**Introducing FRET-based biosensors for the analysis of
cAMP-PKA signaling in the fungal pathogen *Candida
glabrata***

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1 **Introducing FRET-based biosensors for the analysis of cAMP-PKA signaling in the fungal**
2 **pathogen *Candida glabrata***

4 Running title:

5 **Biosensor-based analysis of *C. glabrata* cAMP-PKA pathway**

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Summary

The cAMP-PKA pathway is central to signal transduction in many organisms. In pathogenic fungi such as *Candida albicans*, this signaling cascade has proven to be involved in several processes, such as virulence, indicating its potential importance in antifungal drug discovery. *Candida glabrata* is an upcoming pathogen of the same species, yet, information regarding the role of cAMP-PKA signaling in virulence is largely lacking. To enable efficient monitoring of cAMP-PKA activity in this pathogen, we here present the usage of two FRET-based biosensors. Both variations in the activity of PKA and the quantity of cAMP can be detected in a time-resolved manner, as we exemplify by glucose-induced activation of the pathway. We also present information on how to adequately process and analyze the data in a mathematically correct and physiologically relevant manner. These sensors will be of great benefit for scientists interested in linking the cAMP-PKA signaling cascade to downstream processes, such as virulence, possibly in a host environment.

34 Introduction

35 The limited arsenal of antifungal therapies that is available and the increasing occurrence of resistance
36 against these drugs hamper efficient eradication of fungal infections. The consequences for the patient
37 as well as the economy are substantial. *Candida* species are the most common cause of fungal
38 invasive infections and the fourth- and sixth-most common cause of bloodstream-associated
39 infections in the United States and Europe, respectively (Mean *et al.*, 2008). Also in Asian countries,
40 such as China, these infections are a very significant problem (Chen *et al.*, 2018). The relative
41 frequency with which *Candida glabrata* is encountered in infections is increasing steadily compared
42 to the other *Candida* species. Over the period of 1989 to 1999, the incidence of *C. glabrata* in
43 bloodstream-associated infections has more than doubled (Trick *et al.*, 2002).

44 Fungal pathogens of the *Candida* genus can elicit a wide range of virulence factors,
45 depending on the human or animal niche in which they reside. Environmental elements are sensed by
46 the fungal cells and signal to specific downstream events. One of the signaling pathways central to
47 virulence in many fungal pathogens is the protein kinase A (PKA) pathway (Choi *et al.*, 2015; Hogan
48 and Sundstrom 2009; Kronstad *et al.*, 2011). Very little is known, however, about the involvement of
49 this pathway in *C. glabrata* pathogenesis. Additionally, the commonly-used methods to determine
50 PKA activity or the level of the secondary messenger, cyclic adenosine monophosphate (cAMP),
51 suffer from their mostly indirect way of reporting (Colombo *et al.*, 2017; Colombo *et al.*, 1998;
52 Griffioen *et al.*, 1996; Martinez-Pastor *et al.*, 1996; Pernambuco *et al.*, 1996; Schepers *et al.*, 2012).
53 These techniques are often expensive, time-consuming or unable to signal events in an *in vivo* and
54 dynamic way. The lack of proper tools for in-depth and efficient analysis of the cAMP-PKA pathway,
55 slows down fundamental research and, by extension, the potential of using the acquired knowledge in
56 drug discovery. To overcome the limitations of existing read-outs, we aimed to establish state-of-the-
57 art tools for determination of cAMP-PKA pathway activity in *C. glabrata* (Allen and Zhang 2006;
58 Colombo *et al.*, 2017; Nikolaev *et al.*, 2004). We present the use of functional fluorescence resonance
59 energy transfer (FRET)-based biosensors for determination of PKA activity and the level of the
60 secondary messenger cAMP in the fungal pathogen. Using the AKAR3 and EPAC2 biosensors, we
61 confirm that stimulation of starved cells with glucose increases the level of cAMP and activates PKA,

indicating that, similar to the model yeast *Saccharomyces cerevisiae*, glucose is one of the environmental factors triggering this pathway. The established FRET-based biosensors will allow fast and adequate analysis of the cAMP-PKA pathway and thorough examination of its constituents as potential targets for new antifungal drugs.

