Analysis of age-associated alternation of SCSA sperm DNA fragmentation index and semen characteristics of 1790 subfertile males in China

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Abstract

Background: It has been identified that incidence of infertility was about 20% among couples worldwide, about 50% caused by male elements. However, conventional semen laboratory detections could not handle clinical needs, which led to more comprehensive parameters for male fertility evaluation. We aimed to investigate the clinical relationship of age-linked changes and the sperm chromatin structure assay (SCSA) sperm DNA fragmentation index (DFI), and routine semen characteristics among subfertile Chinese males.

Methods: 1790 clinical semen specimens were enrolled from February 2018 to October 2019. Clinical and laboratory data including routine semen analyses, sperm DFI, and sperm morphology were collected and showed age-related alterations in semen parameters.

Results: Our results, displayed an increase in sperm DFI with age, were demonstrated in three age-groups, particularly within the ≥35-year cohort. There were positive and inverse correlations of sperm DFI with abnormal semen characteristics and with normal morphological parameters, respectively. Furthermore, age, sperm morphology, concentration, and progressive motility, immotile sperm percentage, semen volume, sperm survival, and high acidine orange DNA stainability (indicating immature forms) were found to be independent risk factors affecting sperm DNA integrity. Likewise, men aged ≥35 years had a higher sperm DFI than did normozoospermic men in the overall cohort. Routine semen characteristics, sperm DFI, and morphology tended to alter with age.

Conclusions: The SCSA sperm DFI showed the greatest clinical application in the assessment of male fertility in this study, which should help infertility clinics decide on reproductive options for the treatment of older infertile couples.
1 | INTRODUCTION

The incidence of infertility is 10-20% among couples worldwide. Of all cases, nearly 50% are caused by male factors. Clinically, male infertility is diagnosed by semen quality, which in turn seriously affects fertilization capacity. However, semen quality has declined gradually over the past decades globally. Generally, the evaluation of male fertility includes physical examinations and semen analysis. Standardized semen analysis consists of the descriptive analysis of sperm motility, morphology, and concentration, and these analyses must surpass certain threshold levels to be considered “normal.” However, these conventional parameters do not meet the clinical demand because 25% of infertility cases worldwide remain unexplained. Therefore, more comprehensive evaluation techniques are required to evaluate potential male fertility.

New laboratory techniques have been established to investigate sperm physiology and functions by monitoring characteristics such as capacitation, the acrosome reaction, reactive oxygen species (ROS), sperm DNA damage, chromatin structure, zona pellucida binding, and sperm-oocyte fusion. In 1980, Evenson et al introduced the concept of sperm DNA fragmentation (DFI) as related to pregnancy outcome and also the sperm chromatin structure assay (SCSA) test. These data showed that subfertile bulls and men attending infertility clinics had two and four times more SCSA-defined sperm DNA damage than fertile bulls and men. The original SCSA test used heat to open DNA strands at sites of strand breaks; however, the revised SCSA test used low pH to open these same sites. Over 12 menstrual cycles, Evenson et al showed that 187 couples without known infertility factors, pregnant over the first three months, had significantly lower SCSA test scores than those pregnant in months 4-12 and again lower scores for the couples that did not achieve a pregnancy over 12 months. In 2006, Bungum et al showed intrauterine insemination (IUI) couples only produced 2% pregnancies with SCSA %DFI values >25%. In a study of 1633 in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) cycles, a SCSA %DFI above the level of 20%-30% was seen as an indication for switching to ICSI fertilization. As an important supplement to routine semen tests, the SCSA-defined DFI might help predict the outcomes of natural conception and IVF, monitor sperm DNA damage caused by environmental pollutants and medical interventions, and assess the effects of male reproductive system diseases and their therapies on male fertility.

