Pathways to Injury in Chronic Pancreatitis: Decoding the Role of the High-Risk SPINK1 N34S Haplotype Using Meta-Analysis

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Abstract

Background: The complex interactions between recurrent trypsin-mediated pancreatic injury, alcohol-associated pancreatic injury and SPINK1 polymorphisms in chronic pancreatitis (CP) are undefined. We hypothesize that CP occurs as a result of multiple pathological mechanisms (pathways) that are initiated by different metabolic or environmental factors (etiologies) and may be influenced differentially by downstream genetic risk factors. We tested this hypothesis by evaluating the differences in effect size of the high risk SPINK1 N34S haplotype on CP from multiple etiologies after combining clinical reports of SPINK1 N34S frequency using meta-analysis.

Methods and Findings: The Pubmed and the Embase databases were reviewed. We studied 24 reports of SPINK1 N34S in CP (2,421 cases, 4,857 controls) using reported etiological factors as surrogates for pathways and multiple meta-analyses to determine the differential effects of SPINK1 N34S between alcoholic and non-alcoholic etiologies. Using estimates of between-study heterogeneity, we sub-classified our 24 studies into four specific clusters. We found that SPINK1 N34S is strongly associated with CP overall (OR 11.00; 95% CI: 7.59–15.93), but the effect of SPINK1 N34S in alcoholic CP (OR 4.98, 95% CI: 3.16–7.85) was significantly smaller than in idiopathic CP (OR 14.97, 95% CI = 9.09–24.67) or tropical CP (OR 19.15, 95% CI = 8.83–41.56). Studies analyzing familial CP showed very high heterogeneity suggestive of a complex etiology with an I² = 80.95%.

Conclusion: The small effect of SPINK1 N34S in alcoholic subjects suggests that CP is driven through a different pathway that is largely trypsin-independent. The results also suggest that large effect sizes of SPINK1 N34S in small candidate gene studies in CP may be related to a mixture of multiple etiologic pathways leading to the same clinical endpoint.

Introduction

Chronic pancreatitis (CP) is a common chronic inflammatory syndrome of the pancreas that has been defined by clinical signs and symptoms linked to end-stage pathological criteria[1]. While acute pancreatitis is recognized as an acute inflammatory response to pancreatic injury [2], the pathophysiological mechanisms underlying the development and progression of CP in humans have yet to be discerned.

CP is defined by the presence of chronic inflammatory cells within the pancreas, progressive fibrosis, sclerosis and parenchymal atrophy [1,3,4,5]. The pancreatic stellate cell (PSC) is the mediator of fibrosis, and is ubiquitous in chronic pancreatitis [6,7]. The diagnosis of CP requires greater than six month duration of inflammation, permanent loss of exocrine function, and evidence on abdominal imaging studies of duct distortion, fibrosis or calcification. Abdominal pain and diabetes mellitus are clinical features that are also common to CP [1,3,4,5].

CP is sub-classified according to epidemiologic risk factors (e.g. alcoholism, family history, living in certain tropical regions, autoimmune disorders), although the relative risk of recognized environmental factors may be diminutive [3,8]. If no inciting factor can be identified, CP is termed ‘idiopathic’ and sub-classified as early versus late-onset idiopathic CP by age of diagnosis. Although excessive alcohol consumption is a risk factor for CP, fewer than 5% of alcoholics develop pancreatitis, and many patients with CP do not drink alcohol [8,9]. Furthermore, the pathophysiological pathways that link the normal pancreas to the end-stage pathology of CP have not been clearly defined.

Several recent human genetic studies have provided insight into components of the pathogenic mechanisms leading to human CP, all of which are related to failure to regulate intrapancreatic...
trypsin activity and the associated pancreatic injury. The three primary susceptibility genes for CP include the cationic trypsinogen gene (PRSS1) [10], the cystic fibrosis transmembrane conductance regulator gene (CFTR) [11,12], and the pancreatic secretory trypsin inhibitor gene, also known as serine protease inhibitor Kazal type 1 (SPINK1) [13,14,15]. SPINK1 is an acute phase protein whose gene expression and protein concentrations are markedly upregulated by inflammation [16,17,18]. SPINK1 gene mutations are thought to diminish protection against prematurely activated trypsin, and are thereby linked to trypsin-related pancreatic injury [10,15]. PRSS1, CFTR and SPINK1 seem to play complementary roles in protecting the pancreas from the damage incurred by prematurely activated trypsin.