Results and discussion

Generation of C. glabrata optimized plasmids containing EPAC2 and AKAR3 sequences

We transferred the sequence encoding the AKAR3 and EPAC2 sensors from the pYX212-CFP-AKAR3-cpVenus and pYX212-YFP-EPAC2-CFP plasmids (Colombo *et al.*, 2017) to a *C. glabrata* compatible plasmid, pESg03, behind the *CgPGK1* promoter. The resulting plasmids pLDg01 and pLDg02, containing the *AKAR3* and *EPAC2* sequences, respectively, were further adapted by replacing the *CgHIS3* marker for the dominant *hphNT1* gene, resulting in plasmids pLDg03 and pLDg04. These plasmids allow for monitoring of PKA activity and cAMP levels in virtually all types of background strains, because specific auxotrophies are not required. Details on the experimental properties are given in the Experimental procedure section of this manuscript.

Glucose-induced activation of PKA in C. glabrata using AKAR3

The AKAR3 biosensor monitors PKA activity in a living cell. Upon phosphorylation of the sensor domain and subsequent binding to the ligand, the FRET donor and acceptor approach each other, thereby increasing the transfer of energy (Figure 1A). We used ratiometric FRET (FRET_R) monitoring to follow activation of PKA. The FRET_R response of AKAR3-transformed *C. glabrata* cells was monitored upon re-addition of glucose to glucose-starved cells. We grew the strains expressing the sensors in synthetic complete SC_{glu} medium containing glucose and hygromycin for 24 hours and collected the cells. After washing the cell pellet three times with MES buffer, it was resuspended in SC medium without glucose at an OD₆₀₀ of 2 and incubated for another two hours at 30°C. The cells were loaded in a microtiter plate of the CellASIC® ONIX2 microfluidics device of Merck. Imaging of the FRET donor, CFP, and the FRET acceptor, cpVenus, was performed continuously. After ten minutes of imaging under continuous SC medium (without glucose) flux, the same medium with 2%

glucose was perfused. Images were acquired continuously for another 30 minutes. The FRET_R values were calculated for every time point using the following formulas⁽¹⁾.

$$FRET_R = \frac{FRET^F - Corr(FRET^F)}{Donor^F - Corr(Donor^F)} \tag{1}$$

$$Corr(FRET^F) = FRET^{EV} - Bgd(FRET^{EV}) + Bgd(FRET^F)$$
$$Corr(Donor^F) = Donor^{EV} - Bgd(Donor^{EV}) + Bgd(Donor^F)$$

The following abbreviations and terms were used in the formulas:

<i>FRET_R</i>	Acceptor/donor emission upon donor excitation
<i>FRET^F</i>	Acceptor signal of FRET sample, donor excitation and acceptor emission
<i>Donor^F</i>	Donor signal of FRET sample, donor excitation and donor emission
<i>Corr(FRET^F)</i>	Correction factor for FRET sample, donor excitation and acceptor emission
<i>Corr(Donor^F)</i>	Correction factor for FRET sample, donor excitation and donor emission
<i>FRET^{EV}</i>	Acceptor signal of control sample, donor excitation and acceptor emission
<i>Donor^{EV}</i>	Donor signal of control sample, donor excitation and donor emission
<i>Bgd(FRET^{EV})</i>	Background signal control sample, donor excitation and acceptor emission
<i>Bgd(FRET^F)</i>	Background signal FRET sample, donor excitation and acceptor emission
<i>Bgd(Donor^{EV})</i>	Background signal control sample, donor excitation and donor emission
<i>Bgd(Donor^F)</i>	Background signal FRET sample, donor excitation and donor emission
<i>EV</i>	Empty vector control sample

Figure 1C shows the results obtained for the FRET_R ratio, after correction for background and autofluorescence. It can be appreciated that the FRET_R signal of the AKAR3 biosensor increases with approximately 11% relative to the basal level (after 3.7 min) after glucose addition, indicating an increase in PKA activity. The FRET_R signal gradually declines over time. It is, however, not the amplitude of the FRET_R curve that reflects the actual PKA activity in the cell. Since these sensors are saturated rather quickly, the enzyme activity should be deduced from the sigmoid gain of the curve (Kluba *et al.*, 2015). The faster the maximal level is reached, the higher the PKA activity. To

determine the sigmoid gain of the curve, we fitted a sigmoidal curve through our data points using GraphPad Prism. The following general formula⁽²⁾ describes this type of curve.

