Downregulation of Critical Oncogenes by the Selective SK2 Inhibitor ABC294640 Hinders Prostate Cancer Progression

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Abstract
The bioactive sphingolipid sphingosine-1-phosphate (SIP) drives several hallmark processes of cancer, making the enzymes that synthesize SIP, that is, sphingosine kinase 1 and 2 (SK1 and SK2), important molecular targets for cancer drug development. ABC294640 is a first-in-class SK2 small-molecule inhibitor that effectively inhibits cancer cell growth in vitro and in vivo. Given that AR and Myc are two of the most widely implicated oncogenes in prostate cancer, and that sphingolipids affect signaling by both proteins, the therapeutic potential for using ABC294640 in the treatment of prostate cancer was evaluated. This study demonstrates that ABC294640 abrogates signaling pathways requisite for prostate cancer growth and proliferation. Key findings validate that ABC294640 treatment of early-stage and advanced prostate cancer models downregulate Myc and AR expression and activity. This corresponds with significant inhibition of growth, proliferation, and cell-cycle progression. Finally, oral administration of ABC294640 was found to dramatically impede xenograft tumor growth. Together, these pre-clinical findings support the hypotheses that SK2 activity is required for prostate cancer function and that ABC294640 represents a new pharmacological agent for treatment of early stage and aggressive prostate cancer.

Implications: Sphingosine kinase inhibition disrupts multiple oncogenic signaling pathways that are deregulated in prostate cancer.

Introduction
Prostate cancer is the most commonly diagnosed noncutaneous malignancy and the second leading cause of cancer-related deaths in U.S. males. Patients presenting with locally confined disease have a favorable survival outlook due to surgical intervention, whereas patients with disseminated prostate cancer are typically treated with androgen deprivation therapy (ADT) that is transiently effective. Recent FDA-approved therapies for advanced prostate cancer, such as Enzalutamide, Abiraterone Acetate, Sipuleucel-T, and Radium-223 (1), have extended the therapeutic window; however, resistance still occurs and these treatments typically only provide a modest survival advantage. Therefore, novel therapeutic targets, increased therapeutic penetrance and improved overall durability of response are paramount for new treatment options.

Two prominent oncogenes driving prostate cancer initiation, progression, and resistance are androgen receptor (AR) and c-Myc. All stages of prostate cancer are dependent on AR activity, and as such, most treatments focus on targeting this pathway. For example, ADT or direct AR antagonists are often administered for disseminated disease, which affords patients with 24 to 36 months of disease control. However, recurrent prostate cancer (termed castrate-resistant, CRPC) often results due to aberrant reactivation of AR. Myc amplification occurs frequently (~30%) in prostate cancer and correlates with advanced disease, biochemical recurrence, and poor prognosis (2–5). Notably, animal models of Myc-mediated prostate cancer require functional AR for tumorigenesis (6), indicating that both AR and Myc oncogenes are essential for prostate cancer progression. Therefore, the ability to simultaneously suppress the activities of Myc and AR could profoundly inhibit tumor cell growth and dramatically impact clinical options.

Bioactive sphingolipids are dynamic signal transducers that regulate numerous cellular processes, including host inflammation, angiogenesis, cell migration, and regulation of tumor growth (7). As such, manipulating the ceramide/sphingosine-1-phosphate (SIP) rheostat within cancer cells is of pharmacologic interest. Sphingosine kinases 1 and 2 (SK1 and SK2, respectively) catalyze phosphorylation of sphingosine to generate mitogenic SIP and are frequently overexpressed in a variety of human cancers, including prostate cancer (8, 9). SK1 is a cytosolic protein that translocates to the cell membrane upon activation and is necessary for tumor progression (10). Although SK1 and SK2 shares kinase homology and are 80% similar, overall, SK2 contains distinct nuclear localization and export signals, which differentiate cellular localization and biologic functions from SK1 (11, 12). SK2 also contains a proapoptotic BH3 domain that promotes apoptosis when overexpressed (13); however, depletion of SK2 inhibits tumor cell proliferation and migration (14).
and xenograft tumor growth (15). More recently, isozyme-specific inhibitors have elucidated the nonredundant role of SK2 in tumor biology. In particular, ABC294640 is a first in class SK2-specific inhibitor that inactivates AKT and ERK, decreases Myc expression, elicits antiestrogenic effects on estrogen receptor-α, and promotes autophagy (16–20). The ability of ABC294640 to inhibit AR-positive prostate cancer proliferation and signaling pathways has not been previously investigated.

