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Molecular evidence for the involvement of alternative splicing in jejunum epithelial cells in broiler chickens fed dietary supplementation with olive mill wastewater

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ABSTRACT

There is no doubt that alternative splicing is conserved in chickens and mammals, but evaluating the effects of nutrition on alternative splicing in chickens is crucial in a wide range of fields. Although the olive diet has been extensively studied in human, mouse, and chicken systems, little is known about its impact on chicken alternative splicing systems. Hence, the current study aimed to assess the effect of feeding polyphenol-enriched olive mill wastewater to female broiler chickens via alternative splicing by analyzing high-throughput sequencing raw reads of RNA utilizing genomics and bioinformatics methodologies. It also aimed to look for differences in isoform expression and discover molecular functions and biological processes linked to differentially transcribed genes. The findings of our study revealed that 51 genes involved in isoform switching and alternative splicing events were not used evenly. This is due to the reduced use of ATSS in olive mill wastewater groups compared to control groups. Furthermore, the gene ontology analysis revealed that 25 GO terms were enriched in biological processes, 16 GO terms were enriched in molecular function, and 25 GO terms were enriched in cellular components. Kinase and adenylyltransferase activities were significantly enriched in terms. The molecular analysis presented herein provides valuable insight into the role of phenolics in alternative gene-splicing mechanisms in chickens, demonstrating how an industrial waste product can be repurposed as a feed supplement with a satisfactory outcome.

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Introduction

A critical aspect of gene expression analysis is how phenotype and genotype correlate with nutrition and evolutionary biology (1,2). Most research has so far focused on understanding the effect of nutrition variables at gene expression levels (i.e., quantitative polymerase chain reaction, subtractive hybridization, microarrays, sequencing technologies and bioinformatics tools) (3–5). However, most research has not considered how a single genome might manifest many phenotypes. This subject can be addressed at the cellular level, for example, how a single gene can produce both skin and blood cells; also, at the molecular and biological levels, one gene in multiple animals can show a variety of phenotypes depending on environmental factors (5,6). This mechanism, known as "phenotypic plasticity", generates phenotypic variation in the absence of underlying genetic diversity. For instance, due to the nutritional value of royal jelly, a single genome can produce both the queen and worker castes in honeybees (7,8). However, changes in a gene's overall expression are not the only way expression can be affected. For example, the difference between head lice (harmless) and body lice (disease vector) is caused by alternative splicing of an identical genome, not differential gene expression (9). Together, these studies indicate that most biological diversity is due to mechanisms of alternative splicing rather than genomic variation (10,11). Chicken genes undergo alternative splicing in 23 to 56% of genes (12–14) compared to 68% and 57% in human and mouse, respectively (14,15). Alternative splicing is one of the most fundamental processes of gene expression regulation, producing a vast number of mRNA and protein isoforms (proteomic diversity) from a single gene (8,11). In this process, a particular exon of a gene is either included or excluded from the final, processed messenger RNA (mRNA) (16). Thus, it increases protein diversity and allows the system to cope with the increasingly broad spectrum of functional and behavioral complexity (8,11,16). Many cellular activities crucial for biological function, such as cell proliferation, motility, and medication responsiveness, are profoundly affected by the expression of alternative or even tumor-specific splice variants (17). It is still unclear, however, to what extent alternative splicing functionally contributes to the initiation and increase of poultry production. Here we will describe general changes to the splicing machinery in chickens and discuss the impact of the olive extract on chicken genes. The Mediterranean diet is regarded as one

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of the healthiest eating habits, and olive oil is a significant component in reducing the risk of cancer and metabolic diseases (18,19). There is growing interest in utilizing the wastewater from olive mills, which are rich in polyphenols, for animal nutrition. Olive mill wastewater (OMWW) is thought to improve chicken performance and the quality of products derived from food (20–22). Moreover, it has been concluded that OMWW dietary supplementation can be used as a nutritional strategy to improve chicken performance and health, prevent intestinal damage, enhance innate immunity, and regulate cholesterol metabolism and fat deposition (22). However, the effect of dietary supplementation with OMWW on alternative splicing or changes in expression at the isoform level has not been studied yet. Hence, we propose that feeding OMWW to broiler chickens could stimulate and result in different transcript exon usage. Accordingly, the current study aimed to investigate the effect of feeding OMWW, waste products enriched with polyphenols, on jejunum epithelial cells in broiler chickens via alternative splicing (differential transcript usage) by analyzing RNA data using genomics and bioinformatics analyses. The study also aimed to scan changes in isoform expression and find molecular function and biological processes related to genes differentially transcribed as an effect of dietary supplementation with OMWW in female broiler chickens.

