatory disorders, and neurodegenerative disease.¹⁵ Thus, the therapeutic applications of ASCs nicely parallel those of PRP. Recent efforts to apply adult human ASCs in regenerative medicine therapies have faltered in the United States, in part due to Food and Drug Administration (FDA) objections to excessive primary human cell and tissue manipulation during stem cell isolation and expansion techniques. Further objections to the use of xenogeneic fetal bovine serum (FBS) for human stem cell expansion have evoked the use of autologous PRP as an alternative medium for stem cell culture and adipose tissue rejuvenation. PRP, however, has been shown to contain osteo-inductive growth factors such as platelet derived epidermal growth factor (PDEGF), fibroblast growth factor (FGF), insulin growth factor (IGF), and transforming growth factor (TGF- β) which can upregulate osteogenic markers in bone marrow cells,^{16,17} suggesting that exposure to PRP may stimulate stem cells to undergo undesired osteogenic differentiation. Considering the ever increasing clinical incorporation of PRP for adipose tissue rejuvenation and fat grafting, this risk represents a significant detriment to cosmetic surgery applications.⁵⁻⁹ Despite these concerns, recent data have supported a role for PRP in safely augmenting the proliferation and differentiation capacities of stem cells.¹⁸⁻²⁰

Given the potential contribution of ASCs and PRP to cosmetic surgery and rejuvenative medicine, and plastic surgery's significant history of innovation in these fields, we believe it is critical to investigate the effect of PRP on primary human ASC growth and osteogenic differentiation. Here, we compare the effect of fresh non-activated PRP (fPRP), flash frozen PRP (ffPRP), and cryopreserved PRP (cPRP) on cell adherence and proliferation in primary human ASCs isolated from whole adipose tissue from patients of varying ages.

METHODS

ASC Isolation

All procedures were conducted with approval from the IRB of the University of Pennsylvania and informed consent. Subcutaneous adipose was excised during consecutive abdominoplasties from four healthy females (mean age, 53.25 ± 6.9 years) between September and December of

2014. No exclusion criteria were used. Tissue was cryopreserved at -70°C without added buffers or preservation media. The stromal vascular fraction (SVF) was isolated using a standard collagenase protocol.²¹ Cell quantification was performed using a Countess automated cell counter (Invitrogen, Carlsbad, CA). Equivalent numbers of cells were seeded and incubated in standard growth media (DMEM/F12, Gibco of Life Technologies, Norfolk, CT, supplemented with 10% FBS, Serum Source International, Charlotte, NC, and penicillin/streptomycin, Gibco) at 37°C in 5% CO_2 for 4 hours prior to the addition of PRP.

PRP Collection and Preparation

Blood (25 cc) was drawn via standard venipuncture from two healthy volunteer females (62 ± 2 years old) between June and August of 2015. No exclusion criteria were used. Whole blood was processed using the Magellan Autologous Platelet Separator System (Arteriocyte Cellular Therapies, Hopkinton, MA). Seven mL of PRP from each patient with platelet enrichment of 4 to $6 \times$ baseline (per Arteriocyte protocol), were pooled in a 1:1 ratio. PRP was prepared as follows: 1) fresh PRP (fPRP) immediately applied to cells; 2) flash frozen PRP (ffPRP) subject to 15 min incubation at -70°C with no added buffers or preservatives followed by thawing at 37°C ; and 3) one-month cryopreservation (cPRP) at -70°C . Experimental ASCs were incubated in 10% PRP and 90% standard growth media.

PRP Feeding Supplementation

Three different feeding protocols were applied to the PRP preparations described above: 1) Single Administration (SA) PRP plus standard growth media on Day 0 left unchanged for 7 days or until quantification; 2) Repeated Administration (RA) PRP plus media on Days 0, 2, and 4; and 3) Repeated Control (RC) PRP plus media on Day 0, with media only supplementation on days 2 and 4. Single Administration and Repeated Administration media-only controls (C) were fed in parallel with SA and RA experimental protocols (Figure 1). All standard growth media contained 10% FBS.

ASC Adherence and Proliferation

Proliferation assays were carried out on Day 1, 2, 4, and 7 (Figure 1) using the CyQUANT NF Cell Proliferation Assay Kit (Molecular Probes of Life Technologies Co., Norwalk, CT) according to manufacturer's instructions. On Day 1, fluorescence was measured and compared between groups to quantify initial cell adherence. Measurements were normalized to media only control and Day 1 values to determine population doubling (PD).