(2)

$$S(t) = S_1 + \frac{(S_2 - S_1)}{1 + e^{-\alpha(t-t_0)}}$$

S_1 represents the lower limit of the curve and S_2 the upper limit or maximum value. The t (for time) value where the curve is at half maximum is depicted as t_0 . The sigmoid gain of the curve at t_0 is α , which linearly correlates with the slope. When PKA activity is higher, the maximum is reached faster and α increases. The sigmoid gain (α) of the maximum in our experiment is 8.59 s^{-1} .

Glucose-induced increase in cAMP levels in *C. glabrata* using EPAC2

The EPAC2 biosensor monitors the level of cAMP in a living cell. Upon increased binding of cAMP to the Epac domain, the conformational change induces separation of the FRET pair, thereby lowering the energy transfer between both fluorophores (Figure 1B). The FRET_R response of EPAC2-transformed *C. glabrata* cells was monitored similarly as performed for AKAR3. Cells were starved for glucose, washed and loaded in the microfluidics device. After initial imaging of the basal fluorescence levels, glucose was added under continuous monitoring of donor and acceptor fluorescence for another 30 minutes. Figure 1D shows the results obtained for the FRET_R ratio, after correction for background and autofluorescence as described in formula (1). For the EPAC-type sensors, the amount of cAMP in the cell is reflected by the amplitude of the curve. The ratio in EPAC2 showed a decrease of 6% (after 3 min).

To enable efficient monitoring of cAMP-PKA activity in *C. glabrata*, we here present the usage of two FRET-based biosensors. Both variations in the activity of PKA and the quantity of cAMP can be detected in a time-resolved manner, as exemplified by glucose-induced activation of the pathway. We also present adequate methods to analyze the FRET_R data in a mathematically correct and relevant manner. These sensors will be of great benefit for scientists interested in linking the cAMP-PKA

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signaling cascade to downstream processes, such as virulence, and will also provide information of other environmental triggers activating the pathway. Experiments using these sensors to unravel the role of the cAMP-PKA pathway in the physiology and pathogenicity of the fungus, are performed in our laboratory at the moment.

Experimental procedures

Plasmids used in this study

The plasmids used in this study are depicted in table 1. The pESg03 plasmid was generated by inserting an alternative PCR amplified multiple cloning site in the SacI- SphI-linearized pCgACH plasmid using standard ligation (Kitada *et al.*, 1996). The *C. glabrata* plasmids containing the sequences encoding EPAC2 or AKAR3, were generated by amplification of the sensor sequences from the pAKAR3 and pEPAC2 plasmids and the *CgPGK1* terminator into the SalI- NotI-linearized pESg03 plasmid and insertion via Gibson cloning (NEB). The primers used are shown in table 2. Further replacement of the auxotrophic marker gene by a hygromycin resistance gene was also conducted. The latter gene was amplified starting from the pFA6a-*hphNTI* plasmid (Janke *et al.*, 2004) and inserted by restriction digest, using ScaI and XcmI, and ligation.

Strains and growth/starvation conditions

The *C. glabrata* BG2 strain was used for all experiments (Cormack and Falkow 1999). For growth, SC_{glu} containing 400 mg/l hygromycin was used. To measure activation of PKA or the cAMP level, cells were starved for glucose using SC medium lacking glucose. The protocols for starvation of the particular nutrient are mentioned in the main text. 2-(N-morpholino)ethanesulfonic acid (MES) buffer was used at a concentration of 25 mM and pH of 6.5 to wash the cells before FRET measurement.