Materials and Methods

High throughput sequencing of RNA raw reads

High throughput sequencing paired-end raw reads (2x150bp) of RNA-Seq transcriptomic of the Illumina HiSeq 4000 platform of broiler chickens (*Gallus gallus*) (jejunum epithelial cells in two conditions) were retrieved from the NCBI database under BioProject number PRJNA450504 and SRR numbers (SRR7012468 to SRR7012486) (22) using the SRA Toolkit (23). In the study described in (22), total RNA was isolated from jejunum epithelial cell samples from two experimental groups of female broilers fed two different diets for a period of 20 days. A commercial feed (CTRL) was given to one group, whereas a CTRL diet enriched with 0.03% olive mill wastewater (OMWW) was fed to the other group. Herein, in our study, in total, RNA raw reads from 19 transcriptome libraries (54.56GB on average) of jejunum epithelial cells from female broiler chickens belonging to both groups were retrieved from the NCBI database and used for genomics and bioinformatics analyses.

Read processing and gene expression analysis

Base calling and quality scoring of the raw reads were visualized using fastQC v 0.11.8. The poor-quality reads and adapters were removed using trimmomatic v0.38 (24). Clean raw sequences were mapped to the chicken maternal broiler reference genome (bGalGal1.mat.broiler.GRCg7b (GCA 016699485.1) and the transcript abundance quantification was performed using Kallisto with default parameters (25). Alternatively spliced genes between two groups, CTRL and OMWW were identified using *DEXSeq* (26) implemented by isoformswitchanalyzeR (27). This package provides a general linear model for each gene, testing the relative proportion of expression of each exon under each condition. Then Lowly expressed genes with less than one TPM (Transcripts Per Million) and lowly expressed isoforms that did not contribute to gene expression were excluded from further investigation. After adjusting for multiple testing using the Benjamini-Hochberg procedure, a gene was considered differentially alternatively spliced if it had an absolute isoform usage difference of 10% and a p-value <0.05 (28).

Gene enrichment and functional analysis

The Database for Annotation, Visualization, and Integrated Discovery (DAVID) was used to perform gene-set enrichment analysis (GSEA) (29). Fisher`s exact test was applied on genes that were differentially alternatively spliced, and GO terms enrichment analysis identified three subsets of GO annotations, namely biological process (BP), molecular function (MF), and cellular component (CC), with a p-value <0.05. Results were visualized with REVIGO (30).

Results

Data quality and quantification

After excluding low-quality reads and adapters, on average 15.6 million reads were obtained. The range of the percentage of GC content was between 37.9% and 40%, and the probability of an inaccurate base call under the quality score (Q30%) was 1 in 1000. The N (poor quality) content across all bases was nil, and the quality score error rates across all bases were less than 0.1%. At the isoform level, the amount of expression was measured using a TPM, and 15984 genes expressed 40594 isoforms. Furthermore, after removing genes with TPM less than 3 from all libraries, there remained 4608 genes with the expression of 11663 isoforms.

Differentiations of isoforms

About 51 genes with differential isoform usage (DIU) between CTRL and OMWW conditions at a p-value of 0.05 were identified (Supplementary Table 1). The top five genes with differentially spliced isoforms between CTRL and OMWW were MALRD1, DYNC1H1, NFATC3, SLC6A6, and MCCC1 (Supplementary Figures 1-5).

