

*DNA Research*, 2017, 24(3), 327–332

doi: 10.1093/dnares/dsw044

Advance Access Publication Date: 17 April 2017

Full Paper

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# ChloroMitoCU: Codon patterns across organelle genomes for functional genomics and evolutionary applications

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Edited by Prof. Kenta Nakai

Received 18 March 2016; Editorial decision 13 September 2016; Accepted 14 September 2016

## Abstract

Organelle genomes are widely thought to have arisen from reduction events involving cyanobacterial and archaeal genomes, in the case of chloroplasts, or  $\alpha$ -proteobacterial genomes, in the case of mitochondria. Heterogeneity in base composition and codon preference has long been the subject of investigation of topics ranging from phylogenetic distortion to the design of overexpression cassettes for transgenic expression. From the overexpression point of view, it is critical to systematically analyze the codon usage patterns of the organelle genomes. In light of the importance of codon usage patterns in the development of hyper-expression organelle transgenics, we present ChloroMitoCU, the first-ever curated, web-based reference catalog of the codon usage patterns in organelle genomes. ChloroMitoCU contains the pre-compiled codon usage patterns of 328 chloroplast genomes (29,960 CDS) and 3,502 mitochondrial genomes (49,066 CDS), enabling genome-wide exploration and comparative analysis of codon usage patterns across species. ChloroMitoCU allows the phylogenetic comparison of codon usage patterns across organelle genomes, the prediction of codon usage patterns based on user-submitted transcripts or assembled organelle genes, and comparative analysis with the pre-compiled patterns across species of interest. ChloroMitoCU can increase our understanding of the biased patterns of codon usage in organelle genomes across multiple clades. ChloroMitoCU can be accessed at: <http://chloromitocu.cgu.edu.tw/>

**Key words:** chloroplast, codon usage, functional genomics, evolution, mitochondria, web application

## 1. Introduction

Organelle genomes have been the focus of much research for several decades because of their biological activity linked to photosynthetic efficiency, energy storage, and the regulation of gene expression.<sup>1</sup> They have evolved independently, and their genetic architecture has been shaped as a consequence of evolutionary forces.<sup>2</sup> One such evolutionary force is codon usage, which has evolved independently in organelle genomes. Codon usage, a pattern that describes the degeneracy of the genetic code and illustrates differential usage of synonymous codons, is thought to be under the influence of several factors such as mutational pressure,<sup>3</sup> translational selection,<sup>4</sup> and tRNA dependencies.<sup>5,6</sup> In model organisms, the evolutionary forces shaping codon usage have been assessed using either genome-wide sets of predicted coding regions or subsets of highly expressed ribosomal proteins.<sup>7</sup> In non-model organisms for which complete genome sequences are not available, codon usage has often been estimated using expressed sequence tags<sup>4,8,9</sup> or organelle genomes,<sup>3</sup> providing clues about the mechanistic evolution of the nuclear and organelle genomes, respectively. From an evolutionary point of view, codon usage plays an important role in defining phylogenies and within-taxon or among-taxa variance.<sup>10</sup> Previous studies demonstrated the clade-specific molecular selection and codon adaptation of *rbcL*, which encodes the large subunit of ribulose 1,5-bisphosphate carboxylase, or RuBisCO.<sup>11</sup> Furthermore, chloroplast genomes replicate through a D-loop mechanism and transcribe genes in both the sense and antisense orientation, like the nuclear genome. Codon usage patterns and strand asymmetry have been linked to transcription selection. The highly expressed genes tend to be localized on the leading strand, thus creating a coding bias, which orients the directionality of replication and transcription.<sup>12</sup>

