

TRAF3 and NBR1 both influence the effect of the disease-causing CYLD(Arg936X) mutation on NF- κ B activity

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Abstract

Recently described Hungarian and Anglo-Saxon pedigrees that are affected by CYLD cutaneous syndrome (syn: Brooke-Spiegler syndrome (BSS)) carry the same disease-causing mutation (c.2806C>T, p.Arg936X) of the *cylindromatosis* (CYLD) gene but exhibit striking phenotypic differences. Using whole exome sequencing, missense genetic variants of the *TRAF3* and *NBR1* genes were identified in the affected family members of the Hungarian pedigree that are not present in the Anglo-Saxon pedigree. This suggested that the affected proteins (TRAF3 and NBR1) are putative phenotype-modifying factors. An in vitro experimental system was set up to clarify how wild type and mutant TRAF3 and NBR1 modify the effect of CYLD on the NF- κ B signal transduction pathway. Our study revealed that the combined expression of mutant CYLD(Arg936X) with TRAF3 and NBR1 caused increased NF- κ B activity, regardless of the presence or absence of mutations in TRAF3 and NBR1. We concluded that increased expression levels of these proteins further strengthen the effect of the CYLD(Arg936X) mutation on NF- κ B activity in HEK293 cells and may explain the phenotype-modifying effect of these genes in CYLD cutaneous syndrome. These results raise the potential that detecting the levels of TRAF3 and NBR1 might help explaining phenotypic differences and prognosis of CCS.

KEYWORDS

Brooke-Spiegler syndrome, CYLD cutaneous syndrome, CYLD gene, NBR1 gene, NF κ B activation, TRAF3 gene

1 | BACKGROUND

CYLD cutaneous syndrome (CCS) is characterized by the development of a wide variety of skin appendage tumors, such as cylindromas, trichoepitheliomas and spiradenomas, and are inherited in an autosomal dominant manner. Historically, descriptive names, including Brooke-Spiegler syndrome (BSS; OMIM 605041), familial

cylindromatosis (FC; OMIM: 132700), and multiple familial trichoepithelioma (MFT; OMIM: 601606), were assigned on the basis of the predominant tumor type and location, but were recently recognized to be a clinical spectrum of allelic conditions driven by CYLD pathogenic variants.^{1,2}

CYLD was localized to chromosome 16q12-13 by genetic linkage analysis and fine mapping.^{3,4} It encodes a 956 aa tumor suppressor that

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contains three Cap-Gly domains involved in protein–protein interactions and has a ubiquitin carboxyl-terminal hydrolase activity, with a preferential targeting of Lys-63 (K63) linked and Met1 ubiquitin chains.⁵⁻⁷

The main function of CYLD is to negatively regulate activation of NF- κ B mediated by the tumor necrosis factor receptor (TNFR). The most important protein interaction partners of CYLD are NF- κ B essential modulator (NEMO) and tumor necrosis factor receptor-associated factor 2 (TRAF2), both of which are target substrates of the deubiquitinating activity of CYLD.^{8,9}

Distinct pathogenic variants in *CYLD* have been reported in >100 families; strikingly some unrelated pedigrees with the same mutation have been observed to develop symptoms with different severity.¹⁰⁻¹³ One such hotspot is the disease-causing (rs121908390, c.2806C>T, p. Arg936X) pathogenic variant, resulting in a truncated form of the protein (p. Arg936X) in which the ubiquitin-specific processing protease histidine box is deleted.¹⁴⁻¹⁶ A recent study of two families of Hungarian and Anglo-Saxon origins carrying this truncating mutation demonstrated that the same disease-causing mutation can lead to striking differences in severity of disease.¹⁶ To understand how the same underlying mutation results in differing symptoms in the two families, whole exome sequencing was carried out to identify the possible phenotype-modifying genetic factors causing the observed clinical differences.¹⁷ Comparing the data obtained from the two families Pap et al¹⁷ identified three polymorphisms as potential phenotype-modifying variants: the rs1053023 SNP of the *signal transducer and activator of transcription 3 (STAT3)* gene, the rs1131877 SNP of the *tumor necrosis factor receptor-associated factor 3 (TRAF3)* gene and the rs202122812 SNP of the *neighbour of BRCA1 gene 1 (NBR1)* gene. While the *STAT3* variant is located in the 3' untranslated region, the *TRAF3* and *NBR1* variants cause amino acid changes in the protein.