In the past decades, age-related declines in semen quality have been reported in men aged >35 years, and these changes become more significant with age >40 years. Moreover, reproductive outcomes can get worse with a male age of >40 or even >35 years, commonly classified as “advanced” age. Deterioration in semen quality is evident not only in terms of ejaculate volume, sperm count, motility, vitality, and sperm morphology, but also in sperm chromatin quality. Thus, sperm DNA damage is more often observed in infertile men, which might be related to poor assisted reproductive technology treatment (ART) outcomes such as miscarriage. With the growing interest in the effect of sperm DNA damage on reproductive outcomes, it is important to demonstrate the influence of age on sperm DNA damage. Here, we comprehensively analyzed the correlations between age and SCSA-defined sperm DNA fragmentation index (DFI), high DNA stainability (HDS) with acridine orange, and routine semen characteristics and sperm morphological alternations among 1790 subfertile Chinese men.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

This study was approved by the Ethics Committee of the Shenzhen Baao Women’s and Children’s Hospital, Jinan University (Shenzhen, China), and written informed consents were obtained from all the participants. All specimens were handled and made anonymous in line with the ethical and legal standards.

2.2 | Patients and specimens

This retrospective study used the clinical and laboratory data of 1790 subfertile Chinese men who visited the Department of Reproductive Health of Shenzhen Baao Women’s and Children’s Hospital, Jinan University, Shenzhen, China, as outpatients for pre-pregnancy checkups between February 2018 and October 2019. Exclusion criteria for this study included azospermia and any medical history of tumors, testicular torsion, cryptorchidism, testicular injuries, varicocele, mumps orchitis, and other relevant systemic diseases. We included 1790 adult men aged 21-58 years (median 31 years) with their clinical semen parameters listed below.

2.3 | Analysis of routinely semen parameters

After several days of sexual abstinence, generally 3-5 days was recommended, a clean ejaculate produced by masturbation was collected into a sterile container in an assigned room and rapidly transferred to the laboratory. First, the specimens were weighed to determine volume and then kept in a 37°C water bath for 30 minutes for liquefaction. Then, pH was determined using pH strips. Sperm concentration, motility, and survival rates were...
measured using an SAA-II computerized system (Suijia), using 2-2.5 μL well-mixed aliquots. Duplicate sperm smears were prepared using 10-20 μL well-mixed aliquots and stained with Diff-Quik staining solution (Boruide). Slides were observed using an optical microscope (×100; BX51; Olympus), and the morphology of the acrosome, head, midpiece, and principal piece was assessed.

2.4 | Flow cytometry analysis of DFI and HDS

These sperm lack proper exchange of histones for protamines. Since AO-stained histone-complexed DNA fluoresces 2.3× than protamine-complexed DNA, these sperm are easily identified by the SCSA test. Sperm DFI and HDS were measured using the sperm chromatin structure assay (SCSA) via flow cytometry based on the manufacturer’s kit instructions (Anda Biotech). According to recommended protocols, specimens were diluted to a concentration of (1-2) × 10⁶ cells/mL and then treated with a solution of Triton X-100, NaCl, and Tris-HCl in an acidic environment for 30 seconds. Subsequently, staining buffer with various concentrations of acridine orange (AO), NaCl, NaHPO₄, and EDTA was added to the detection tubes and then measured using fluorescence-activated cell sorting (FACS) in a Canto II flow cytometer (Becton Dickinson). Each sample needed a minimum of 5000 spermatozoa for FACS data analysis. More importantly, we need to ensure the flow rates ranging from 100/second to 300/second with measured flow cytometry, otherwise re-dilution and re-detection, when treated via AO. Using AO staining, normal double-stranded DNA fluoresces green, and single-stranded DNA fluoresces red upon excitation of blue laser (488 nm) light. Proportion of sperm with increased red fluorescence indicating damaged chromatin, which defined as DFI. HDS was defined as spermatozoa with an abnormally high level of green fluorescence, condensation with a lack of exchange of nuclear histones for protamines.

2.5 | Clinical definitions

Normozoospermia was defined as follows: sperm concentration ≥15 × 10⁶/mL, total sperm count ≥39 × 10⁶/mL, sperm progressive motility ≥32%, and normal morphology ≥4%, according to the 5th guidelines of the World Health Organization. The teratozoospermia index (TZI) was defined as abnormal levels of morphological defects in the head, neck, midpiece, and principal piece, and included oversized residual cytoplasmic droplets. The multiple abnormalities index (MAI) signifies the mean value of sperm defects including defects in the head, neck, and tail. The sperm deformity index (SDI) was used to express the overall prevalence of defective sperm morphology within the total sperm population. In a study of 45 men who

![Figure 1](image-url)
produced a monthly semen sample over eight months showed a very high repeatability of SCSA data over each month even though the scattergram patterns were different between many of the men.\textsuperscript{23} This study also showed comparisons between SCSA parameters and classical semen parameters.