On the basis of these studies and the unresolved need to foster durable therapeutic options for advanced prostate cancer, it was hypothesized that inhibiting SK2 activity with ABC294640 is a viable option for treating prostate cancer by inhibiting several pathways implicit for disease progression. Findings described herein demonstrate that ABC294640 abrogates prostate cancer tumor maintenance by disrupting signaling events regulated in vivo. The ability of ABC294640 to inhibit AR-positive prostate cancer proliferation and signaling pathways has been previously investigated.

In this study, we demonstrate that ABC294640 abrogates prostate cancer tumor maintenance by disrupting signaling events regulated in vivo. The ability of ABC294640 to inhibit AR-positive prostate cancer proliferation and signaling pathways has been previously investigated.

Flow cytometry

Cells were treated and seeded in hormone-proficient or -depleted conditions, as indicated, and labeled with bromodeoxyuridine (BrdUrd; Invitrogen) 2 hours before harvest. Cells were fixed in 100% ethanol, stained with FITC-conjugated anti-BrdUrd antibody (BD Biosciences), and processed using FACS Calibur (BD Biosciences).

Western blot analysis and proteasome degradation

Cells were lysed in buffer containing 25 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS (G Biosciences). Total cell lysates were resolved by 10% or 4% SDS PAGE and immunoblotted with the following antibodies: AR-N20, Myc, pAKT S473, total AKT (Cell Signal Technology), and HRP-GAPDH (Genetex). For analyzing proteasomal contribution, cells were treated with 10 μmol/L MG132 (Enzo) for 24 hours.

In vivo analysis

All procedures involving mice were performed in accordance with Pennsylvania State University IACUC protocols. Seven-week-old male NCR-nude mice (Charles River Laboratories) were s.c. injected in the flank with 3 × 106 cells in 10 μL total saline/ Matrigel (BD Biosciences). When tumors reached approximately 100 to 150 mm3 mice were administered 50 mg/kg ABC294640 or vehicle (46% PEG400/47% Saline/7% EtOH) daily. Tumor volume was measured with calipers.

Statistical analysis

All results were analyzed using the two-tailed Student t test (adjusted for variance) or Mann–Whitney test. For all analyses, a P value of <0.05 was deemed significant.

Results

ABC294640 decreases cell survival of androgen deprivation therapy–sensitive prostate cancer cells

ABC294640 (Fig. 1A) is a first in class, sphingosine kinase 2 inhibitor that selectively inhibits SK2 enzymatic activity, but has no effect on SK1 (23). Previous studies demonstrated the ability of ABC294640 to inhibit cell proliferation of various tumor cell types, including AR-negative DU145 and PC3 prostate cancer cells (23, 24). To determine the effects of ABC294640 on the proliferation of AR-positive, ADT-sensitive LNCaP cells, sulfohydrazine B (SRB), and trypan blue exclusion assays were used. ABC294640 inhibited cell proliferation with an IC50 value of 12 μmol/L (Fig. 1B). On the basis of the IC50 value, cells were treated with 10 and 20 μmol/L ABC294640 to further examine cell growth. Treatments resulted in significantly decreased cell numbers, compared with vehicle treatment, in a dose-dependent manner (Fig. 1C). Furthermore, ABC294640 treatment of LNCaP cells decreased the rate of cell-cycle progression, as evident through a 90% decrease in BrdUrd incorporation, relative to control (Fig. 1D). Therefore, based on cell viability, cell proliferation, and cell-
SK2 inhibition abrogates multiple proliferative pathways in prostate cancer cells