Tumor necrosis factor receptor-associated factor 3 is a ubiquitously expressed protein that functions as an E3 ubiquitin ligase, which ubiquitinates target proteins, either altering their function or targeting them for proteasomal degradation. TRAF3 is a constitutive negative regulator of both canonical and alternative NF- κ B pathway signalling and also acts as a positive regulator of type I interferon production and, thus, is a critical regulator of both innate and adaptive immune responses.¹⁸

The *NBR1* protein is also ubiquitously expressed and is associated with cellular autophagy signalling pathways. *NBR1* interacts with ubiquitin via the ubiquitin-associated (UBA) domain and has been proposed to function as a cargo adapter for autophagic degradation of ubiquitinated substrates.¹⁹

The aim of our study was to determine whether the identified genes and their variants modify CYLD-mediated signal transduction processes.

2 | QUESTIONS ADDRESSED

We examined the possible association of the *TRAF3* rs1131877 variant and the *NBR1* rs202122812 variant with the phenotypic diversity of BSS. Functional studies of the causative *CYLD* mutation and of the previously predicted¹⁷ phenotype-modifying genetic factors

were performed to determine the effect of genetic interactions and their role in modifying the phenotype.

We set up an in vitro experimental system and carried out functional tests to answer the following question:

What are the molecular and cellular consequences of the combined presence of the identified polymorphisms and how do they modify the effect of the *CCS* germline *CYLD*(Arg936X) mutation?

3 | EXPERIMENTAL DESIGN

To clarify the contribution of the identified polymorphisms to modifying *CYLD*'s role in the disease, an in vitro experimental system was designed. The main role of *CYLD* is the regulation of TNFR-mediated NF- κ B activation. To monitor NF- κ B activity, HEK293 cells were co-transfected with the pNF κ B-luc Cis-Reporter plasmid (StrataGene), the pGL4.75 [hRLuc/CMV] plasmid (Promega), which was used as internal control, and different combinations of the pcDNA3.1(+) vector (Invitrogen) carrying the wild type or mutant *CYLD*, *TRAF3* and *NBR1* cDNAs fused with tag sequences, which were added for easy detection by western blot. The viability of the HEK293 cells was not affected by the transfection with any combinations of the plasmids as it was assessed by MTT assay (Figure S1) and followed by regular microscopic control. The expression levels of *CYLD*, *TRAF3* and *NBR1* proteins were determined by western blot analyses.

Detailed description on the materials and methods are found in Appendix S1.

4 | RESULTS

4.1 | Overexpression of *CYLD*(Arg936X) induces higher NF- κ B activation

To assess how wild type (WT) or mutant *CYLD*, *TRAF3* and *NBR1* affects NF- κ B signalling, HEK293 T cells were co-transfected with plasmids harbouring WT or mutant 3xHA-*CYLD*, *TRAF3-HFC* or *NBR1-HFC* fusion constructs together with an NF- κ B firefly-luciferase reporter construct and an internal control plasmid expressing Renilla luciferase. Luciferase activities were measured 24 h after transfection, as described previously.^{20,21} Cells transfected with the empty vector pcDNA3.1(+) and luciferase vectors were used as controls in each experiment.

Overexpression of the WT proteins did not change NF- κ B-induced luciferase activity, indicating that, in the cells, increased levels of these proteins do not affect NF- κ B activity (Figure 1A). In contrast, overexpression of the *CYLD*(Arg936X) mutant protein resulted in an approximately threefold increase in NF- κ B activity. Overexpression of *TRAF3*(Met129 Thr) and *NBR1*(Arg306Gln) mutant proteins alone had no effect (Figure 1A).

To detect the overexpression levels of the different proteins, western blot analyses were carried out. All of the transfected proteins were detected in the transfected cells (Figure 1B).