The high-risk SPINK1 N34S haplotype has been observed in one to three percent of most populations, while the incidence of CP is less than one in ten thousand [14,19]. Furthermore, the reported range of odds ratios (ORs) describing the risk of SPINK1 N34S carriers of developing pancreatitis has varied from nonsignificance [13] to nearly 80 [15]. Wide variations in reported effects of small genetic association studies have been attributed to statistical variation in underpowered studies, poor methodology, publication bias toward studies with large ORs as well as a myriad of other non-biological factors [20,21,22]. However, we hypothesize that in the case of the SPINK1 N34S high-risk haplotype, the variation in reported effect sizes may be a result of differences in the proportion of subjects with pathogenic pathways linking environmental stressors to pancreatic stellate cell (PSC) activation through recurrent trypsinogen activation and inadequate trypsin inhibition by SPINK1. The basic model is illustrated in Figure 1. In this model, SPINK1-regulated pathways would include all upstream etiologic factors associated with recurrent trypsinogen activation (e.g. PRSS1 and CFTR mutations) while SPINK1-independent pathways would include factors that drive PSC to produce fibrosis through mechanisms that are generally independent of recurrent trypsinogen activation (e.g. autoimmune pancreatitis, toxins, pancreatic cancer). Although a number of functionally different risk factors have been statistically associated with alcoholic CP [9], it is not clear if the PSC and fibrosis in alcoholic CP is driven by trypsin-dependent, or independent pathways.

In order to address questions of heterogeneity, and specifically the role of alcohol and SPINK1 N34S in human CP, we employed meta-analysis in a manner illustrative of the growing realization that meta-analysis is most effective in examining relationships in complex diseases by evaluating between-study heterogeneity [23,24,25]. We tested the hypothesis that the true effect size of the functional SPINK1 N34S haplotype is pathway-dependent by reviewing the world literature and gathering data from case-control studies that evaluated the association of SPINK1 N34S with CP. When possible, we reclassified patients by reported etiology and performed a series of meta-analyses on each category to determine the effect size of the high-risk SPINK1 N34S haplotype within each etiologic sub-classification. We hypothesized that etiology-based categories defining a trypsin-dependent pathway would be associated with a larger SPINK1 N34S effect than those that were trypsin-independent.

Methods

We systematically reviewed the world’s literature on SPINK1 polymorphisms. Only case-control studies were considered. When possible, subjects were classified according to reported etiology, with the following categories as presented in Table 1: Alcoholic chronic pancreatitis (ACP), idiopathic chronic pancreatitis (ICP), familial/hereditary chronic pancreatitis (FCP) and tropical pancreatitis (TP). We then conducted a series of meta-analyses combining subjects within etiology-based sub-classifications as described below.

Study selection criteria

The literature search and study review was performed by two separate authors (EA, DCW). Genetic association studies of the SPINK1 N34S high-risk haplotype in pancreatitis that were published prior to May 2007 were identified by searching the PubMed and the EMBASE databases. Search terms included polymorphism(s), SPINK1, Serine Protease Inhibitor Kazal type 1, PSTI, N34S and pancreatitis. The reference list of citations in the identified publications was reviewed to identify additional published articles not indexed by the major databases. Genetic association studies that reported the frequency of the SPINK1 N34S high-risk haplotype in patients with acute and/or chronic pancreatitis and in a control population were selected. When more than one published report used data from the same case and control population, we included only the largest study with extractable data in the meta-analysis. Corresponding authors were contacted in some cases to clarify issues of possible data duplication. Only studies that used validated genotyping methods, such as direct gene sequencing, polymerase chain reaction paired with restricted fragment length polymorphism and denaturing gradient gel electrophoresis, were included. Studies based on linkage results (pedigree studies), case reports, editorials, review articles and studies published in a language other than English were excluded.