Osteogenic Differentiation with ffPRP Supplementation

ffPRP supplemented cells demonstrated the most robust cell adherence and growth curves of all groups and were subsequently tested for osteogenic differentiation. ASCs were divided into 4 groups: 1) standard growth media; 2) differentiation control with osteogenic media (standard growth media supplemented with: 0.1 $\mu\text{mol/L}$ Dexamethasone, 5 $\mu\text{g/mL}$ β -glycerol phosphate, and 50 $\mu\text{mol/L}$ ascorbic acid, each from Sigma, Bloomington, MN); 3) PRP control with 10% PRP in standard growth media; 4) PRP + Differentiation with 10% PRP and osteogenic media. ASCs were incubated with or without 10% ffPRP for 7 days prior to differentiation then incubated in standard or osteogenic media for 21 days followed by staining for alkaline phosphatase (ALP) activity using Fast

Blue RR Salt and Naphthol AS-MX phosphate (Sigma, Bloomington, MN) as per manufacturer's protocol.

Statistical Analysis

Statistical analysis of cell adherence results was carried out via one-way ANOVA with Tukey post hoc comparing all values to media only controls. Proliferation results were analyzed via one-way ANOVA using a Bonferroni post hoc comparing all groups in a pairwise manner using Prism 5 software (GraphPad Software Inc. La Jolla, CA).

RESULTS

Flash Frozen PRP Preparation Best Maintains Initial Stem Cell Adherence

To determine whether PRP affects initial ASC adherence post SVF plating, cell adherence for all PRP preparation groups was measured on Day 1 after plating. fPRP-RA (1205.8 \pm 368.0 AFU), fPRP-RC (999.9 \pm 479.1 AFU), and fPRP-SA (1164.3 \pm 636.2 AFU) all demonstrated significantly lower cell adherence than control (2499.3 \pm 760.7 AFU, $P < 0.001$, Figure 2A). In contrast, ffPRP-RA (2999.8 \pm 1033.9 AFU), ffPRP-RC (2318.0 \pm 936.3 AFU) and ffPRP-SA (1955.3 \pm 834.4 AFU) groups demonstrated similar cell adherence compared to media only controls (2499.3 \pm 760.7 AFU, Figure 2A). When cPRP was analyzed, cPRP-RA (1895.4 \pm 1022.3 AFU), cPRP-RC (1370.8 \pm 287.8 AFU), and cPRP-SA (1622.0 \pm 687.1 AFU) all demonstrated significantly lower cell adherence compared to control (3474.0 \pm 907.4 AFU, $P < 0.001$, Figure 2B).

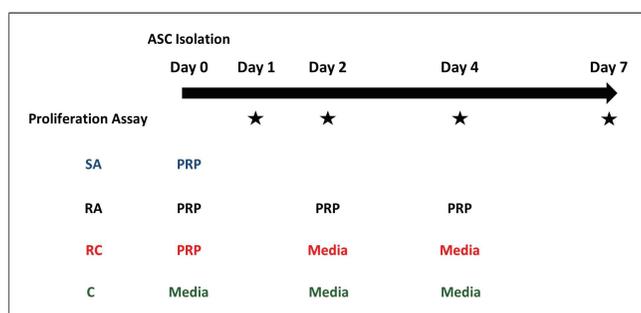


Figure 1. PRP supplementation protocols for ASC growth. Diagram depicting the PRP feeding and ASC assay time points. ★ denotes proliferation assays. C: media-only control; Media denotes when standard growth media was added to cells; PRP denotes when PRP was added to cells; RA, repeated administration; RC, repeated control; SA, single administration.

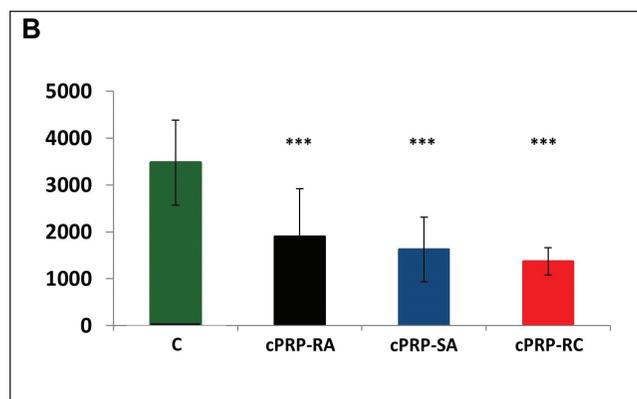
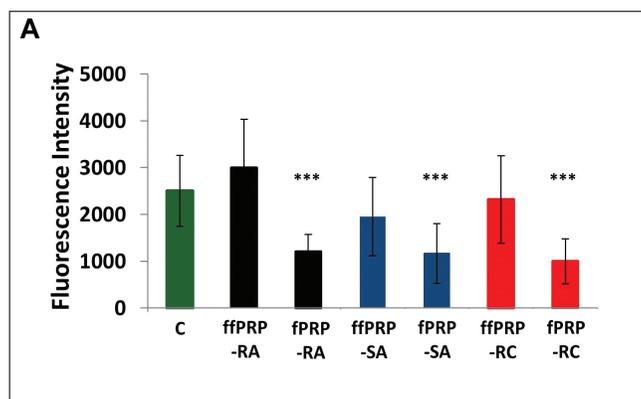