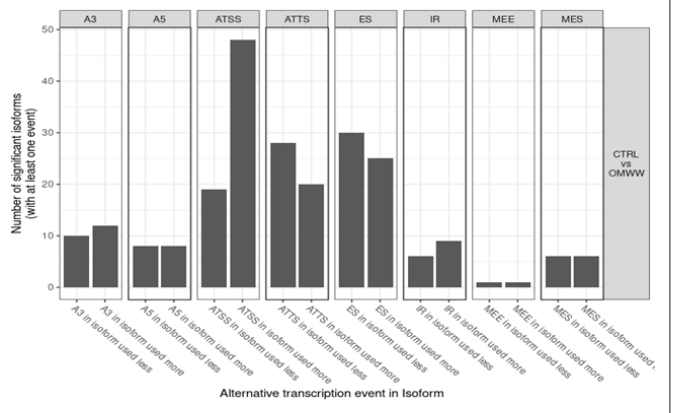

Figure 1. The number of isoforms differentially used between OMWW and CTRL (rows of. sub-plots) resulting in at least one splice event. Alternative 3` splice site (A3), alternative 5` splice site (A5), alternative transcription start site (ATSS) and alternative transcription termination site (ATTS), exon skipping (ES), intron retention (IR), mutually exclusive exon (MEE) and multiple exon skipping (MES).

Genome-wide analysis of alternative splicing

Genome-wide analysis revealed that alternative splicing events showed various forms, including intron retention (IR), exon skipping (ES), multiple exon skipping (MES), alternative transcription start site (ATSS) and alternative transcription termination site (ATTS). As seen in Figure 1, some alternative splicing events are not used evenly, as evidenced by the utilization of ATSS, ATTS, and ES in OMWW samples with more gains than losses. When comparing OMWW to CTRL, considerable enrichment was observed for the loss of ATSS in OMWW samples. Furthermore, there was a significant difference in ATTS gain at a q-value of less than 0.05 in using more than 40 genes between control and treatment (Figure 2).

Gene enrichment and functional analysis

As shown in Table 1, from differentially spliced isoforms, we discovered 25 enriched biological process (BP) Go terms, 16 enriched molecular function (MF) GO terms, and 25 enriched cellular component (CC) GO terms with p-values less than 0.05. The most common biological activities are amide transport, membrane organization, nucleoside phosphate biosynthetic processes, and GTPase activity regulation (Supplementary Figure 6). As a cellular component, significant Go terms are associated with the *trans*-Golgi network, cytoplasm, intracellular anatomical structure, endomembrane system, and protein-containing complex (Supplementary Figure 7). At the molecular function level, most GO terms were significantly enriched with 1 phosphatidylinositol binding, anion-cation symporter activity, (anion, lipid, and ion) binding, and adenylyltransferase activity, which were considerably enriched in terms of molecular function (Supplementary Figure 8).

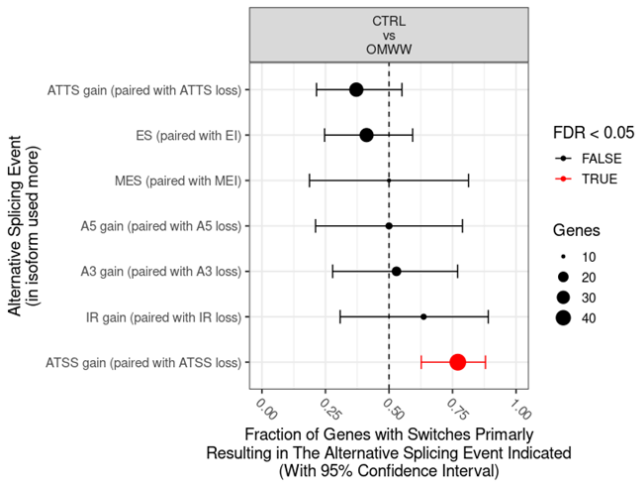

Figure 2. Comparison of enrichment and depletion in isoform switch consequences between CTRL and OMWW conditions (y-axis). The x-axis depicts the fraction (with a 95% confidence interval) of switches from control to OMWW that result in the outcome indicated by the y-axis. The fraction (and 95% CI) of isoform switches (x-axis) that result in the gain of a specific alternative splice event (indicated by the y-axis) in the switch from CTRL to OMWW. A dashed line indicates no enrichment or depletion. The color indicates whether the FDR is less than 0.05 (red) or not (black).