S1P activates multiple mitogenic signaling cascades that drive tumor growth (7). For example, ABC294640 attenuates S1P-mediated AKT activation, which is one mechanism for cell growth suppression (17). To understand whether additional mechanisms exist by which ABC294640 inhibits early-stage AR-positive prostate cancer cell growth, further biochemical analyses were performed on LNCaP cells. Consistent with previous reports, ABC294640 decreased AKT phosphorylation in a time- and dose-dependent manner (Fig. 2A), suggesting that ABC294640 inhibits known S1P-mediated pathways in prostate cancer. Myc expression is often deregulated in prostate cancer progression and promotes aggressive phenotypes and resistance to therapies (4, 25, 26). Previous reports demonstrate that ABC294640 downregulates Myc expression in hematologic cancers (18, 19); however, this has not been previously assessed in epithelial tumor cells. Therefore, Myc expression was analyzed in LNCaP cells following ABC294640 treatment (10–20 μmol/L for 24 and 48 hours). Similar to results in hematologic cancers, Myc protein levels were significantly decreased at doses that inhibit cell growth (Fig. 2A). To determine whether Myc transcriptional activity was affected by ABC294640 treatment, levels of Myc transcript and the expression of Myc target genes CDK1 and ornithine decarboxylase (ODC) were examined by qRT-PCR. Myc mRNA and CDK1 expression were significantly decreased in response to ABC294640 treatment, although ODC was not altered in response to the drug, demonstrating a degree of selectivity for Myc perturbation (Fig. 2B). Therefore, on the basis of these data, ABC294640 treatment of ADT-sensitive prostate cancer inhibits AKT and Myc activity in prostate cancer.

AR-positive, ADT-sensitive prostate cancer cells represent early-stage disease as androgen signaling is required for cell growth and proliferation (27). Because SKI-II, a dual SK1/SK2 inhibitor, was found to modulate AR expression (28), it was hypothesized that ABC294640-mediated SK2 inhibition could disrupt AR signaling and contribute to growth suppression. To address this, AR protein levels in LNCaP cells treated with ABC294640 were examined and found to be decreased by 24 hours and remained depleted throughout ABC294640 treatment (Fig. 2A). Next, AR transcriptional activity was measured by qRT-PCR for three clinically relevant AR target genes, PSA/KLK3, TMPRSS2, and FKBP5, and found to be decreased by 73%, 67%, and 90%, respectively, relative to control. Surprisingly, AR transcript was also decreased following ABC294640 treatment (Fig. 2C). These results demonstrate that in consort with AKT and Myc deregulation, ABC294640 treatment also disrupts AR activity, which is indispensable for early-stage prostate cancer.

To determine whether the ABC294640-mediated decreases in AR and Myc expression are specifically due to inhibition of SK2 activity, LNCaP cells were depleted of SK2 by siRNA and AR and Myc levels were evaluated. siRNA-mediated SK2 depletion reduced mRNA expression 60%, which corresponded with a loss of Myc protein but not mRNA expression (Supplementary Fig. S1). These results are consistent with previous validations of ABC294640-mediated Myc depletion with siRNA approaches that demonstrate SK2 siRNA decreases Myc protein but not mRNA (18, 19). Surprisingly, siRNA-mediated SK2 depletion did not
alter AR mRNA or protein expression (Supplementary Fig. S1). This suggests that ABC294640 could be directly affecting AR activity or that Myc mRNA depletion following treatment is required for AR inhibition.

PF543 potently inhibits SK1 activity ($K_i = 6 \text{ nmol/L}$), blocks S1P production, but, interestingly, has no effect on cancer cell proliferation (29). Therefore, the ability of PF543 to inhibit AR signaling with AR or Myc was analyzed. On the basis of colony formation assay, 10 nmol/L ABC294640 or vehicle for 24 or 48 hours, as indicated. Cell lysates were immunoblotted with Myc, AR, pAKT, AKT, and GAPDH antibodies; B, LNCaP cells were cultured in androgen-depleted media and mRNA transcript levels of Myc, CDK1, and ODC were analyzed by qRT-PCR. C, LNCaP cells were treated as in B and mRNA transcripts of AR, PSA, TMPRSS2, and FKBP5 were analyzed by qRT-PCR. D, LNCaP cells were plated at low density and treated with 10 nmol/L ABC294640, 100 nmol/L PF543, or vehicle for 2 weeks. Cell colonies with fixed, stained with crystal violet, and colonies with 50 or greater cells were counted. **, P < 0.01 compared with DMSO and PF543 conditions. E, LNCaP cells were treated with 10 nmol/L PF543, 100 nmol/L PF543, 1 μmol/L PF543 or vehicle for 72 hours. Cell lysates were immunoblotted with AR, Myc, and GAPDH antibodies; **, P < 0.01.