Recently, there has been considerable focus on the exploitation of organelles as a source of pharmaceuticals and as biofactories,<sup>13</sup> in which foreign genes are reverse engineered and overexpressed.<sup>14</sup> This paves the way for hyper-expression-based genetic engineering using either a single gene, such as Toc cyclase or  $\gamma$ -Toc methyltransferase in lettuce,<sup>15</sup> or an entire pathway, such as the mevalonate pathway in tobacco.<sup>14,16</sup> For efficient transgenic overexpression in chloroplasts, well-established protocols have been widely demonstrated<sup>17</sup> based on the alternations of codon usage patterns between organelle genomes, which have predominantly A-ending and/or U-ending codons, and nuclear genomes, which have predominantly G-ending, C-ending, or U-ending codons.<sup>18</sup> Despite the relevance of codon usage patterns in phylogenomics and the design of optimal gene-expression experiments, relatively few open-source repositories have been developed for the wide exploration of synonymous codon usage across organelle genomes. Only a few of the repositories in existence encompass even a modest range of taxa; most are either organism-centric, with compiled codon usage tables for microbial organisms,<sup>19</sup> or simple, single-organism, web-based displays of codon usage statistics (available at: [http://www.cmbi.uga.edu/software/codon\\_usage.html](http://www.cmbi.uga.edu/software/codon_usage.html)).

In the case of eukaryotes, the majority of the resources available to date are focused on either a single model organism, such as [http://crumb.stanford.edu/community/codon\\_usage.shtml](http://crumb.stanford.edu/community/codon_usage.shtml) for *Saccharomyces cerevisiae*, which limits the exploration of codon usage patterns across multiple species. It is worthwhile to mention that the Kazusa Codon Usage Database (<http://www.kazusa.or.jp/codon>) is the only repository that hosts the pre-compiled codon usage counts for a relatively large number of species, including the organelle genomes.<sup>20</sup> The latest update of the Kazusa database dates back to 2007 (NCBI-GenBank Flat File Release), which presents a challenge for the exploration and cross-species comparison of codon patterns. The Kazusa database also lacks

features such as the cross-comparison of organelle genomes and the comparison of codon usage patterns between user-submitted genes and the pre-compiled organelle genomes. Additionally, the Kazusa database contains a relatively small number of organelle genomes, limiting its ability to explore the recently published organelle genomes and the applicability of those genomes as transgenic vehicles. In a web report on the NCBI Genbank release, Richmond stated that one of the top priorities in codon-usage research was a necessary update of the pre-compiled codon tables, urging that such tables include data from non-model species.<sup>21</sup> In light of the limited resources available for the study of organelle genome-specific patterns of codon usage and the critical importance of those patterns to applications to enhance organelle-based bio-factories, multi-gene targeting, and recombinant protein expression<sup>22</sup>, we developed ChloroMitoCU, which provides simple access to patterns of codon usage in 328 chloroplast genomes (29,960 CDS) and 3,502 mitochondrial genomes (49,066 CDS). ChloroMitoCU allows the comparison of organelle-specific codon patterns across multiple species with the same translational table and also allows comparative assessments of the codon usage patterns between user-submitted transcripts or genes and the pre-compiled organelle genomes.

## 2. Material and methods

### 2.1 Genome data retrieval and codon usage patterns

We retrieved all of the chloroplast (328) and mitochondrial (3,502) genomes from the NCBI organelle genomes repository.<sup>23</sup> The coding regions of the respective genomes were downloaded and subsequently parsed for a length threshold. For the identification of the codon usage patterns, we maintained a threshold of 300 bp. We used CodonW (available from [codonw.sourceforge.net](http://codonw.sourceforge.net)) to identify the codon usage patterns in each of the genes with a minimum length of 300 bp. To identify the strand-specific codon usage patterns, genes localized on the positive (+) and negative (−) strands were separated based on the organelle genome annotation available in NCBI RefSeq. The current version of ChloroMitoCU contains 29,960 complete (full-length) protein-coding genes (CDSs) from chloroplasts and 49,066 CDSs from mitochondria. The relative synonymous codon usage (RSCU) index was calculated as previously described.<sup>3</sup> RSCU values represent the ratio between the observed usage frequency of a codon in a sample and the expected usage frequency if all of the codons in the synonymous codon family for the particular amino acid are used equally.<sup>3</sup> To predict potential CDSs in assembled transcripts or organelle genomes, we deployed a modified version of the Transdecoder prediction pipeline (available from <https://transdecoder.github.io>), which incorporates all of the available translational tables for wider predictions of CDSs across many organisms.