### 2.6 Statistical analysis

All data were analyzed using SPSS software (v. 17.0; SPSS Inc, Chicago, IL, USA). Quantitative variables are displayed as means ± standard deviations (SDs). Variations between parameters were evaluated by one-way analysis of variance (ANOVA) for normally distributed data or the Mann-Whitney nonparametric U test for non-normally distributed data. Correlations between DFI and other semen parameters were analyzed using Spearman’s correlation for skewed data distributions or Pearson’s test for normal data distributions. Multivariate logistic regression analysis was utilized to evaluate interactions of DFI with other semen parameters. We also calculated the odds ratio (OR) values when attempting to identify the risk factors for a higher sperm DFI; \( P < .05 \) was assumed to be statistically significant.

### 3 RESULTS

#### 3.1 Patient characteristics

Laboratory data produced from SCSA test were displayed in supplementary Figure 1, including DFI and HDS. Among 1790 semen specimens analyzed, the clinical routine semen parameters, along with sperm DFI and HDS, were listed by age span, respectively, in Table 1 in which normal form morphology, progressive motility, prevalence of sperm DFI, sperm survival, semen volume, immotile sperm, abnormal head, SDI, T2I, MAI, and abnormal main defects of sperm showed statistically significant differences across the age-group >35 years, compared with age <30-year and 30- to 35-year cohorts, respectively \( (P < .05) \).

#### 3.2 Age positively correlated to the prevalence of sperm DFI in subfertile men

Sperm DFI obtained from 1790 cases of subfertile men before pregnancy were settled with the diversity of age distribution. As shown in Figure 1A, sperm DFI with age >35 years was significantly higher
TABLE 2  Correlations between DFI and other clinical semen parameters based on age span

| Semen parameters | Correlations | <30 years | 30-35 years | >35 years |
|------------------|--------------|-----------|-------------|-----------|
|                  | r            | P         | r           | P         | r          | P          |
| Normal form morphology (%) | -.21         | <.0001**** | -.15       | .0003***  | -.23       | <.0001     | -.24       | <.0001**** |
| Concentration (10⁶/mL) | -.03         | .13       | -.08       | .06       | -.06       | .09         | .02         | .61        |
| Progressive motility (%) | -.43         | <.0001**** | -.41       | <.0001*** | -.39       | <.0001     | -.53       | <.0001***  |
| Total sperm count (10⁶) | -.02         | .31       | -.01       | .78       | -.04       | .22         | .09         | .06        |
| Abstinence time (day) | .15          | <.0001**** | .08        | .06       | .17        | <.0001     | .14         | .001***    |
| Sperm survival (%) | -.43         | <.0001**** | -.42       | <.0001*** | -.43       | <.0001     | -.53       | <.0001***  |
| Semen pH | -.14         | <.0001**** | -.02       | .52       | -.04       | .24         | -.02       | .71        |
| Volume of semen (mL) | .15          | <.0001**** | .12        | .005**    | .16        | <.0001     | .09         | .06        |
| Non-progressive motility (%) | -.18         | <.0001**** | -.15       | .0003**   | -.16       | <.0001     | -.22       | <.0001***  |
| Immotile sperm (%) | .45          | <.0001**** | .43        | <.0001**** | .41        | <.0001     | .54         | <.0001***  |
| Abnormal head (%) | .21          | <.0001**** | .15        | .0003***  | .22        | <.0001     | .24         | <.0001***  |
| SDI | .20          | <.0001**** | .15        | .0005**   | .22        | <.0001     | .11         | .03**      |
| TZI | .15          | <.0001**** | .11        | .008**    | .17        | <.0001     | .11         | .03**      |
| MAI | .15          | <.0001**** | .10        | .01**     | .1         | .001**     | .11         | .03**      |
| Abnormal main (%) | .06          | .01**      | .07        | .07       | .04        | .20         | .04         | .39        |
| Abnormal principle (%) | .13          | <.0001**** | .08        | .04*      | .14        | <.0001     | .11         | .02*       |
| ERC sperm count | .07          | .003**     | .03        | .44       | .04        | .21         | .09         | .06        |
| HDS | -.001        | .81        | -.03       | .49       | .002       | .95         | -.02       | .31        |

Abbreviations: DFI, DNA fragmentation index; SDI, sperm deformity index; TZI, teratozoospermia index; MAI, multiple abnormalities index; ERC, excess residual cytoplasmic; HDS, high stainability.