Discussion

Previous research has demonstrated the significance of alternative splicing of precursor mRNA in increasing the complexity of gene expression and playing an important role in cellular differentiation and organism development. Methods and applications of high-throughput sequencing in transcriptome research have revolutionized our knowledge of alternative splicing. Hence, high-throughput sequencing paired-end raw reads of RNA-Seq transcriptomic (2x150 bp) on the Illumina HiSeq 4000 platform of broiler chickens with the aid of genomics and bioinformatics techniques were used to investigate the effect of feeding dietary supplementation with OMWW on jejunum epithelial cells in broiler chickens via alternative splicing. The first part of our data analysis revealed that the average expression was 7.2 transcripts per gene, which is higher than previous studies (31) but lower than the human average of 10 transcripts (32). There are many explanations for these differences; they might be due to different genetic or tissue backgrounds or the methods for RNA extraction and platforms of sequencing technologies. For instance, in chicken, transcript expression has been measured across tissues, and two brain tissues, the cortex and the cerebellum, expressed the greatest number of transcripts and locations. Furthermore, around 22% of the transcripts were tissue-specific, with reproductive tissues having the most tissue-specific transcripts (31). The use of Nanopore sequencing highlighted the utility of long-read data in the discovery of new loci and the resolution of complex or lengthy transcripts (31). Hence, we believe that a meta-analysis using several datasets can corroborate and support the findings of our study. The switch plots in Supplementary Figures (1–5) demonstrate that 51 genes with no differences in gene expression showed significant isoform switching. We discovered that various types of isoform switch consequences were either enriched or depleted in isoform switches as a result of dietary supplementation with OMWW. These findings suggest the involvement of natural products in enhancing the complexity of gene expression for several essential genes associated with the fundamental structure of the cellular membrane, ATP, and ion binding. The most common changes found in genome-wide analyses were those impacting ATSS and ATTS (Figures 1 and 2). Switching isoforms at ATTS differences may have altered gene expression regulation at isoform usage. More research is required on the stability of this region's genome throughout the impact of nutrition in the chicken module.

Among the genes with differentially spliced isoforms were three that encode the ubiquitin protein (Supplementary Table 1). Ubiquitin has been postulated to be implicated in the induction of a group of proteins that act during the cellular response to different types of environmental stress, as well as a role in chromatin structure and cellular proteolytic degradation (33,34). Also, there were more than three genes related to kinase activity and enzymatic activity: methylcrotonoyl carboxylase, galactose mutarotase, ligase, endonuclease, methyltransferase, phosphatase, lysine acetyltransferase 6A, isomerase, and GTPase (Supplementary Table 1). Kinases that phosphorylate certain carboxyl-terminal domain (CTD) serine residues have been found and are components of the protein machinery driving the specific function. Ser2 phosphorylation, for instance, is preferentially linked with CTD activity at the 3'-end of genes through CDK9 of the positive transcription elongation factor b. Ser5 phosphorylation, on the other hand, is associated with transcriptional initiation

Table 1. The outcomes of all Gene Ontology term enrichment analyses performed on DIU genes in response to OMWW.