## 3. Results and discussion

### 3.1 Development and applications of ChloroMitoCU

For efficient access, ChloroMitoCU was developed and is hosted on a 64-bit Linux server pre-installed with Python version 2.7.3, Apache (<http://www.apache.org>), and PHP (<http://www.php.net/>). ChloroMitoCU runs all analyses in real time via Python modules (version 2.7.10), and the parsed results integrate with PHP modules to provide a web-based display. For each organelle genome, the codon information is further sorted into hierarchical levels of information such as the genomic GC content of the first, second, and third codon positions, the number of encoded proteins, the genome length, the number

ChloroMitoCU organelle specific codon usage database and webserver

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ChloroMitoCU provides a platform for the extensive and comparative exploration of the codon usage patterns across the organelles genomes including chloroplast and mitochondrial genomes. Enhancing the expression pattern through the selection of the appropriate codon usage patterns has been used for transforming several key traits for disease management including viral diseases (Daniell et al. 2003) and as edible vaccines (Trengging et al. 2003). Codon usage patterns also play an important role in studying the effect of the Nucleotide bias and gives an estimate of the selection pressure acting on the genome through gain or loss of mutations. ChloroMitoCU provides a comprehensive platform for more than 3000 organelle genomes from all domains of life covering 328 chloroplast and 49,666 mitochondrial genomes (Truu et al. 2019). ChloroMitoCU provides specific codon usage patterns for the chloroplast and the mitochondrial genomes using the translational tables. In addition, ChloroMitoCU provides additional webserver as an added utility for the estimation of the codon usage in accordance with the translational table in user uploaded sequence dataset. ChloroMitoCU supports the alternative translational tables for the estimation of the codon usage in the user uploaded sequence of model and non-model species.

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**Statistics**

Version of NCBI  
3,502 mitochondrial genomes  
49,666 CDS analyzed

328 chloroplast genomes  
29,960 of CDS analyzed

**Quick Search**  
Here we provide a quick search pattern for the chloroplast and mitochondrial genomes. On the basis of the selection of the category (Chloroplast or Mitochondrial genomes), the genomes category will be updated accordingly for the efficient browsing of the codon usage patterns.

Select a chloroplast genome  
Acidosa purpurea chloroplast [11]

Select a mitochondrial genome  
Abalates stellaris [2]

**Quick browsing of pre-compiled codon usage tables for 3,502 mitochondrial and 328 chloroplast genomes. The number in the parenthesis displays the translational tables used by the organism.**

**Strand specific evaluation of codon choices**

Positive strand Negative strand Both strands

**Browse ChloroCU**

Species	Acc.	Len	Prot	RNA	GC	GCC1	GCC2	GCC3	Translational codon table
<i>Acidosa purpurea</i>	NC_015820	139,697	82	46	0.3933	0.4748	0.3972	0.3079	11
<i>Acacia americana</i>	NC_018099	153,819	84	46	0.3885	0.4659	0.3872	0.3124	11
<i>Acacia catenata plastid</i>	NC_007967	153,821	84	46	0.3887	0.4657	0.3874	0.3130	11

Species page are hyperlinked to NCBI RefSeq record.

Mitochondrial and Chloroplast specific codon usage browsing along with the GC content at first, second and third codon position, number of proteins, RNA and the translational table for each species.

**Browse MitoCU**

Positive strand Negative strand Both strands

Species	Acc.	Len	Prot	RNA	GC	GCC1	GCC2	GCC3	Translational codon table
<i>Abalates stellaris</i>	NC_011942	16,502	13	24	0.4399	0.4731	0.4127	0.4339	2
<i>Abalates profunda</i>	NC_015799	15,277	13	24	0.1904	0.2165	0.2165	0.1443	5
<i>Alnus sepium</i>	NC_011520	16,953	13	28	0.1945	0.2167	0.2429	0.0820	5

-License Agreement: Please use this license agreement to obtain a local copy of the ChloroMitoCU.

**Figure 1.** The efficient browsing system of ChloroMitoCU.

of tRNAs, and the usage patterns and relative frequencies of codons (Fig. 1). Figure 1 shows the user-friendly interface of ChloroMitoCU, which offers a two-way search for browsing chloroplast-specific codon usage patterns (Browse ChloroCU) and mitochondria-specific codon usage patterns (Browse MitoCU). In addition, a dropdown menu provides the alphabetically sorted list of the chloroplast and mitochondrial genomes with associated translational codes. Lastly, all of the alphabets in the quick-search forms are hyperlinked to the corresponding codon usage-pattern page of the selected species.