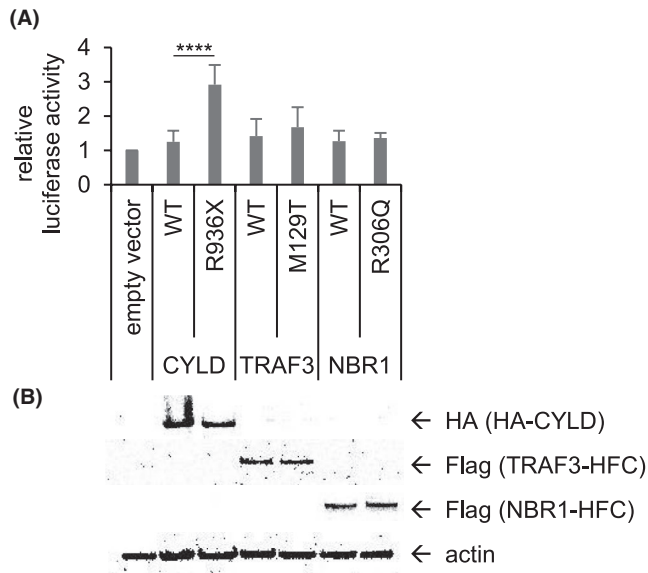


FIGURE 1 Overexpression of *CYLD*(Arg936X) induces higher NF- κ B activation. HEK293 cells were co-transfected with empty vector or plasmid constructs containing 3x-haemagglutinin (HA)-tagged wild type (WT) or Arg936X mutant *CYLD*, C-terminal His-Flag (HFC)-tagged WT or Met129 Thr mutant *TRAF3* or HFC-tagged WT or Arg306Gln mutant *NBR1*, together with an NF- κ B responsive firefly-luciferase reporter construct and a Renilla luciferase construct as an internal control. (A) Firefly luciferase activity was normalized to Renilla luciferase activity, and normalized luciferase activities were compared to the normalized luciferase activity of the empty vector transfected control samples. Statistical analysis was carried out by ANOVA and post hoc testing to compare luciferase activities of samples transfected by each mutant construct to their corresponding WT-transfected sample pairs. Data are represented as mean \pm SD, $n = 3$, **** $p < 0.0001$. (B) Overexpression of the tagged proteins was demonstrated by western blot. To overcome possible differences between experiments, the same nitrocellulose membrane was probed with all antibodies, stripping the antibodies between. Anti-HA was used for visualization of *CYLD*, anti-FLAG was used for visualization of *TRAF3* and *NBR1*, and anti-human-actin antibodies were used to demonstrate equal loading. Data is representative for three independent experiments

4.2 | Wild type and mutant *TRAF3* or *NBR1* further increase the effect of *CYLD*(Arg936X) mutation on NF- κ B activity in HEK293 cells

In a similar experimental setup, pairwise co-expression of WT *CYLD* with either WT or mutant sequences of *TRAF3* or *NBR1* resulted in no difference in NF- κ B activity as compared to the control. In addition, levels in these experiments were similar to levels observed when these proteins were overexpressed alone. However, co-expression of both WT or mutant *TRAF3* with *CYLD*(Arg936X) resulted in a further 2 to 2.5-fold increase in NF- κ B activity compared to the levels observed when mutant *CYLD*(Arg936X) was expressed alone (Figure 2A). Similarly, co-expressing *CYLD*(Arg936X) with *NBR1* resulted in 1.5- to 2-fold higher NF- κ B activity, regardless of whether the WT or mutant *NBR1* sequence was used (Figure 2C).

Co-expression as detected by western blot of the combinations of *CYLD* and *TRAF3* or *CYLD* and *NBR1* proteins resulted in similar expression levels to those observed when the constructs were expressed individually (Figure 2B,D).

4.3 | The combination of *CYLD*(Arg936X), *TRAF3*(Met129 Thr) and *NBR1*(Arg306Gln) mutations does not cause further NF- κ B activation

Co-transfection of HEK 293 T cells with WT *CYLD* combined with any other plasmid construct resulted in no difference in NF- κ B activity. The combined expression of *CYLD*(Arg936X) both with *TRAF3* and *NBR1* caused increased NF- κ B activity, regardless of the latter two proteins were WT or mutant. However, the measured activity never exceeded the levels detected in the cells transfected with pairwise combinations of mutant *CYLD* and *TRAF3* or *NBR1* constructs (Figure 3A).

Similar to the pairwise combinations, in cells co-expressing all three transgenes (*CYLD*, *TRAF3* and *NBR1*), protein levels were the same as in cells expressing only one construct (Figure 3B).