*P < .05
**P < .01
***P < .001
****P < .0001

Correlations between routine semen parameters associated with sperm DFI

Correlations between DFI and other clinical semen parameters based on age span

than the residual cohorts of age <30 and 30-35 years, respectively (28.11 ± 14.36 vs 24.78 ± 12.47, P < .0001; 28.11 ± 14.36 vs 23.66 ± 12.43, P < .0011). However, there was no significant difference between age <30 and 30-35 years groups of sperm DFI (24.78 ± 12.47 vs 23.66 ± 12.43, P > .05). Moreover, Figure 1B clearly illustrated that the sperm DFI was positively associated with age additions (r = .129, P < .0001).

We also evaluated the correlations between age alternations and sperm DFI displayed in Figure 1. Likewise, sperm DFI with subfertile men aged >35 years was positively related to the age (r = 0.248, P < .0001, Figure 1E). These results implied the potential influence of the elder populations on damage of sperm DNA integrity, particularly verified within male age more than 35 y.

3.3 Correlations between routine semen parameters and sperm DFI

Next, we continuously analyzed the associations between sperm DFI and routine semen characteristics via Spearman’s correlations revealed in Table 2 and Figure 2. Positive associations between sperm DFI and abstinence time, semen volume, immotile sperm, abnormal head, SDI, TZI, MAI, abnormal main, and excess residual cytoplasm in sperm count were clearly displayed (P < .05, Figure 2, Table 2). However, morphology, progressive motility, total sperm counts, sperm survival, pH, and non-progressive motility were inversely related to the corresponding sperm DFI additions (P < .05; Figure 2, Table 2).

We further assessed the correlations of age to sperm DFI and semen routine parameters, respectively. As listed in Table 2, it is noticed that progressive motility (r = -.53 vs r = -.39, r = -.41), sperm survival (r = -.53 vs r = -.43, r = -.42), and immotile sperm (r = .54 vs r = .41, r = -.43) in the age >35-year cohort manifested stronger associations than male age <30- and 30- to 35-year groups in sperm DNA damage. Given these age-related correlations on semen parameters and sperm DNA damage, further verification of influencing factor on age should be done.

3.4 Multivariate logistic regression analysis of semen characteristics associated with sperm DFI

Despite association analysis illustrated that various semen parameters were positively or inversely related to sperm DNA integrity, some of these clinical variables might be confounders. Therefore, we subsequently evaluated the independently significant variables correlated to sperm DFI via multivariate logistic regression analysis. As
shown in Table 3, it is demonstrated that age, morphology, concentration, progressive motility, immotile sperm, semen volume, sperm survival, and HDS were independent risk factors in regard to sperm DNA integrity.

Meanwhile, we simultaneously assessed the effects with age on sperm DNA damage and semen characteristics by multivariate logistic regression analysis. As listed in Table 4, the independent risk factors including age, total sperm counts, immotile sperm, and abnormal head affected the sperm DNA integrity among male age >35-year cohorts (P < .05; Table 4). However, except for age, the other semen parameters involving sperm volume, immotile sperm, sperm survival, concentration, and HDS were independent risk factors in line with sperm DFI manifested within male age <30- and 30- to 35-year groups (P < .05; Table S1 and S2). Therefore, it is understandable the significance of age alternations in concert to sperm DNA integrity, especially demonstrated in male age >35-year cohorts.