ABC294640 inhibits CRPC signaling and growth

Advanced prostate cancer is initially managed by blocking the AR signaling axis, which typically progresses to incurable CRPC due to aberrant AR reactivation. Therefore, CRPC cell models were used to determine whether ABC294640 treatment affects Myc and AR signaling within this incurable disease state. To begin, CRPC C4-2 cells, which are metastatic derivatives of LNCaP cells, were treated with ABC294640 and protein levels of Myc and AR were analyzed. Consistent with LNCaP cells, ABC294640 treatment of C4-2 cells decreased Myc protein expression at all doses and time points (Fig. 3A). Next, the consequence of ABC294640 treatment on Myc transcriptional output was determined by qPCR. Myc gene expression in C4-2 cells was decreased 80%, relative to control, and expression of Myc target genes CDK1 and ODC were significantly decreased 40% and 65%, respectively, relative to control (Fig. 3B). Therefore, consistent with LNCaP cells, ABC294640 appreciably disrupts Myc signaling in a disease model where Myc promotes aggressiveness.

CRPC cells, despite survival in the absence of androgens, still require AR signaling for survival (30). Therefore, the ability to negatively regulate AR in CRPC through ABC294640 treatment would directly address an important clinical need. Similar to the results in LNCaP cells, AR protein was depleted in C4-2 cells following ABC294640 treatment in a dose- and time-dependent manner (Fig. 3A). Furthermore, AR mRNA levels were decreased 80%, compared with control, following ABC294640 treatment, and AR transcriptional output was negatively regulated on the basis of 94%, 57%, and 49% decreases in the levels of PSA/KLK3, TMPRSS2, and FKBP5, respectively (Fig. 3C). Finally, cell proliferation was measured to determine the biologic consequence of ABC294640-mediated Myc and AR deregulation. Cell proliferation was significantly inhibited by ABC294640 treatment in a dose-dependent fashion, which was consistent with decreased
IC50 value for ABC294640-induced cytotoxicity was determined within a model expressing AR-SVs, 22Rv1 cells were used. The AR-SVs, which lack the ligand-binding domain and are constitutively active, drive castration resistance, correlate with decreased AR signaling, and as such, represents a potentially active mitogenic signaling, and as such, represents a potential therapy for CRPC.

Numerous mechanisms contribute to AR dysregulation in CRPC, including AR mutations, cofactor perturbation, and intracrine androgen production (30). In addition, AR splice variants (AR-SV) are clinically detectable, AR transcriptional output as demonstrated by significantly decreased mRNA levels of PSA, TMPRSS2, and FKBP5 (Fig. 4F). On the basis of these data, ABC294640 significantly inhibits CRPC cell growth, oncogene expression and activity, and abrogates constitutively active mitogenic signaling, and as such, represents a potential therapy for CRPC.

Figure 3.
ABC294640 negatively regulates CRPC signaling and proliferation. A, C4-2 cells were cultured in hormone-proliferant media and treated with 10 and 20 μmol/L ABC294640 or vehicle for 24 and 48 hours. Cell lysates were immunoblotted with AR, Myc, and GAPDH. B, C4-2 cells were cultured in hormone-depleted media and treated with 10 and 20 μmol/L (△) ABC294640 or vehicle (■) for 72 hours. Cell growth was determined by trypan blue exclusion cell counting; *, P < 0.05; **, P < 0.01; #, P < 0.05, relative to 10 μmol/L.
Figure 4.
ABC294640 inhibits AR-SV CRPC cell signaling and growth. A, 22Rv1 cells were treated with the indicated concentrations of ABC294640 for 72 hours, and cell survival was quantified by the SRB assay. B, 22Rv1 cells were cultured in hormone-proficient media and treated with 20 μmol/L (▲), 40 μmol/L ABC294640 (■) or vehicle (▲) over 96 hours. Cell growth was determined by trypan blue exclusion. C, 22Rv1 cells cultured as in B were treated with 40 μmol/L ABC294640 for 72 hours and analyzed for BrdUrd incorporation. D, 22Rv1 cells were cultured in hormone-proficient media and treated with 10, 20, and 50 μmol/L ABC294640 or vehicle for 72 or 96 hours. Cell lysates were immunoblotted with AR (detecting full-length and splice variant), Myc, and GAPDH. E, 22Rv1 cells were cultured in hormone-depleted media and treated with 30 μmol/L ABC294640 for 72 hours. mRNA transcripts levels of Myc, CDK1 and ODC were analyzed by qRT-PCR. F, 22Rv1 cells were cultured as in E and mRNA transcript levels of AR, AR-V7, AR-V567es, PSA, TMPRSS2, FKBP5 were analyzed by qRT-PCR; *, P < 0.05; **, P < 0.01.
Proteasomal degradation plays a role in AR/Myc protein depletion