5 | DISCUSSION AND CONCLUSIONS

The phenotypic variation in CCS is incompletely explained by variation in *CYLD* itself, and there is a clinical need to delineate modifier genes that may help prognosticate disease severity for affected patients. Recently, unrelated family members of pedigrees of Hungarian and Anglo-Saxon origin affected by CCS were found to carry the same disease-causing mutation (c.2806C>T, p. Arg936X) of the *CYLD* gene, but exhibited striking differences in their phenotypes.¹⁶ In this work, we aimed to reveal the putative phenotype-modifying effects of polymorphisms of the *TRAF3* and *NBR1* genes, which were previously identified based on whole exome sequencing of the Hungarian and Anglo-Saxon family members.¹⁷

The rs1131877 common SNP (global MAF = 0.266) of the *TRAF3* gene causes an amino acid change (Met129 Thr) on the N-terminal part of the *TRAF3* protein which exhibit homology with RING domains found in many E3 ubiquitin ligases and is important for ligase activity.²² The role of *TRAF3* in cellular signalling is mediated mostly by direct interactions with various receptors to transmit the receptor signals to downstream proteins. The most prominent downstream interactor of *TRAF3* is the TRAF-interacting protein (TRAIPI), which is also an interacting partner of *CYLD*.²³ One of the main functions of both *CYLD* and *TRAF3* proteins is the negative regulation of non-canonical NF- κ B activity.²⁴ It has also been described that mutations of *CYLD* and *TRAF3*, identified in different type of cancers, together can lead to constitutive activation of NF- κ B pathway.²⁵ Moreover, the rs1131877 SNP of the *TRAF3* gene is highly predictive for the development of grade ≥ 2 acute esophageal postradiotherapy toxicity,²⁶ and its association with the phenotypic diversity of CCS has been recently predicted.¹⁷