Data Abstraction

Data abstraction was performed by two separate authors (EA, CC) and differences were resolved by discussion. The data elements included the first author, journal, year of publication, country of origin, racial background of the study population (when mentioned), demographics, reported etiology of CP (alcoholic, idiopathic, familial, tropical, etc) and the number of cases and controls. Allelic frequency, genotypic distribution and genotyping methods were recorded. All statistical analysis was based on the number of alleles—as opposed to number of patients, in order to better quantify homozygous cases.

Statistical Methods

For each study, we initially evaluated the association of SPINK1 N34S high-risk haplotype with CP separately. A preliminary meta-analysis combining all studies regardless of etiology was conducted. We then conducted a series of meta-analyses assessing the degree of risk of various categories of CP with SPINK1 N34S. Effect size was expressed as an OR with the corresponding 95% confidence

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Figure 1. Hypothesis of etiology-defined pathways to pancreatic fibrosis. Hypothetical influence diagram illustrating pathologic pathways linking proximal factor (Factor A and B) to PSC (pancreatic stellate cell) and fibrosis through multiple steps (e.g. $a_1$, $a_2$, $a_3$, $b_1$, $b_2$, $b_3$). Etiological factors of type B activate trypsinogen to trypsin, and therefore their pathologic pathway to the PSC can be interrupted by SPINK1. Etiological factors of type A are independent of trypsin, and therefore will not be influenced by variations in SPINK1 expression or function.

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interval (CI). Heterogeneity between studies was tested using the Cochran Q statistic and the I^2 value. Because all statistical tests for heterogeneity are weak, we also included the 95% confidence interval for I^2 [26], which was calculated based on previously described methods[27]. When heterogeneity was not overtly evident, we performed the meta-analysis using both the fixed-effect (Mantel-Hanszel method) and the random-effect models (DerSimonian and Laird method). When heterogeneity was present, we only reported the results using the random-effect model. The Mantel-Hanszel (MH) method was selected over other fixed-effect methods because of potential small sample sizes of mutation carriers. An I^2 value of 50 was considered the threshold, above which studies were considered too heterogeneous to be combined. The Haldane continuity correction (adding 0.5 to each cell) was used if the quantity of N34S-containing genotypes was equal to zero in either the cases or the controls. The presence of publication bias and small study effects were assessed using the Egger regression asymmetry test for funnel plot and the Begg-Mazumdar adjusted rank correlation test. Statistical significance was considered at a p-value of ≤0.05. In cases where the polymorphism was not detected in either cases or controls, the study was omitted from the meta-analyses as it carried a relative weight of zero. All meta-analyses were conducted using Stata version 8.2 (Stata, Inc., College Station, Texas) and Comprehensive Meta-Analysis version 2.0 (Biostat Inc. Englewood, New Jersey).

Table 1. Case control studies considered for inclusion in the meta-analyses and patient subclassification within each study.