These studies demonstrate that ABC294640 decreases Myc and AR expression and signaling in multiple prostate cancer models. This is consistent with a report that SK2 regulates Myc mRNA and protein by altering HDAC activity (19). Alternatively, effects of ABC294640 may be mediated by ceramide accumulation following SK2 inhibition as ceramide is reported to activate protein phosphatase 2A (PP2A), resulting in decreased Myc expression, which is reversible by PP2A inhibitors (35). To determine whether ABC294640 decreases Myc through the ceramide-PP2A pathway, LNCaP cells were treated with vehicle, ABC294640 alone, or ABC294640 in combination with PP2A inhibitor, okadaic acid. ABC294640 treatment resulted in decreased Myc and AR protein expression (Fig. 5A, compare lane 1 with 2 and 3); however, okadaic acid treatment did not alter the effects of ABC294640 (lanes 4 and 5). This indicates that Myc depletion is not due to PP2A activation by ceramide accumulation. Next, the role of the proteasome was explored because Myc and AR are degraded by the ubiquitin–proteasome pathway and proteasome inhibitors can in some cases rescue SK inhibitor–induced Myc depletion (18, 28). Treatment of LNCaP cells with the proteasome inhibitor, MG132, alone increased the steady-state levels of AR and Myc (Fig. 5B, compare lanes 1 and 2); whereas, ABC294640 decreased AR and Myc protein levels (compare lanes 1 and 3). Importantly, MG132 rescued ABC294640-mediated depletion of both AR and Myc.
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Treated animals progressively grew, requiring sacrifices by calipers three times per week. Although tumors of vehicle-orally gavage (or equivalent vehicle volume) and tumors were measured. Prostate cancer tumor growth by simultaneously inhibiting two key signaling pathways fundamental to prostate cancer growth and proliferation. Key findings demonstrate that ABC294640 treatment of ADT-sensitive prostate cancer cells significantly inhibits growth, proliferation, and cell-cycle progression, which is accompanied by inactivation of the AKT, AR, and Myc signaling pathways. In particular, Myc and AR expression levels and subsequent transcriptional activity are both significantly decreased following ABC294640 treatment. Similarly, within two models of CRPC, ABC294640 treatment is sufficient to downregulate Myc and AR expression and activity, including constitutively active AR splice variants, which corresponds with significant attenuation of cell growth and proliferation. Finally, oral administration of ABC294640 was found to dramatically impede xenograft tumor growth. Together, these results support the hypotheses that SK2 activity is required for prostate cancer function and proliferation.

**Discussion**

The bioactive sphingolipid S1P drives several hallmark processes of cancer, making the enzymes that synthesize S1P, that is, SK1 and SK2, important new targets for cancer drug development. ABC294640 is a first-in-class SK2 inhibitor that effectively inhibits cancer cell growth in vitro and in vivo, and has recently completed phase 1 clinical testing in patients with advanced solid tumors (NCT01488513). Previous studies highlight the ability of ABC294640 to modulate sphingolipid metabolism and downregulate mitogenic AKT and MAPK pathways. This study presents novel data that ABC294640 abrogates several additional signaling pathways fundamental to prostate cancer growth and proliferation. Key findings demonstrate that ABC294640 treatment of ADT-sensitive prostate cancer cells significantly inhibits growth, proliferation, and cell-cycle progression, which is accompanied by inactivation of the AKT, AR, and Myc signaling pathways. In particular, Myc and AR expression levels and subsequent transcriptional activity are both significantly decreased following ABC294640 treatment. Similarly, within two models of CRPC, ABC294640 treatment is sufficient to downregulate Myc and AR expression and activity, including constitutively active AR splice variants, which corresponds with significant attenuation of cell growth and proliferation. Finally, oral administration of ABC294640 was found to dramatically impede xenograft tumor growth. Together, these results support the hypotheses that SK2 activity is required for prostate cancer function and proliferation.