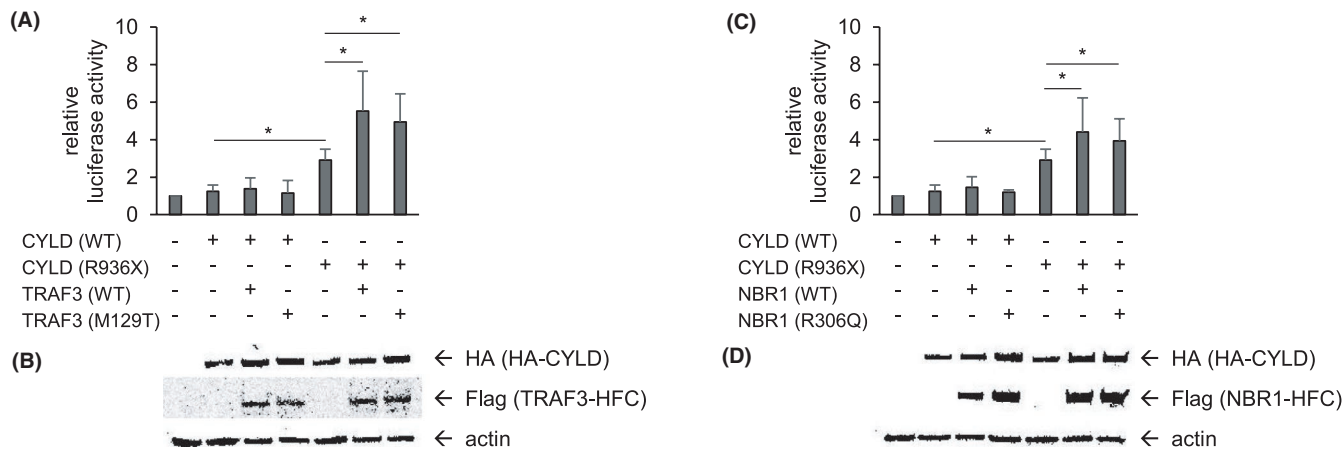


FIGURE 2 The combined effect of CYLD(Arg936X) and TRAF3(Met129 Thr) or CYLD(Arg936X) and NBR1(Arg306Gln) mutations on NF- κ B signalling. HEK293 cells were co-transfected with an NF- κ B responsive firefly-luciferase reporter construct and a Renilla luciferase construct as an internal control, in pairwise combination with HA-tagged WT or Arg936X mutant CYLD either with HFC-tagged WT or Met129 Thr mutant TRAF3 (A, B), or with HFC-tagged WT or Arg306Gln mutant NBR1 plasmid constructs (C, D). Co-transfection with the empty pcDNA3.1(+) vector served as control. (A, C) Firefly luciferase activity was normalized to Renilla luciferase activity, and normalized luciferase activities were compared to the normalized luciferase activity of the empty vector transfected control samples. Statistical analysis was carried out by ANOVA and post hoc testing. Data are represented as mean \pm SD, $n = 3$, $*p < 0.05$. (B, D) Overexpression of the tagged proteins was demonstrated by western blot. To overcome possible differences between experiments, the same nitrocellulose membrane was probed with all antibodies, which were stripped between analyses. Anti-HA was used for visualization of CYLD, anti-FLAG was used for visualization of TRAF3 and NBR1, and anti-human-actin antibodies were used to demonstrate equal loading. Data is representative for three independent experiments

These data supported our hypothesis that the identified TRAF3 polymorphism may act as a phenotype-modifying genetic factor in CCS and prompted us to test this in an in vitro system.

Our results interestingly show that the increased level of TRAF3 protein resulted in higher NF- κ B activity only in cases when mutant CYLD(Arg936X) protein was also overexpressed in the cells. We observed this phenomenon when either WT or mutant TRAF3 protein was co-expressed with the mutant CYLD(Arg936X). Our data indicate that increased levels of TRAF3 are important for higher NF- κ B activity, regardless of the presence of the polymorphism, and higher levels of TRAF3 potentiate the effect of the CYLD(Arg936X) mutation. This synergistic effect might be mediated through the interaction of both CYLD and TRAF3 with TRAIIP. TRAIIP is an E3 ubiquitin ligase and inhibits TNF α mediated NF- κ B activation in a manner similar to CYLD.²³ The interaction between CYLD, TRAF3 and TRAIIP proteins might mutually influence the ubiquitination levels of the three proteins, leading to increased NF- κ B activity. Besides this, elevated expression level of TRAF3 was shown to predispose for salivary gland tumors²⁷ where decreased CYLD expression and function has also been detected.^{28,29} These findings together with our current results suggest an interplay of higher TRAF3 expression and decreased CYLD functions in CCS, but might also be of interest in other cancer types.

The identified rare polymorphism of the NBR1 gene (rs202122812, global MAF = 0.0005) causes an amino acid change (Arg306Gln), but it does not affect any domain with annotated function, and it has not been associated with any human diseases previously. However, NBR1 is a functional homologue of the sequestosome1 (SQSTM1) protein, also known as the ubiquitin-binding protein p62.³⁰ SQSTM1 functions as a bridge between polyubiquitinated cargo and autophagosomes,

and it may regulate the activation of NF- κ B by TNF α . Moreover, the interaction of CYLD with TRAF proteins that drives the deubiquitinating activity of CYLD is reduced in the absence of SQSTM1.³¹ CYLD has several putative direct targets in the NF- κ B pathway, including NEMO, TRAF2, transforming growth factor- β activated kinase 1 (TAK1), and TRAF6.^{5,6,8,32} Deubiquitinating these molecules, particularly NEMO by CYLD, results in decreased NF- κ B activity.⁶

Our experiments revealed that higher NBR1 levels further increase the NF- κ B activity only in cells expressing the mutant CYLD(Arg936X) protein to similar extents, regardless of whether the WT or mutant NBR1 sequence was used. Based on these data, we conclude that NBR1 may also be a modifier of CYLD function in the regulation of NF- κ B activity. We hypothesize that the effect of the CYLD mutation and the increased NBR1 level might both lead to decreased CYLD deubiquitinase activity on the NEMO protein and, as a consequence, increased NF- κ B activity. Moreover, accumulation of NBR1 protein has been observed in breast cancer. Elevated NBR1 expression can prevent autophagy thus elicit metastasis in cancer cells indicating a more general role of NBR1 in tumorous diseases.³³

Since co-expression of mutant CYLD(Arg 936X) with either WT or mutant sequences of the TRAF3 and NBR1 proteins in triple combinations did not increase NF- κ B activity beyond NF- κ B levels of double combinations, we assume that there is no cooperation between TRAF3 and NBR1 in modifying the effects of the mutant CYLD(Arg936X).