Figure 2. Fresh and cryopreserved PRP reduce ASC Adherence while flash frozen PRP does not. PRP isolated from healthy female patients (61 \pm 2 years old) was added to ASCs isolated from healthy female patients (53.25 \pm 6.9 years old, $n = 4$) according to reported storage and feeding conditions and compared to media only controls. Cell adherence was quantified 24 hours after cell isolation and plating for (A) fPRP and ffPRP experiments and (B) cPRP experiments and compared to media only controls. ***- $P < 0.001$ vs media only control. ASCs, adipose-derived stem cells; C, media-only control; cPRP, one month cryopreserved PRP; ffPRP, flash frozen PRP; fPRP, fresh PRP; RA, repeated administration; RC, repeated control; SA, single administration.

Single Administration PRP Supplementation Protocols Augment ASC Proliferation

Subsequently, we investigated whether distinct PRP supplementation protocols differentially affect ASC proliferation. Seven days post-ASC expansion, the fPRP-SA (4.11 ± 1.67 PD, Figure 3A) and ffPRP-SA (3.85 ± 2.32 PD, Figure 3B) groups exhibited significantly higher cell populations than all other groups ($P < 0.01$, Figure 3). fPRP-RA (1.5 ± 0.4 PD), ffPRP-RA (0.64 ± 0.24 PD), cPRP-RA (1.41 ± 0.5 PD), fPRP-RC (2.65 ± 2.11 PD), and ffPRP-RC (1.67 ± 1.06 PD) groups did not differ significantly in population size. On Day 7, no significant difference was observed between cPRP-RA (1.41 ± 0.5 PD) and cPRP-SA (1.4 ± 0.53 PD) ($P > 0.05$). However, cPRP-RC resulted in significantly lower cellular populations compared to either of the other groups (0.36 ± 0.27 PD, $P < 0.001$).

Flash Frozen PRP May Spontaneously Induce Osteogenesis in ASCs

To determine whether ASC exposure to PRP can result in spontaneous differentiation towards the osteocyte lineage, as has been intermittently reported by others,^{20,22} we grew ASCs in osteogenic media in the presence and absence of ffPRP, the PRP preparation that resulted in most robust ASC growth in our initial experiments. As predicted, we observed little alkaline phosphatase (ALP) staining in ASCs grown in control standard growth media (negative control) and strong ALP signal in cells grown in differentiation media (positive control). ASCs grown in differentiation media with supplementation of flash frozen PRP also demonstrated strong ALP signal, confirming that ffPRP supports the capacity for osteogenic differentiation in ASCs. We also observed strong ALP staining in one out

of three ASCs grown with ffPRP without differentiation media, suggesting that PRP supplementation may indeed be sufficient to induce osteogenesis in primary ASCs derived from specific patients (Figure 4).

DISCUSSION

ASCs are increasingly applied in combination with PRP in cosmetic surgery and regenerative medicine applications. Yet, despite the upsurge in applied PRP therapies, the mechanisms responsible for the beneficial effects of PRP remain poorly understood. Here we investigated the effect of PRP on ASC growth to further elucidate the mechanism of PRP activity in adipose tissue rejuvenation and regeneration. We characterized the impact of different PRP supplementation and preparation protocols on ASC isolation, expansion, and differentiation. Specifically, we compared the effect of fPRP, ffPRP, and one month cPRP on cell adherence, proliferation, and osteogenic differentiation in primary human ASCs isolated from whole adipose tissue from patients of varying ages.