| Author et al | Year | Journal | Genotyping Method | Population | ACP | ICP | FCP | TCP |
|-------------|------|---------|-------------------|------------|-----|-----|-----|-----|
| Chen et al[13] | 2000 | J Med Genet | PCR-DGGE | France | Excluded | | | |
| Witt et al[15] | 2000 | Nat Gen | Direct sequencing | Germany - Austria | + | | | |
| Pfutzer et al[14] | 2000 | Gastro | Direct sequencing | USA - Europac | + | + | | |
| Plendl et al[46] | 2001 | Am J Med Gen | PCR-RFLP | Germany | + | | | |
| Witt et al[47] | 2001 | JAMA | Direct sequencing | UK - Germany - Switzerland | + | | | |
| Rossi et al[30] | 2001 | Pancreatology | Direct sequencing | Bangladesh | Excluded** | | | |
| Chen et al[48] | 2001 | Gastro | PCR-DGGE | France | + | | | |
| Kaneko et al[49] | 2001 | J Hum Genet | Direct sequencing+RFLP | Japan | + | | | |
| Threadgold et al[50] | 2002 | Gut | Direct sequencing+RFLP | Europac | + | + | + | |
| Chandak et al[51] | 2002 | J Med Genet | Direct sequencing | India | + | | | |
| Drenth et al[52] | 2002 | Gut | Direct sequencing | Netherlands | + | + | + | |
| Bhadia et al[53] | 2002 | Gastro | Direct sequencing | India | + | | | |
| Hassan et al[54] | 2002 | Am J Hum Gen | PCR-RFLP | Bangladesh - India | + | | | |
| Schneider et al[55] | 2002 | Gastro | Direct sequencing | Bangladesh | + | | | |
| Audrezet et al[56] | 2002 | Eur J Hum Gen | PCR-DGGE | France | + | | | |
| Truninger et al[57] | 2002 | Am J Gastro | Direct sequencing | Europe | + | | | |
| Schneider et al[58] | 2003 | Dig Dis Sci | Direct sequencing | USA | + | | | |
| Gomez-Lira et al[59] | 2003 | Eur J Hum Gen | Direct sequencing | Italy | + | | | |
| Perri et al[60] | 2003 | Eur J Hum Gen | Direct sequencing | Italy | + | | | |
| Bernardino et al[61] | 2003 | JOP | PCR-RFLP | Brazil | + | + | + | |
| Matsubayash et al[61] | 2003 | Cancer Biol Ther | PCR-RFLP | USA | | + | | |
| Chandak et al[62] | 2004 | Gut | Direct sequencing | India | + | + | + | |
| Lempinen et al[63] | 2005 | Scand J Gastro | Direct sequencing | Finland | + | + | | |
| Kume et al[29] | 2005 | Pancreatology | Direct sequencing | Japan | Excluded*** | | | |
| Lee et al[64] | 2005 | Dig Dis Sci | PCR-RFLP | Korea | + | | | |
| Kelles et al[28] | 2006 | Pancreas | Direct sequencing | USA | Excluded* | | | |
| Shimosegawa et al[65] | 2006 | J Gastro Hepatol | PCR-RFLP | Japan | + | + | + | |
| S-Tomaszewsk[66] | 2006 | J Pediatr Gastroenterol Nutr | PCR-RFLP | Poland | | | | |
| Tzetis et al[67] | 2007 | Clin Genet | PCR-DGGE | Greece | + | | | |
| Masamune et al[31] | 2007 | J Gastroenterol | Direct sequencing | Japan | Excluded**** | | | |

*Prevalence of the N34S polymorphism was not reported in the control population. 
**Data duplicated in Schneider et al [55]. 
***Data duplicated in Shimosegawa et al [65]. 
****Data duplicated in Kume et al[29]. 
•Dropped from the analysis because it did not identify the N34S polymorphism in either the cases or the controls therefore resulting in a null relative weight. 
••Unable to subclassify patients into the mentioned categories due to missing data.
PCR: Polymerase Chain Reaction, RFLP: Restricted Fragment Length Polymorphism, DGGE: Denaturing Gradient Gel Electrophoresis.
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Results

Figure 2 is a flow diagram illustrating the studies included. Thirty case-control studies evaluating the association of the \textit{SPINK1} N34S polymorphism with CP were identified (Table 1). Two studies were excluded because the prevalence of the polymorphism was not reported in the control population [13,28]. An additional three studies were excluded due to data duplication in other publications [29,30,31]. Additionally, The report by Bernardino et al [32] was excluded from the analysis because it did not identify the N34S polymorphism in either cases or controls. A total of 24 studies were therefore evaluated.

Chronic Pancreatitis–All etiologies combined

Figure 3 summarizes the results of the initial meta-analysis with all etiologies of CP combined. The total number of patients from these studies was 2,421 with 4,857 controls. The mutation was detected in 469 of 4,842 patient alleles and in 96 of 9,714 control alleles. Significant heterogeneity was detected (Q = 41.05, df = 23, p = 0.01, I^2 = 43.97%). The random effect model showed a combined OR of 11.00 (95% C.I. = 7.59–15.93). Both the Egger and the Begg-Mazumdar tests were not statistically significant with p = 0.65 and p = 0.94 respectively.

Alcoholic Chronic Pancreatitis

We identified patients with alcoholic CP from 9 studies (737 patients, 2,033 controls). Overall, alcohol was the etiologic factor leading to chronic pancreatitis in 31% of the patients that we were able to classify. Six of nine studies failed to identify a statistically significant association between the \textit{SPINK1} N34S polymorphism and alcoholic CP. The \textit{SPINK1} N34S high-risk haplotype was reported in 49 of 1,474 patient alleles and in 37 of 4,066 control alleles. There was no heterogeneity detected (Q = 7.36, df = 8, p = 0.50, I^2 = 0%). Both the fixed and the random-effect model showed a pooled OR of 4.98 (95% C.I. = 3.16–7.85)–the lowest among the different categories that we analyzed. Figure 4 summarizes the meta-analysis results pertaining to patients with alcoholic CP.