Ratiometric FRET measurements: microfluidics and imaging

We used the CellASIC® ONIX2 Microfluidic Platform developed by Merck for automated addition of compounds to cells during continuous imaging by microscopy. The cells are loaded into a chamber of a microtiter plate (Y04C plate, Merck), where they are trapped spatially by an elastic ceiling. In a

continuous manner, liquid is fluxed through the cell chamber. The first well is filled with medium without glucose. The second well is filled with the same liquid, supplemented with glucose. Images are taken during 15 minutes while the cell chamber is fluxed with the first liquid, in order to calculate the basal FRET_R ratio and monitor whether the signal is stable. Later, the liquid present in the second well is fluxed through the chamber over a period of 30 minutes. The temperature was stable at 24 +/- 1°C, the pressure applied to the system was constant at 34.5 kPa. In case no microfluidics system is present, data can also be collected by continuous imaging of cells during the manual addition of glucose to glucose-starved cells in microscopy dishes. Ratiometric FRET measurements were performed by continuously monitoring the emission of donor and acceptor molecules, upon donor excitation, representing donor fluorescence and FRET, respectively. The donor was visualized, using the 458-nm laser line, excitation DM458/515, SDM490 and band pass BA480-495 filter. FRET was monitored, using the 458-nm laser line, DM458/515, SDM560 and band pass BA505-605 filter. Images were taken by scanning at 8.0 µs/pixel. The 60x UPlanSApo (NA 1.35) objective lens was used in combination with a digital zoom of 1.5x. The microscope was set to take images in the donor, acceptor and DIC channel, every 20 seconds, for up to one hour.

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174 *Ratiometric FRET measurements: data analysis*

After acquisition, the images were compiled in one file and on average 20 cells (or ROIs) were selected to calculate FRET_R ratios over the course of the experiment. We selected the cells empirically with a preference for moderate or highly fluorescent ROIs, avoiding saturated regions. This selection was made in the first frame of the experiment before any FRET measurements were performed, indicating that it was purely based on fluorescence intensity and not on FRET_R data. The FRET_R ratios presented, were calculated by subtracting both the background and autofluorescence from the sample intensity of every cell, in both the donor and acceptor channel. The corrected acceptor value was then divided by the corrected donor value to obtain the corrected FRET_R ratio for that particular cell. To be able to visually compare the responses of all cells, we averaged the corrected ratios for one cell over a particular time period before addition of the compound (-10 to -5 min). We divided all values by this factor to obtain a trace that starts from a FRET_R ratio of '1' on

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186 average. Lastly, for every time point, the relative and corrected ratios of all cells were averaged for all
187 selected ROIs and displayed. A sigmoidal curve was fitted to the AKAR3 data points and the sigmoid
188 gain was calculated using GraphPad Prism 7.
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For Peer Review