**Multiple mechanisms to modify Myc expression**

ABC294640 inhibits cell growth in several tumor types, including hematologic acute lymphoblastic leukemia (ALL), multiple myeloma, and KHSV-associated primary lymphoma, and solid tumors of the ovaries, breast, liver, and prostate (16–19, 40, 41). Within many of these cell models ABC294640 treatment downregulates Myc protein and/or mRNA expression. Multiple mechanisms exist to explain how ABC294640 treatment may decrease Myc. In one case, SK2 regulates histone deacetylase (HDAC) in the nucleus, with nuclear S1P-inhibiting HDAC activity and promoting accumulation of histone H3 lysine 9 acetylation (H3K9ac) at MYC promoter elements, which often represents sites of active transcription (19, 42). ABC294640 treatment decreases nuclear S1P, gene-specific H3K9ac and MYC
transcription, demonstrating the ability of ABC294640 to reverse SK2-mediated gene expression. In other models, ABC294640 promotes cell apoptosis and downregulates Myc protein in a p53-mediated fashion that is rescued by MG132 and implicates a role for the proteasome (18). Finally, ceramide has been reported to decrease Myc protein expression in prostate cancer cells by a protein phosphatase 2A-dependent mechanism (35). However, our data argue this mechanism is not active in prostate cancer cells as inhibition of PP2A did not attenuate ABC294640-induced downregulation of Myc or AR. Our studies do suggest there are multiple mechanisms by which ABC294640 affects Myc and AR expression as treatment decreased mRNA and enhanced proteasomal degradation of both proteins.

Myc/AR crosstalk

Myc and AR regulate expression of numerous genes involved in cell growth and proliferation, are required for prostate cancer progression, and are commonly perturbed in aggressive disease. Interestingly, it is not clearly understood how these molecules impinge on signaling of one another. For example, AR suppresses Myc expression in normal prostate epithelial cells, but increases Myc in cancer cells (43). Myc overexpression confers castrate-resistant cell growth independent of AR signaling whereas Myc inhibition impairs ADT-sensitive and CRPC cell growth (44, 45). Early studies indicate that Myc binding to AR is required for AR autoregulation (46). In AR-positive molecular apocrine breast cancer, AR and Myc are involved in a feed-forward mechanism whereby AR activates Myc and Myc reinforces AR signaling by silencing causes tumor regression; however, heterogeneity within tumor samples allows for escape from the oncogene dependence (50, 51). Therefore, in addition to disrupting oncogenic signaling of Myc and AR in prostate cancer, targeting tumors that are Myc addicted could be a feasible therapeutic option with widespread ramifications.

Oncogene addiction could also help explain the ability of ABC294640 to inhibit AR-negative prostate cancer cells. DU145 and PC-3 are both growth inhibited, with IC50 values approximately 2-fold higher than LNCaP but similar to aggressive 22Rv1 cells (20, 23). PC3 and DU145 prostate cancer cells express constitutively active AKT and ERK (52), respectively, and PC3 expresses elevated levels of Myc (44). Therefore, ABC294640 treatment should be efficacious in blocking cell growth despite the absence of AR expression. Overall, ABC294640 represents a potent pharmacologic agent that effectively attenuates AR-positive and Myc-dependent prostate cancer cells.

Disclosure of Potential Conflicts of Interest

C.D. Smith is a president and CEO at Apogee Biotechnology Corporation and has ownership interest (including patents) Apogee Biotechnology Corporation.

Authors' Contributions

Conception and design: R.S. Schrecengost, K.E. Knudsen, C.D. Smith. Development of methodology: R.S. Schrecengost, K.E. Knudsen. Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R.S. Schrecengost, S.N. Keller, M.J. Schierer. Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R.S. Schrecengost, K.E. Knudsen. Writing, review, and/or revision of the manuscript: R.S. Schrecengost, M.J. Schierer, K.E. Knudsen, C.D. Smith. Study supervision: C.D. Smith.

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