Tropical Chronic Pancreatitis

Four studies assessed patients with tropical pancreatitis (351 patients, 973 controls). The high-risk haplotype was detected in 168 of 702 patient alleles and in 44 of 1,946 control alleles. The heterogeneity testing showed Q = 7.36, df = 8, p = 0.50, I^2 = 43.97%. The pooled OR calculated using the random-effect model was 19.15 (95% C.I. = 8.83–41.56). Figure 5 summarizes the meta-analysis results pertaining to patients with tropical pancreatitis.

Idiopathic Chronic Pancreatitis

Fourteen studies were included in this analysis (963 patients, 3,015 controls), only two of which did not detect a statistically significant association with \textit{SPINK1} N34S (figure 6). The Cochran’s Q statistics was calculated at 20.96 with a p value of 0.08 and an I^2 of 37.98%. The pooled OR was 14.97 (95% C.I. = 9.09–24.67).

Figure 2. Flow diagram of the studies included in the meta-analysis. * The report by Bernardino et al was excluded from the meta-analysis because it did not detect the N34S haplotype in neither the cases nor the controls and was therefore assigned a weight of zero.

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Figure 3. Meta-analysis results for chronic pancreatitis all etiologies combined based on allelic frequency. Heterogeneity testing: Q-value = 41.05, df = 23, p = 0.01, I^2 = 43.97 (95% CI: 10.56–64.90). doi:10.1371/journal.pone.0002003.g003

Figure 4. Meta-analysis results for alcoholic chronic pancreatitis based on allelic frequency. Heterogeneity testing: Q-value = 7.36, df = 8, p = 0.5, I^2 = 0 (95% CI: 0.00–62.01). doi:10.1371/journal.pone.0002003.g004

Figure 5. Meta-analysis results for tropical pancreatitis based on allelic frequency. Heterogeneity testing: Q-value = 5.72, df = 3, p = 0.13, I^2 = 47.56 (95% CI: 20.96–78.99). doi:10.1371/journal.pone.0002003.g005
Discussion

Familial and Hereditary Chronic Pancreatitis

Six studies (249 patients, 955 controls) were analyzed. Significant heterogeneity was detected by the Cochran Q statistic (Q = 26, df = 5, p = 0.00). Additionally, the I-squared value was 80.94%. Due to the high degree of heterogeneity, these studies were not combined.

Another potential explanation is that there are multiple subpopulations with different complex, multi-step etiologic pathways that all lead to the same phenotype (e.g., end-stage organ fibrosis). Thus, a genetic factor that is critical in one etiologic pathway will only be shown to have a large effect size in populations in which that pathways dominates [25,33]. In other populations, in which the dominant etiological pathway is independent of the gene variant in question, the measured effect size of the genetic variant may be small.

Alcohol is a toxin that damages the pancreas by altering key regulatory processes, causing direct injury to acinar cells and driving stellate cells to produce fibrosis. Specifically, studies have shown that alcohol can act directly on the brainstem [34], acinar cells [35], immune system [36], and PSCs [37]. The association of alcohol with TP than controls [40]. Cathepsin B is a lysosomal enzyme present in pancreatic acinar cells that appears to be important in protecting the pancreas from premature trypsinogen activation [41,42], suggesting that SPINK1 N34S polymorphisms may increase the risk of alcoholic CP.

When meta-analysis is applied to complex genetic syndromes, the measured effect size of a genetic variable between studies is often widely discrepant [20,21]. A way to interpret the observation that very large effect sizes in small genetic studies are often not “reproducible” in subsequent studies has been attributed to a combination of false-positive findings (e.g., related to multiple testing without correction) and publication bias toward reports with large effect sizes.

Figure 6. Meta-analysis results for idiopathic chronic pancreatitis based on allelic frequency. Heterogeneity testing: Q-value = 20.86, df = 13, p = 0.08, I² = 37.98 (95% CI: 0.00–65.00).