For each organism and organelle, ChloroMitoCU hosts organism-specific and organelle-specific web layouts, which display the species name and corresponding translational table, the amino acid organization, information about synonymous codons from the species-specific translational table (Fig. 1). Organism specific webpages in both Browse ChloroCU and Browse MitoCU displays the species-specific distribution of GC content in the CDSs at the first, second, and third positions, and color-coded codons display of the RSCU values, with stop codons indicated in white and marked by asterisk (\*) (Fig. 2). Additionally, it displays the summary of the codon usage statistics with information on the synonymous codons as per the species specific translational table (Fig. 2). Finally, the last module in each organelle-specific or organism-specific web page in Browse ChloroCU and BrowseMitoCU provides real-time comparisons of the codon usage-frequency profiles of the user-selected plastome and any phylogenetically related chloroplast genome (Fig. 3). The inference of RSCU values can help the end-user to investigate the codon usage independently of the amino acid composition. RSCU values  $> 1$  (red horizontal bars), as compared with those  $< 1$  (green horizontal bars), for a codon imply that the

codon is used more frequently than expected based on the amino acid content of the gene or genome (Fig. 2).

An additional module (Fig. 4) implemented in ChloroMitoCU allows the user to either analyze the CDSs for codon usage patterns or predict the CDSs from stranded or non-stranded assemblies or assembled genes. It further allows the user to select the translational table according to the organelle and also to select between complete CDS predictions or all reported CDSs within the assembled regions. The results page of the prediction module allows the comparative assessment of the codon usage patterns across other pre-embedded organelle genomes using the same codon translational table. The Python module (version 2.7.10) allows the user to simultaneously view the codon usage patterns of the user-input gene of interest and the organelle-specific codon patterns of the selected species in order to understand the codon-specific patterns according to the specific organelle of interest.

### 3.2 Functional genomics and evolutionary applications of ChloroMitoCU

Functional genomics is at the forefront of the accelerated use of plants as models for antibody expression and oral vaccine development.<sup>24</sup> Recent reports demonstrate the role of codon optimization in the enhancement of chloroplast gene expression and translation rates.<sup>25</sup> Taking that perspective into account, ChloroMitoCU provides comparative analysis of the codon usage patterns of a wide range of chloroplast genomes, which holds great potential for the optimization of gene expression and for heterologous gene expression. Recently, the investigation of a hypothesis coupling codon usage and