Category	GO ID	GO Term	Gene Count	Genes %	P-value	FET	Fold Enrichment
BP	GO:0071705	nitrogen compound transport	11	21.57	0.01391	0.0057	2.339
BP	GO:0042886	amide transport	10	19.61	0.01629	0.0064	2.441
BP	GO:0071702	organic substance transport	12	23.53	0.01695	0.0076	2.145
BP	GO:1901293	nucleoside phosphate biosynthetic process	4	7.84	0.02724	0.0043	6.004
BP	GO:0051641	Transport	13	25.49	0.03069	0.016	1.881
BP	GO:0006996	organelle organization	17	33.33	0.03284	0.019	1.64
BP	GO:0015031	protein transport	9	17.65	0.0334	0.014	2.313
BP	GO:0048193	Golgi vesicle transport	4	7.84	0.03823	0.0069	5.254
BP	GO:0065003	macromolecular complex assembly	9	17.65	0.039	0.016	2.244
BP	GO:0015833	peptide transport	9	17.65	0.04005	0.017	2.232
BP	GO:0044802	single-organism membrane organization	6	11.76	0.04108	0.013	3.073
BP	GO:0045184	establishment of protein localization	9	17.65	0.04716	0.021	2.16
BP	GO:0043087	regulation of GTPase activity	4	7.84	0.0475	0.012	4.45
BP	GO:0006753	nucleoside phosphate metabolic process	5	9.8	0.04894	0.017	3.322
BP	GO:0016192	vesicle-mediated transport	8	15.69	0.04129	0.026	2.204
BP	GO:0008104	protein localization	11	21.57	0.04615	0.034	1.809
BP	GO:0007009	plasma membrane organization	4	7.84	0.04946	0.016	4.112
BP	GO:0055086	nucleobase-containing small molecule metabolic process	5	9.8	0.04282	0.022	3.09
BP	GO:0006810	Transport	16	31.37	0.04526	0.047	1.513
BP	GO:0043933	macromolecular complex subunit organization	9	17.65	0.04656	0.037	1.952
BP	GO:0034613	cellular protein localization	9	17.65	0.04013	0.039	1.933
BP	GO:0070727	cellular macromolecule localization	9	17.65	0.04265	0.04	1.92
BP	GO:0046907	intracellular transport	8	15.69	0.04402	0.044	1.991
BP	GO:0010256	endomembrane system organization	5	9.8	0.04442	0.031	2.82
BP	GO:0051649	establishment of localization in cell	9	17.65	0.09788	0.049	1.849
MF	GO:0043168	anion binding	14	27.45	0.01041	0.0049	2.038
MF	GO:0005543	phospholipid binding	5	9.8	0.02005	0.0041	4.65
MF	GO:0005524	ATP binding	9	17.65	0.02311	0.009	2.447
MF	GO:0035091	phosphatidylinositol binding	4	7.84	0.02545	0.004	6.121
MF	GO:0032559	adenyl ribonucleotide binding	9	17.65	0.02552	0.01	2.403
MF	GO:0030554	adenyl nucleotide binding	9	17.65	0.0265	0.011	2.386
MF	GO:0005545	1-phosphatidylinositol binding	2	3.92	0.03139	0.00046	61.212
MF	GO:0043167	ion binding	19	37.25	0.0327	0.042	1.427
MF	GO:0070566	adenylyltransferase activity	2	3.92	0.04725	0.0023	28.056
MF	GO:0008289	lipid binding	5	9.8	0.02247	0.022	3.072
MF	GO:0035639	purine ribonucleoside triphosphate binding	9	17.65	0.0333	0.035	1.949
MF	GO:0032555	purine ribonucleotide binding	9	17.65	0.01037	0.039	1.91
MF	GO:0032553	ribonucleotide binding	9	17.65	0.0437	0.041	1.894
MF	GO:0017076	purine nucleotide binding	9	17.65	0.02467	0.042	1.889
MF	GO:0015291	secondary active transmembrane transporter activity	3	5.88	0.03447	0.016	5.642
MF	GO:0015296	anion:cation symporter activity	2	3.92	0.02921	0.0051	18.704
CC	GO:0005829	Cytosol	17	33.33	0.00089	0.00037	2.328
CC	GO:0005737	Cytoplasm	34	66.67	0.00091	0.00037	1.46
CC	GO:0044444	cytoplasmic part	28	54.9	0.00285	0.0018	1.553
CC	GO:0005622	Intracellular	39	76.47	0.00616	0.0047	1.234
CC	GO:0044424	intracellular part	39	76.47	0.00616	0.0048	1.234
CC	GO:0044433	cytoplasmic vesicle part	6	11.76	0.01257	0.0029	4.173
CC	GO:0044431	Golgi apparatus part	6	11.76	0.01288	0.003	4.148
CC	GO:0005794	Golgi apparatus part	8	15.69	0.02114	0.0073	2.757

CC	GO:0012505	endomembrane system	14	27.45	0.03421	0.018	1.922
CC	GO:0031410	cytoplasmic vesicle part	8	15.69	0.04424	0.0029	2.359
CC	GO:0044446	intracellular organelle part	25	49.02	0.04501	0.032	1.353
CC	GO:0097708	intracellular vesicle	8	15.69	0.04512	0.018	2.349
CC	GO:0044427	chromosomal part	6	11.76	0.04578	0.026	2.969
CC	GO:0044464	cell part	41	80.39	0.04659	0.04	1.107
CC	GO:0005623	cell	41	80.39	0.04659	0.042	1.107
CC	GO:0031090	organelle membrane	9	17.65	0.04026	0.028	2.043
CC	GO:0043229	intracellular organelle part	33	64.71	0.04061	0.032	1.205
CC	GO:0044422	organelle part	25	49.02	0.06545	0.048	1.311
CC	GO:0031982	Vesicle	8	15.69	0.06576	0.026	2.158
CC	GO:0005694	Chromosome	6	11.76	0.07207	0.029	2.607
CC	GO:0043234	protein complex	12	23.53	0.07685	0.043	1.682
CC	GO:0005802	trans-Golgi network	3	5.88	0.07737	0.012	6.363
CC	GO:0043228	non-membrane-bounded organelle	15	29.41	0.07853	0.047	1.529
CC	GO:0043232	intracellular non-membrane-bounded organelle	15	29.41	0.07853	0.048	1.529
CC	GO:0000785	Chromatin	4	7.84	0.09315	0.024	3.614