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enrichment of this group with subjects carrying PRSS1 and CFTR mutations and the large effect of SPINK1 polymorphisms with progression to CP is consistent with our proposed model. Of note, the highest reported OR of any study (OR = ~80) was that of Witt et al that was conducted in a relatively homogenous group of children with early onset idiopathic CP where few, if any, environmental exposures played a role, especially tobacco smoking and alcohol [15]. Thus, the high OR reflected an enrichment of the pathway that is most strongly regulated by SPINK1 rather than the random effects of chance.

The risk of familial and hereditary CP is strongly associated with SPINK1 N34S, but these data should be interpreted with caution. Hereditary pancreatitis is an autosomal dominant disorder with very high penetrance that is caused by gain-of-function mutation in the cationic trypsinogen gene [10]. The etiology of hereditary pancreatitis is unequivocally linked to trypsin-associated pathway of recurrent acute pancreatitis leading to CP, but the effect is to such a degree that normal expression of SPINK is not sufficient to prevent it. On the other hand, a major proportion of familial chronic pancreatitis that is not autosomal dominant is associated with multiple family members with homozygous SPINK1 mutations. In this case, the etiology-based classification system is biased toward an association with SPINK1. These factors likely contribute to the high heterogeneity of effects in this classification.

The current study has several limitations. One of the challenges in understanding CP in human subjects is distinguishing trypsin-dependent and trypsin-independent pathways in CP from the central role of trypsin in acute pancreatitis. Indeed, AP may be necessary to initiate CP by activating the immune system within the pancreas (including PSCs), thereby initiating the fibrosing process [44, 45]. Thus, although CP is driven by trypsin-dependent and trypsin-independent factors, the fact that an individual is initially susceptible to the first episode of AP may blur the distinction between trypsin-dependent and trypsin-independent pathways to CP. Another limitation is that the published reports did not include or classify pancreatic fibrosis caused by autoimmune pancreatitis or pancreatic cancer, which, because they are thought to be trypsin-independent, would have been an important comparison group for SPINK1 N34S effects. Furthermore, the effect of ethnicity and race was usually not reported, although the country of origin was available (Table 1) and the etiologies of interest were clearly reported. Genetic polymorphisms often vary by race or ethnicity. Further studies are warranted to more thoroughly evaluate racial and/or ethnic variation in SPINK1 polymorphisms. Another limitation relies in the fact that classification of the patients into etiology-based subgroups was carried based on the etiologies listed in the manuscripts included in the analysis. It is unlikely that all twenty-four centers used the same criteria to diagnose and categorize these patients and therefore variations in the diagnostic criteria may explain some of the heterogeneity observed. Furthermore, a certain degree of misclassification may have occurred as a result and there is considerable residual heterogeneity beyond what the etiologic grouping can explain. Additionally, the presence of potential confounding variables or modifying factors could not be completely ruled out due to the limited amount of information and data assessing such factors in each study. Despite these challenges, clear differences in the effect of SPINK1 in different etiologies were observed.

In conclusion, meta-analysis of association studies examining the SPINK1 N34S polymorphism in CP confirms a significant overall pathologic association, although the reported effect size varies significantly depending on the etiology of CP. Modeling CP as a complex syndrome resulting from various pathogenic pathways with or without recurrent trypsinogen activation allows for the direct assessment of the effect of SPINK1 polymorphisms in the development of CP. While the subgroup analysis needs to be interpreted with caution, our results suggest that much of the variance in reported ORs between small studies of candidate genes in complex disorders may be attributed to a mixture of multiple etiologic pathways leading to a single clinical endpoint (e.g. organ fibrosis in CP). Alcohol appears to drive fibrosis in alcoholic CP primarily through a trypsin-independent pathway as reflected by the significantly lower association of SPINK1 N34S with this etiology. Additional studies are warranted to further assess the presence of any confounding or modifying factors and to elucidate the various pathophysiological mechanisms involved and their implications in the etiology of CP.

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Author Contributions

Conceived and designed the experiments: DW EA. Performed the experiments: EA CC. Analyzed the data: DW EA CC MB GP. Wrote the paper: DW EA JG.

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