Our experiments specifically address the observations that PRP produces growth factors that positively influence ASC growth^{2,3,19,20,22,23} in an attempt to elucidate why data in support for PRP are inconsistent.^{19,20,22-35} Accordingly, we subjected PRP to minimal manipulation avoiding exogenous reagents such as detergents and exogenous enzymatic lysis buffers in order to minimize the potential detrimental effects of such variables on PRP and ASC biology that may have influenced prior studies.²⁵⁻²⁷ We utilized a single fifteen-minute freeze-thaw cycle (ffPRP) to activate platelets rather than additional activating factors.^{26,27} Furthermore, we pooled the two independent patients' PRP samples to mitigate inter-patient variability in platelet count, activity, and enrichment. We hypothesized that this approach permitted us to investigate the effects of PRP

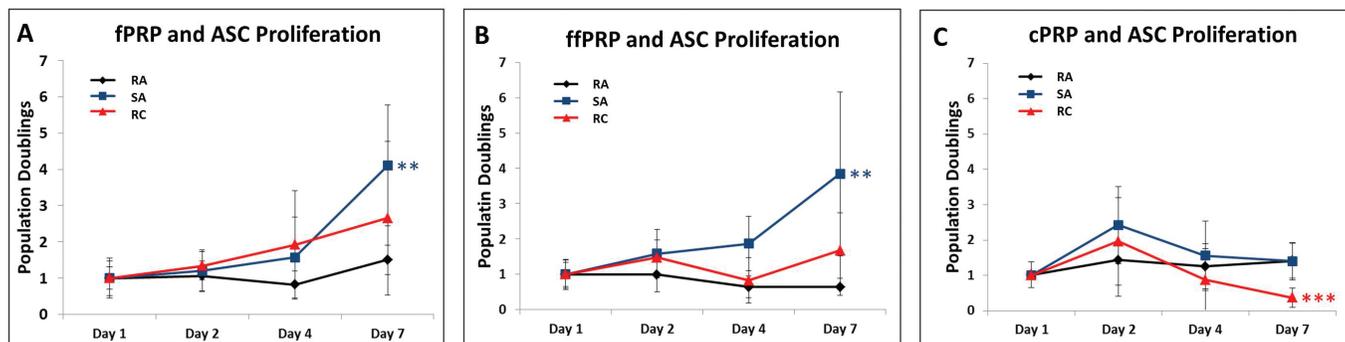


Figure 3. A single administration fPRP and ffPRP supplementation improves ASC population growth. (A) fPRP, (B) ffPRP, and (C) cPRP isolated from healthy female patients (61 ± 2 years old) was added to ASCs isolated from healthy female patients (53.25 ± 6.9 years old, $n = 4$) according to stated storage and feeding conditions. The ASC population was quantified throughout a 7-day time course. $^{**}P < 0.01$ for ffPRP-SA and fPRP-SA on Day 7 vs all other groups. $^{***}P < 0.001$ for cPRP-RC on Day 7 vs all other groups. ASCs, adipose-derived stem cells; C, media-only control; cPRP, one month cryopreserved PRP; ffPRP, flash frozen PRP; fPRP, fresh PRP; RA, repeated administration; RC, repeated control; SA, single administration.

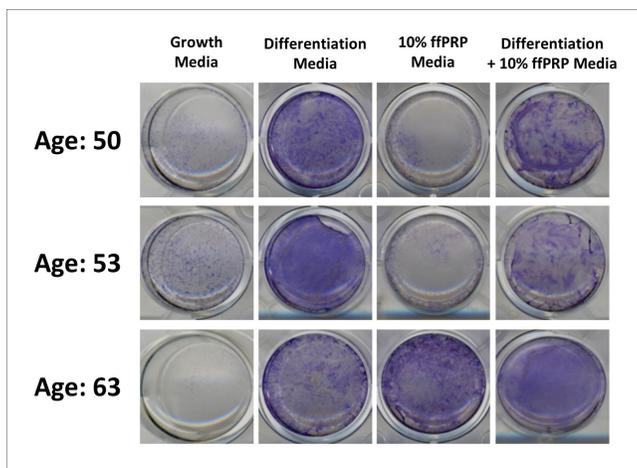


Figure 4. Flash frozen PRP can induce spontaneous osteogenic differentiation in ASCs. ASCs were isolated from tissue derived from healthy patients ($n = 3$). Cells were grown to confluence and incubated with or without 10% ffPRP for 7 days. Cells were then allowed to differentiate in standard growth media or differentiation media throughout a 21-day time course. Cells were fixed and then stained for alkaline phosphatase. ASCs, adipose-derived stem cells; ffPRP, flash frozen PRP.

on ASC in a neutral, clinically relevant, and translatable manner.