3.5 | Odds ratio with Age regarding to sperm DFI

As shown in Figure 3, sperm DFI of men aged 35-39 y tend to raised spite no significant differences with male age 21- to 24-,
TABLE 4 Clinical semen parameters correlated with DFI by multivariate logistic regression analysis among male age >35-year group

| Semen parameters | Odds ratio | 95% CI       | P value |
|------------------|------------|--------------|---------|
| Age              | 1.101      | 1.041-1.164  | .001    |
| Total sperm counts | 1.003     | 1.001-1.004  | .0001   |
| Immotile sperm   | 1.060      | 1.045-1.076  | .0001   |
| Abnormal head    | 1.190      | 1.040-1.362  | .01     |

25- to 29-, and 30- to 34-year groups (P > .05). Inversely, sperm DFI with male age 40-44 and ≥45 years showed significant differences with groups of aged 21-24, 25-29, and 30-34 years (P < .01; P < .001; Figure 3), which driven us to define the male age 35 y as interval point, better for assessing the OR values for sperm DNA damage.

According to practically clinical diagnosis and treatment experience, 30% of sperm DFI were considered as cutoff point, implied lower fertility potential. Subfertile men aged ≥35 years had an OR with a higher level of sperm DNA damage than group of men <35 years in whole cohorts, also accompanied with groups of men within normozoospermia, respectively (OR = 1.576; OR = 1.596; Tables 5 and 6). Likewise, the morphology, progressive motility, and sperm survival, might be protective elements with lower OR value with men aged ≥35 years (OR = 0.703; OR = 0.643; OR = 0.594; P < .05; Table 5). These age-related results, particularly male age ≥35 years, confirmed that aging had significantly risk influences on sperm DNA damage, further urged us to compare the time point of 35 y as cutoff point for men fertility.

4 | DISCUSSION

Aging leads to a general decline in the function of tissues and organs, including reproductive tissues and organs. Male reproductive system functional declines caused by aging including decreased sex hormone levels, disorders of the histological structure of the testes, oxidative stress, and de novo mutations are deleterious to male fertility. It has been shown that semen quality is inversely correlated with age. Moreover, several studies have reported that advanced paternal age can increase the risk of embryo implantation failure and abortions, pregnancy problems, and poor live birth outcome. Frattarelli et al found that advanced male age negatively affected embryo development in assisted reproductive technology (ART) treatments. Ferreyra et al also found that advanced paternal age increased the aneuploidy rates in embryos derived from donated oocytes. However, the effects of paternal age on fertility remain controversial. Wu et al found that paternal age had no effect on fertilization rate, embryo quality at the cleavage stage, or miscarriage rates. Ferreira et al reported no influence of paternal age on implantation or pregnancy rates among couples with normozoospermic men. Alfaraj and Yunus found no significant association between advanced paternal age and the outcomes of IVF in 451 couples. Because of these unclear, ambiguous, and controversial data, we need more sensitive and accurate indicators to assess the effect of age on male fertility.

In this study, routine semen parameters along with SCSA-defined sperm DFI and HDS of 1790 semen specimens were analyzed according to the men's age ranges. No statistically significant difference in the sperm concentration, sperm volume, total sperm count, non-progressive motility, TZI, multiple anomalies index, SDI, or HDS was observed among the different age-groups, which was consistent with other studies. However, there was a significant increase in sperm DFI, and significant decreases in normal sperm morphology, progressive motility, and sperm survival across increasing age-groups. Furthermore, we found that age was positively correlated with the prevalence of sperm DFI. Similar results have been reported previously. Thus, a study involving 1124 men carried out by Rosiak-Gill found that older men (≥40 years) had higher percentage of DFI than younger men. Similarly, Winkle observed a significantly higher percentage of DFI in men aged ≥40 years compared with those aged 36-39 years. Additionally, Plastira et al showed that the DFI increased significantly with age in patients with oligoastroteratozoospermia. Because the integrity of sperm DNA plays a crucial role in normal embryo development, DFI has been applied in some clinical andrology laboratories. Sperm DFI might directly reflect the degree of sperm DNA destruction, so it has been used to assess sperm DNA damage worldwide. There is increasing evidence for a correlation between DFI and male infertility. Thus, sperm DNA damage is more often observed in infertile men (reviewed in Barratt et al). Moreover, high DFI may be related to decreases in the pregnancy rate in ART treatments as well as the miscarriage rates.