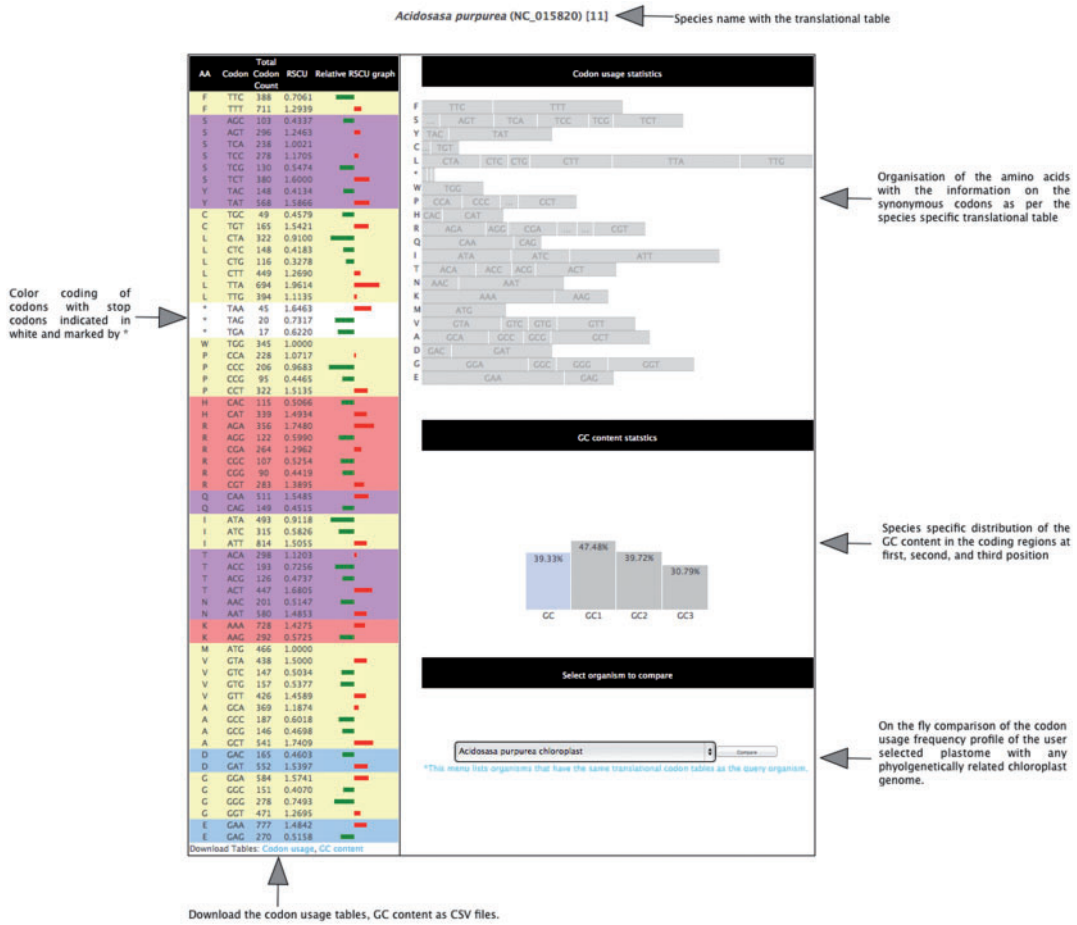


Figure 2. Graphical visualization of the codon counts and real time comparison of the codon counts across several species for comparative assessment across phylogenies.

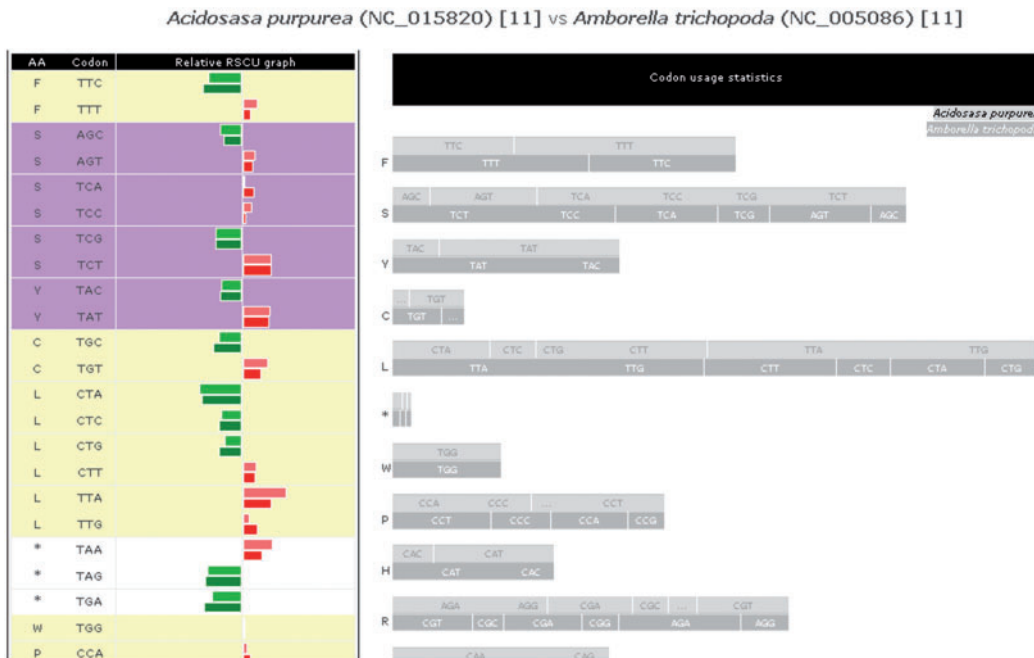


Figure 3. Comparative profiling of the codon usage tables across two organelle genomes.