These results underline the importance of functional studies to clarify the significance of putative phenotype-modifying factors. Although previous WES results suggested the involvement of TRAF3(Met129 Thr) and NBR1(Arg306Gln) in affecting the effect

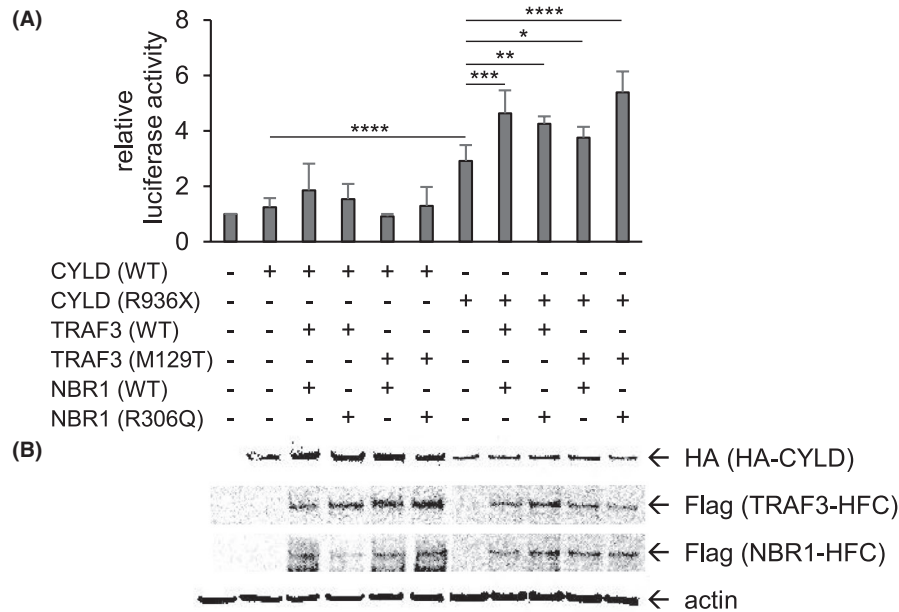


FIGURE 3 The combined effect of CYLD(Arg936X), TRAF3((Met129 Thr) and NBR1(Arg306Gln) mutations on NF- κ B signalling in HEK293 cells. HEK293 cells were co-transfected with an NF- κ B responsive firefly-luciferase reporter construct and a Renilla luciferase construct as an internal control, in the triple combinations of HA-tagged WT or Arg936X mutant CYLD with HFC-tagged WT or Met129 Thr mutant TRAF3, or with HFC-tagged WT or Arg306Gln mutant NBR1 plasmid constructs. Co-transfection with the empty vector pcDNA3.1(+) served as control. (A) Firefly luciferase activity was normalized to Renilla luciferase activity, and normalized luciferase activities were compared to the normalized luciferase activity of the empty vector transfected control samples. Statistical analysis was carried out by ANOVA and post hoc testing. Data are represented as mean \pm SD, $n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (B) Overexpression of the tagged proteins was demonstrated by western blot. Samples transfected with plasmid combinations of WT or Arg936X CYLD constructs were run on separate SDS-PAGE gels, blotted, handled and visualized the same way in the same experiment. To overcome possible differences between experiments, the same nitrocellulose membrane was probed with all antibodies and stripped between analyses. Anti-HA was used for visualization of CYLD, anti-FLAG was used for visualization of TRAF3 and NBR1, and anti-human-actin antibodies were used to demonstrate equal loading. Data is representative for three independent experiments

of the CYLD(Arg936X) in CCS,¹⁷ our functional analyses revealed no role for these variants, but suggested rather the importance of the abundance of TRAF3 and NBR1 next to the germline CYLD(Arg936X) mutation in CCS. Our results clearly demonstrate that only detailed analyses of the role of a protein can reveal clinical utility for explaining phenotypic differences and possibly foresee its effect in the prognosis of the disease. In addition, characterizing these correlations may promote the understanding of their mechanisms and may hopefully contribute to the development of future therapeutic modalities.

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CONFLICT OF INTERESTS

The authors have declared no conflicting interests.

AUTHOR CONTRIBUTIONS

ÉÁ contributed to conception, design, analysis and interpretation and drafted the manuscript. JD contributed to design, analysis and interpretation and critically revised the manuscript. EK carried out experiments. MS contributed to the concept, interpretation, and critically revised the manuscript. NR and NN contributed to the conception and critically revised the manuscript. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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SUPPORTING INFORMATION

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

Appendix S1. Supplementary materials and methods.

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