We demonstrated that fresh PRP (non-activated) and one-month cryopreserved PRP supplementation resulted in reduced initial cell adherence compared to control groups without PRP supplementation ($P < 0.001$), while the flash frozen PRP (activated) groups showed equivalent to or better cell adherence than controls. These data suggest that the factors released from flash frozen platelets are beneficial to initial ASC adherence and that exposure to inactive or long-term cryopreserved platelets may have a detrimental effect to ASC adherence. It remains to be determined whether the detrimental effect observed via SVF supplementation with the latter two platelet categories is secondary to specific factors released from these platelets or to other variables.

Significantly, the beneficial effects of ffPRP continued beyond initial cell adherence and throughout the tested seven-day growth curve as demonstrated by significantly higher ultimate population sizes as compared to non-PRP controls. In addition, ffPRP also demonstrated significantly higher proliferation rates after initial cell adherence, suggesting that while these platelets were not subject to initial cryolysis, they may have undergone spontaneous activation during the cell culture thereby releasing factors beneficial to ASC proliferation. In contrast, no such benefit was observed in the proliferation of ASCs supplemented with one-month cryopreserved PRP, suggesting that either this PRP preparation prevented the subsequent activation

of platelets, or that the specific panel of factors released from this preparation is detrimental to ASC proliferation. Irrespective of PRP preparation, single administration protocols showed significantly higher ultimate population levels than other feeding protocols and controls. It remains to be determined whether the success of single administration is secondary to extended uninterrupted contact between ASCs and PRP or to reduced mechanical cell disruption associated with media aspiration during feeding of the repeated administration group. These data support flash frozen PRP (activated) in a single bolus supplementation for improving initial ASC adherence and subsequent proliferation.

Yet although the single feed flash frozen PRP preparation and feeding protocol demonstrated optimized ASC growth parameters, we observed the potential for spontaneous osteogenesis in one patient, confirming prior studies. Activated PRP releases osteogenic factors^{16,17} and leukocytes within PRP have been suggested to induce osteogenesis via activation of the NF- κ B pathway.³³ Further, TGF- β , a growth factor released by PRP, modulates bone matrix synthesis by stimulating proliferation of osteoblast precursor cells.¹ Likewise, platelet derived growth factor (PDGF), also released by PRP, has been linked to bone formation both in vitro and in vivo.^{1,36} These reports suggest PRP may indeed independently induce ASC differentiation along the osteogenic lineage perhaps secondary to the release of these factors.^{20,22} Correspondingly, microcalcifications resulting from fat grafting³⁷⁻⁴⁰ further caution that additional research must be conducted prior to the use of PRP for ASC growth, or in other clinical applications. Consequently, future molecular experiments are required to characterize specific factors, such as TGF- β and PDGF, released by PRP activation during the quick freeze-thaw cycle and subsequent ASC proliferation, as well as patient-specific factors, that may predispose these cells to undergo undesired spontaneous differentiation.

While this study utilized PRP produced by a single device to reduce methodology variability, it should be noted that there are multiple separator systems, harvesting devices, and protocols for platelet rich plasma isolation as well as platelet lysate reagents on the market. Several studies have compared these systems and protocols and found that there is large variability in resulting PRP products.^{11,27,41,42} Therefore, this study is limited to PRP produced by the Magellan Autologous Platelet Separator System, though the authors have no financial or other interest in this product.

In the 15 years since their discovery, there has yet to be a large-scale study that demonstrates significant improvement in surgical outcomes when cultured ASCs are used compared with processed fat grafting alone. However, there are several small-scale studies that continue to elucidate the therapeutic potential of these cells.^{5,11,14,15} While

additional confirmatory large-scale studies are required, the work presented here illustrates the potential of combining autologous reagents such as PRP and ASCs for synergistic effects. ASCs continue to be broadly utilized for both clinical and research applications. As such, the mechanisms by which we can safely and efficiently optimize ASC use for cosmetic surgery and regenerative medicine applications must be elucidated.

CONCLUSION

We demonstrated here that the addition of flash frozen PRP improves initial ASC adherence while both fresh and flash frozen PRP significantly augment subsequent ASC proliferation. PRP supplementation in the form of a single administration without additional cell feeding or manipulation is optimal to augment ASC adherence and growth. These effects may be responsible for the therapeutic benefits of PRP on adipose tissue rejuvenation and healing. However, our data support prior reports of spontaneous ASC differentiation upon PRP exposure and invoke a critical need for additional molecular studies on PRP activity and patient-specific factors prior to further expansion to clinical applications.

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Dr Gersch and Mr Glahn contributed equally to this work.

Disclosures

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