The screenshot shows the ChoroMitoCU web interface with several sections and annotations:

- Input sequence type:**
  - CDS sequences (Annotation: ← User input coding regions)
  - Assembled organelle sequences:
    - Strand-specific (Annotation: ← User input assembled regions (strand specific or Non-strand specific))
    - Non strand-specific
- Starts with:**
  - Methionine
  - Unusual start codon (according to the codon table) (Annotation: ← Selection of the start codon)
- Dataset:**
  - Complete CDS only (Annotation: ← Selection of the predicted coding regions for the estimation of the codon usage profile of the user input assembled regions)
  - All predicted CDS (the Sprimer partial, internal, 3primer partial and the complete CDS)
- Translational codon table:** A dropdown menu set to '1'.
- Type:**
  - Chloroplast
  - Mitochondrial (Annotation: ← Selection of the Translational tables and the orgarnism type)
- Length Filter:** A dropdown menu set to 'All' (Annotation: ← length filter for the predicted coding regions).
- File Upload:** A 'Choose file' button with the text 'no file selected' and 'Please select a file containing the fasta sequences for uploading (Example file)'. Below it is an 'Upload' button.

**Figure 4.** On-the-fly prediction of CDSs from assembled organelle genome fragments and estimation of the codon counts, taking into account the translational codon table.

tRNA import in the unicellular model algae, *Chlamydomonas reinhardtii*, suggested that co-evolution is the mechanism underlying the dynamic import of tRNAs and that the hypothesis could be adapted to explain the composition of the mitochondrial genome.<sup>26</sup> Comparative analyses of base composition revealed a varying level of mutational pressure acting upon chloroplast and nuclear genes<sup>18</sup> however, base frequencies at fourfold-degenerate sites in mitochondrial genomes displayed a strong context-dependent mutational pattern, indicating correlations between pairs of neighbouring bases.<sup>27</sup> Last, taking into account the relative differences in the strand-specific codon usage patterns in organelle genomes, especially in mitochondrial genomes,<sup>27</sup> ChoroMitoCU, provide the strand specific browsing of the codon usage patterns in both organelle genomes (chloroplast and mitochondria) (Fig. 1) to understand the evolutionary role of the strand asymmetry in shaping the codon usage of the user interested organelle and to infer the difference in strand-specific mutational biases.

Comparative analyses with nuclear genes revealed the dominance of variable patterns of evolutionary constraints, particularly in chloroplast and mitochondrial genes, which were found to be influenced by the translation level.<sup>28</sup> The deciphering of species-specific patterns of codon usage has effectively paved the way for the enhancement of heterologous gene translation in several model and non-model organisms<sup>29,30</sup>. Comparative assessments of codon usage patterns are important for understanding patterns of codon preferences. For this reason, we implemented an additional module in ChoroMitoCU that allows assessment of two organelle genomes if the genomes share the same codon table. When compared with the previously developed Kazusa database (<http://www.kazusa.or.jp/codon/>), ChoroMitoCU provides an easier way to access pre-compiled codon usage tables and has several added functionalities.

#### 4. Conclusion

ChoroMitoCU provides curated information of codon usage patterns in organelle genomes and allows for the comparative assessment of those patterns across organelle genomes. ChoroMitoCU also offers the prediction of CDSs and the comparative assessment of codon usage between the predicted CDSs and the embedded organelle genomes. In the future, ChoroMitoCU will be updated as per the NCBI RefSeq release, and the newly reported chloroplast and mitochondrial genes,

and their corresponding patterns of codon usage, will be incorporated. The enhancement of expression through the selection of appropriate codon usage patterns has been used to transform several key traits to manage diseases, including viral diseases and to create edible vaccines.<sup>29</sup> We believe that ChoroMitoCU will serve as a standard platform to enhance organelle genomics and gene expression and to understand the evolution of the various codon usage patterns in relation to the genomic compositions across the Kingdoms of life.

#### Acknowledgements

GS thanks Department of Biodiversity and Molecular Ecology, Research and Innovation Centre, Fondazione Edmund Mach, Via E. Mach 1, 38010 S. Michele all' Adige (TN), Italy for providing the facility for computational analysis and GS thanks Petrus Tang for supporting for the open access fees.

#### Authors' contributions

G.S., C.V. conceived and designed the research; G.S. and T.W.C. performed the re- search; C.C.L., R.C.G., P.J.H., and P.T. contributed to the design of the web server. G.S. wrote the article, and K.C.N, J.W and N.L.P provided revisions. All authors have read and approved the article.

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