To better understand the relation between DFI and sperm quality, we performed Spearman’s correlation analysis between semen routine characteristics and DFI. There were positive correlations between sperm DFI and days of abstinence, immotile sperm rates, semen volume, TZI, MAI, and SDI. In contrast, normal morphology,
sperm concentration, progressive motility, non-progressive motility, sperm survival, total sperm counts, and percentage of HDS were inversely correlated with DFI. After grouping by age, we found that the stronger correlations between DFI and the normal form morphology, progressive motility, non-motility sperm, and sperm survival in the cohort of ≥35 years. Factors related to sperm DNA damage are known to include age, environmental restrictions, and male reproductive anomalies such as varicocele.

Our data showed that DFI could better predict male fertility, compared with traditional semen parameters, and better reflect the effect of age on semen quality. Because the sperm DFI was related to various semen parameters, sperm DNA damage might be a key factor in age-related or other decreases in semen quality. Surprisingly, the prevalence of HDS showed no significant correlations with sperm DFI in the cohort aged ≥35 years, indicating that how age influences semen parameters and male fertility is complicated.

To explore the associations between male aging and sperm DNA damage, we assessed the OR values for the prevalence of sperm DFI in different age-groups. Based on the results from the sperm DFI assessment and previous reports, sperm DFI was set as a cutoff possibly implying lower fertile potential. We found that men ≥35 years of age had higher OR values with a higher level of sperm DFI than those aged <35 years both in the whole cohort and among groups of men with normozoospermia or abnormal semen variables (OR = 1.142, 1.029 and 1.178, respectively; Tables 4 and 6). Likewise, normal sperm morphology, progressive sperm motility, and sperm survival were identified as risk factors with higher OR values for men aged ≥35 years. This is well established that ROS are important contributors to both pre- and post-meiotic sperm damage. In addition, spermatozoa have sparse antioxidant defense mechanisms and are easily impaired by excessive ROS. Thus, men aged ≥40 years exhibited higher levels of oxidative sperm DNA damage compared with younger men, indicating that oxidative stress might be an important mediator of male age-related DNA damage. Because the underlying mechanisms of age-related sperm DNA damage seem to be complicated and multifactorial, further research is clearly needed.

In summary, age-associated declines in normal sperm morphology, progressive motility, survival, and an increase in sperm DFI along with increased levels of immotile forms were revealed in this study. Our data indicate that sperm DFI has greater clinical utility in the assessment of male fertility than standard semen parameters. Thus, DFI is a sensitive and accurate indicator in evaluating the effect of aging on male fertility, so paying more attention to this measure might lead to better outcomes in ART for older couples.

| Semen parameters               | <35 years | ≥35 years | P value | OR (95% CI) |
|--------------------------------|-----------|-----------|---------|-------------|
|                                | n         | n         |         |             |
| **DFI**                        |           |           |         |             |
| <30%                           | 435       | 64        | .0001   | 1.596 (0.997-2.557) |
| ≥30%                           | 132       | 31        |         |             |

*P = .05.

| Semen parameters               | <35 years | ≥35 years | P value | OR (95% CI) |
|--------------------------------|-----------|-----------|---------|-------------|
|                                | n         | n         |         |             |
| **Normal form morphology**     |           |           |         |             |
| <4%                            | 514       | 739       | .001    | 0.703 (0.574-0.862) |
| ≥4%                            | 267       | 270       |         |             |
| **Progressive motility**       |           |           |         |             |
| <32%                           | 476       | 777       | .0001   | 0.643 (0.524-0.789) |
| ≥32%                           | 262       | 275       |         |             |
| **Sperm survival**             |           |           |         |             |
| <58%                           | 104       | 1149      | .001    | 0.594 (0.431-0.818) |
| ≥58%                           | 71        | 466       |         |             |

**TABLE 5** Odds ratio for DFI in the group of men aged ≥35 years and men aged <35 years in the entire cohorts

**TABLE 6** Odds ratio for DFI in the group of men aged ≥35 years and men aged <35 years within normozoospermia
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CONFLICT OF INTEREST
The authors declare no financial or commercial conflict of interest.

AUTHORS’ CONTRIBUTIONS
Ruijing Lu and Xian Chen participated in analyzing data and drafted the article; Weijian Yu, Fan Jiang, Xiyou Zhou, and Yang Xu carried out laboratory data collecting; Feng Wang conceived the study, participated in its design and coordination, and helped to draft the article. All authors have read and approved the final version of the article and agree with the order of the presentation of the authors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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