190 **References**

- 191 Allen, M. D. and J. Zhang (2006). Subcellular dynamics of protein kinase A activity visualized by
192 FRET-based reporters. *Biochem Biophys Res Commun*, **348**(2): 716-721. DOI:
193 10.1016/j.bbrc.2006.07.136.
- 194 Chen, M., Y. Xu, N. Hong, Y. Yang, W. Lei, L. Du, . . . W. Liao (2018). Epidemiology of fungal
195 infections in China. *Front Med*, **12**(1): 58-75. DOI: 10.1007/s11684-017-0601-0.
- 196 Choi, J., W. H. Jung and J. W. Kronstad (2015). The cAMP/protein kinase A signaling pathway in
197 pathogenic basidiomycete fungi: Connections with iron homeostasis. *J Microbiol*, **53**(9): 579-587.
198 DOI: 10.1007/s12275-015-5247-5.
- 199 Colombo, S., S. Broggi, M. Collini, L. D'Alfonso, G. Chirico and E. Martegani (2017). Detection of
200 cAMP and of PKA activity in *Saccharomyces cerevisiae* single cells using Fluorescence Resonance
201 Energy Transfer (FRET) probes. *Biochem Biophys Res Commun*, **487**(3): 594-599. DOI:
202 10.1016/j.bbrc.2017.04.097.
- 203 Colombo, S., P. Ma, L. Cauwenberg, J. Winderickx, M. Crauwels, A. Teunissen, . . . J. M. Thevelein
204 (1998). Involvement of distinct G-proteins, Gpa2 and Ras, in glucose- and intracellular acidification-
205 induced cAMP signalling in the yeast *Saccharomyces cerevisiae*. *EMBO J*, **17**(12): 3326-3341. DOI:
206 10.1093/emboj/17.12.3326.
- 207 Cormack, B. P. and S. Falkow (1999). Efficient homologous and illegitimate recombination in the
208 opportunistic yeast pathogen *Candida glabrata*. *Genetics*, **151**(3): 979-987. DOI:
- 209 Griffioen, G., R. J. Laan, W. H. Mager and R. J. Planta (1996). Ribosomal protein gene transcription
210 in *Saccharomyces cerevisiae* shows a biphasic response to nutritional changes. *Microbiology*, **142** (Pt
211 **8**): 2279-2287. DOI: 10.1099/13500872-142-8-2279.
- 212 Hogan, D. A. and P. Sundstrom (2009). The Ras/cAMP/PKA signaling pathway and virulence in
213 *Candida albicans*. *Future Microbiol*, **4**(10): 1263-1270. DOI: 10.2217/fmb.09.106.
- 214 Janke, C., M. M. Magiera, N. Rathfelder, C. Taxis, S. Reber, H. Maekawa, . . . M. Knop (2004). A
215 versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and
216 promoter substitution cassettes. *Yeast*, **21**(11): 947-962. DOI: 10.1002/yea.1142.
- 217 Kitada, K., E. Yamaguchi and M. Arisawa (1996). Isolation of a *Candida glabrata* centromere and its
218 use in construction of plasmid vectors. *Gene*, **175**(1-2): 105-108. DOI:
- 219 Kluba, M., Y. Engelborghs, J. Hofkens and H. Mizuno (2015). Inhibition of Receptor Dimerization as
220 a Novel Negative Feedback Mechanism of EGFR Signaling. *PLoS One*, **10**(10): e0139971. DOI:
221 10.1371/journal.pone.0139971.
- 222 Kronstad, J. W., G. Hu and J. Choi (2011). The cAMP/Protein Kinase A Pathway and Virulence in
223 *Cryptococcus neoformans*. *Mycobiology*, **39**(3): 143-150. DOI: 10.5941/MYCO.2011.39.3.143.
- 224 Martinez-Pastor, M. T., G. Marchler, C. Schuller, A. Marchler-Bauer, H. Ruis and F. Estruch (1996).
225 The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional
226 induction through the stress response element (STRE). *EMBO J*, **15**(9): 2227-2235. DOI:
- 227 Mean, M., O. Marchetti and T. Calandra (2008). Bench-to-bedside review: *Candida* infections in the
228 intensive care unit. *Crit Care*, **12**(1): 204. DOI: 10.1186/cc6212.

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229 Nikolaev, V. O., M. Bunemann, L. Hein, A. Hannawacker and M. J. Lohse (2004). Novel single chain
230 cAMP sensors for receptor-induced signal propagation. *J Biol Chem*, **279**(36): 37215-37218. DOI:
231 10.1074/jbc.C400302200.

232 Pernambuco, M. B., J. Winderickx, M. Crauwels, G. Griffioen, W. H. Mager and J. M. Thevelein
233 (1996). Glucose-triggered signalling in *Saccharomyces cerevisiae*: different requirements for sugar
234 phosphorylation between cells grown on glucose and those grown on non-fermentable carbon sources.
235 *Microbiology*, **142 (Pt 7)**: 1775-1782. DOI: 10.1099/13500872-142-7-1775.

236 Schepers, W., G. Van Zeebroeck, M. Pinkse, P. Verhaert and J. M. Thevelein (2012). In vivo
237 phosphorylation of Ser21 and Ser83 during nutrient-induced activation of the yeast protein kinase A
238 (PKA) target trehalase. *J Biol Chem*, **287**(53): 44130-44142. DOI: 10.1074/jbc.M112.421503.