The following information is presented: category (BP: biological process, MF: molecular function), GO ID (GO term accession number), GO term (name of Gene Ontology Term), gene count (number of genes enriched in the list of DIU), gene% (number of genes expressed in DIU belonging to the GO category over the total number of genes expressed in DIU), p-value (raw p-value), FET (Fisher's exact test), fold enrichment (the percentage of genes in the DIU list belonging to a pathway, divided by the corresponding percentage in the background). The category (CC: cellular component) is presented.

through cyclin-dependent kinase 7 (CDK7) of the general transcription factor IIH (35). Moreover, ser7 phosphorylation has been shown to aid in elongation and splicing (36). Hence, phosphorylation is a process that clearly establishes the existence of a functional connection between transcription and alternative splicing. Therefore, kinases are crucial regulators of cell activity (37). The transfer of a phosphate group from a donor ATP molecule to a substrate is catalyzed by kinase enzymes. As a result, phosphorylated products and ADP are formed, and proteins and lipids can both be kinase substrates (38). Here, we can conclude that olive compounds either inhibited or promoted kinase activity. In olive oil mill wastewater, there are at least 30 phenolic compounds (39) that are effective antioxidants and radical scavengers (40,41). Moreover, it has been indicated that polyphenols are known to decrease the level of reactive oxygen species and slow down the progression of cancer, neurodegenerative, and cardiovascular diseases (42), although splicing factors that do not belong to the serine/arginine-rich (SR) protein and hnRNP families and whose expression is cell type-specific were also implicated in cancer through global alternative splicing changes. The epithelial-specific splicing regulator protein 2 ESRP2 is significantly expressed in primary prostate cancer, which is stimulated by androgens. These hormones generate ESPR2 overexpression and, as a result, several alternative splicing events that drive an epithelial splicing program characteristic of prostate cancer cells and associated with cancer growth (43,44). Interestingly, according to recent studies, having olive polyphenols lowers the risk of acquiring cancer. Additionally, the computational research demonstrated that the phenolic chemicals in olives have equivalent inhibitory potential against Bruton's tyrosine kinase (45). In the list of DIU, we also detected two genes that were linked to Nexin sorting (Supplementary Table 1). Sorting nexins are a large group of proteins that are localized in the cytoplasm and have the potential for membrane association either through their lipid-binding PX domain or through protein-protein interactions with membrane-associated protein complexes (46). These proteins are evolutionarily well conserved from yeast to mammals (27,47). Finally, the data collected and processed on alternative splicing is likely to be just the tip of the iceberg, with additional information to be revealed in future research (44).

Following the analysis of RNA data with genomics and bioinformatics techniques, the current study was carried out to investigate the role of alternative splicing and the discovery of molecular function and biological processes related to genes that are differentially transcribed as an impact of dietary supplementation with olive mill wastewaters in female broiler chickens. The results of our study revealed that alternative splicing was common in chickens fed an olive extract-enriched diet. The study's most obvious conclusion is that olive extract changed transcript usage for many important metabolic enzymes. In the genomic structure, alternative splicing was not used equally at ATSS. Here, we can expand our understanding of the discovery of novel interactions between genetic and dietary variables, as ATSS has a potentially critical impact on cell proliferation, differentiation, and adaptation.

Authors' contribution

Pshtiwan Bebane and Sarbast I. Mustafa authors contributed equally to the study's design, work, and bioinformatics analysis, and all authors contributed to the manuscript's writing.

Interest conflict

The authors have stated that no competing interests exist.

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