239 Trick, W. E., S. K. Fridkin, J. R. Edwards, R. A. Hajjeh, R. P. Gaynes and H. National Nosocomial
240 Infections Surveillance System (2002). Secular trend of hospital-acquired candidemia among
241 intensive care unit patients in the United States during 1989-1999. *Clin Infect Dis*, **35**(5): 627-630.
242 DOI: 10.1086/342300.

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245 Table 1. Plasmids used in this study.

Plasmid	Backbone	Insert	Marker	Reference
pAKAR3	pYX212	<i>pTPII-ECFP-AKAR3-cpVenus</i>	<i>ScURA3</i>	(Colombo <i>et al.</i> , 2017)
pEPAC2	pYX212	<i>pTPII-EYFP-EPAC2-ECFP</i>	<i>ScURA3</i>	(Colombo <i>et al.</i> , 2017)
pESg03	pCgACH	<i>New MCS inserted + pPGK1</i>	<i>CgHIS3</i>	This study
pLDg01	pESg03	<i>pPGK1-ECFP-AKAR3-cpVenus</i>	<i>CgHIS3</i>	This study
pLDg02	pESg03	<i>pPGK1-EYFP-EPAC2-ECFP</i>	<i>CgHIS3</i>	This study
pLDg03	pLDg01	<i>pPGK1-ECFP-AKAR3-cpVenus</i>	<i>hphNT1</i>	This study
pLDg04	pLDg02	<i>pPGK1-EYFP-EPAC2-ECFP</i>	<i>hphNT1</i>	This study

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248 Table 2. Primers used in this study.

Primer name	Sequence (5'->3')
Primers for cloning the novel MCS in pCgACH	
MCS_Fw_SphI	CCCGCGGGTCGACGGTACCGGATCCTCTAGACTGCAGCCTAGGG CGGCCGCGCATG
MCS_Rev_SacI	CGCGGCCGCCCTAGGCTGCAGTCTAGAGGATCCGGTACCGTCGA CCCGCGGGAGCT
Primers for cloning the sensor sequences in pESg03	
EPACp2_Fw_EPACp1	ACCACAGTGAAACGGGAGTTAGCAGGTGTTCTCATTTTTG
EPACp2_Rev_tPGK1	CAAGCTAGTCCCGGGTTACTTGTACAGCTCGTCCATG
tPGK1_Fw_EPAC2	AGCTGTACAAGTAACCCGGGACTAGCTTGAGAACTCCCCC
tPGK1_Rev_pCg	CCATGATTACGCCAAGCTTGCATGCGCGCGGCCGCGCCTATTTG AACTAATTAAACTGAGAAAG
AmpR_Fw	TGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAG
AmpR_Rev	GCCTCCATGTCGATATCAGCTACCACCATCAACAATATTTATAT AG
Primers for cloning the <i>hphNT1</i> sequence in pLDg01 and pLDg02	
hphNT1_Fw_ScaI	TGGTGGTAGCTGATATCGACATGGAGGCCCAAGAATAC
hphNT1_Rev_XcmI	GATGGGCAGACCATCCATGTGCGGCGCGCCCTTCGAGCGTCCCA AAACCTTC

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251 **Figure legend**

252 **Figure 1. Schematic representation of the working mechanism of the AKAR3 and EPAC2**
253 **biosensors upon activation and FRET_R results obtained by stimulation with glucose. A.** Upon
254 activation of PKA, the sensor domain of AKAR3 is phosphorylated and binds to the ligand domain.
255 The FRET donor and acceptor approach each other, thereby increasing the transfer of energy. **B.**
256 Upon increased binding of cAMP to the Epac domain of the EPAC2 biosensor, the conformational
257 change induces separation of the FRET donor and acceptor, thereby lowering the FRET between both
258 fluorophores. **C.** AKAR3 and **D.** EPAC2. Fluorescence was visualized in acceptor and donor
259 channels. The ratio acceptor/donor emission upon donor excitation (FRET_R) is represented in the
260 graph: mean values of at least three biological replicates, normalized to the value before glucose
261 addition (0 min), with the standard error of the mean.

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Authors contribution

L.D. designed and performed the experiments. All authors were involved in interpretation and discussion of the results.

Conflict of interest

The authors declare that there are no competing interests in relation to the work described.

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