RESEARCH

AAV8-mediated gene transfer of microRNA-132 improves beta cell function in mice fed a high-fat diet

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Abstract

MicroRNAs have emerged as essential regulators of beta cell function and beta cell proliferation. One of these microRNAs, miR-132, is highly induced in several obesity models and increased expression of miR-132 *in vitro* modulates glucose-stimulated insulin secretion. The aim of this study was to investigate the therapeutic benefits of miR-132 overexpression on beta cell function *in vivo*. To overexpress miR-132 specifically in beta cells, we employed adeno-associated virus (AAV8)-mediated gene transfer using the rat insulin promoter in a double-stranded, self-complementary AAV vector to overexpress miR-132. Treatment of mice with dsAAV8-RIP-mir132 increased miR-132 expression in beta cells without impacting expression of miR-212 or miR-375. Surprisingly, overexpression of miR-132 did not impact glucose homeostasis in chow-fed animals. Overexpression of miR-132 did improve insulin secretion and hence glucose homeostasis in high-fat diet-fed mice. Furthermore, miR-132 overexpression increased beta cell proliferation in mice fed a high-fat diet. In conclusion, our data show that AAV8-mediated gene transfer of miR-132 to beta cells improves beta cell function in mice in response to a high-fat diet. This suggests that increased miR-132 expression is beneficial for beta cell function during hyperglycemia and obesity.

Key Words

- ▶ insulin secretion
- ▶ microRNAs
- ▶ miR-132
- ▶ gene therapy

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Introduction

Decreased beta cell function plays a pivotal role in the development of type 2 diabetes mellitus. Impaired beta cell function is an early step in the course of type 2 diabetes mellitus, and the onset of beta cell dysfunction seemingly occurs long before the development of hyperglycemia (Perley & Kipnis 1967, Kahn *et al.* 2001). miRNAs are a recently discovered class of evolutionarily conserved short noncoding RNAs that regulate gene expression at a posttranscriptional level. miRNAs bind with imperfect complementary to 3'-UTRs of target mRNAs, causing

translational repression of the target gene or degradation of the target mRNA (Bartel 2004). miRNAs are involved in a wide range of processes that includes development, apoptosis, proliferation, differentiation and regulation of metabolism. In beta cells, miRNAs have emerged as essential regulators of beta-function, beta cell proliferation and beta cell survival (Poy *et al.* 2009, Latreille *et al.* 2014, Tattikota *et al.* 2014, Belgardt *et al.* 2015).

Obesity, a major risk factor for type 2 diabetes mellitus, is known to change miRNA expression in islets

(Zhao *et al.* 2009, Nesca *et al.* 2013). One of these miRNAs, miR-132, is of particular interest as expression of this miRNA is highly induced in islets of several obesity models (Zhao *et al.* 2009, Esguerra *et al.* 2011, Nesca *et al.* 2013). This obesity-related increased expression is severely reduced in diabetic-susceptible BTBR ob/ob mice (Zhao *et al.* 2009). Overexpression of miR-132 in rodent beta cells *in vitro* results in enhanced glucose-induced insulin secretion (Nesca *et al.* 2013, Soni *et al.* 2014). This study aims to investigate the therapeutic benefits of miR-132 overexpression on beta cell function *in vivo*. In order to overexpress miR-132 specifically in beta cells, we created an adeno-associated virus (AAV) vector containing miR-132 under control of the insulin promoter. AAV gene transfer has previously shown to efficiently and stably transduce beta cells *in vivo* without impacting beta cell function (Wang *et al.* 2006, Montane *et al.* 2011). Subsequently, the impact of miR-132 overexpression on beta cell function was studied in mice under normal and insulin-resistant conditions.

Material and methods

Generation of viral vectors

The dsAAV8-RIP-GFP vector was constructed on the backbone of the dsAAV8 plasmid (Nathwani *et al.* 2006). The original LP1 promoter and FIX-coding sequences were replaced by the rat insulin promoter (RIP) (Addgene Plasmid 15029) and enhanced green fluorescent protein (eGFP), by amplification using primers in Table 1 and using MscI BstXI and EcoRI HindIII restriction sites. The gene encoding miR-132 was amplified from chromosomal DNA from C57Bl/6J mice, using primers listed in Table 1 and cloned between the RIP and eGFP in dsAAV8-RIP-GFP, using the restriction enzymes BspEI and SbfI (New England Biolabs).

AAV creation

dsAAV viral particles were generated by triple transfection of human embryonic kidney 293 cells using the 25-kDa

linear polyethylenimine (Polysciences Inc., Eppelheim, Germany) transfection method (Reed *et al.* 2006). AAV viral particles were purified by iodixanol gradient centrifugation as previously described (Zolotukhin *et al.* 1999). Viral titers were determined using quantitative PCR (qPCR) with primers specific for RIP and eGFP.

Mice and *in vivo* virus injection

Male C57Bl/6J wild-type, ob/ob and ob/+ mice (9–10 weeks) were obtained from the Harlan Laboratories. C57Bl/6J mice were injected with AAV at the age of 12 weeks. Intraductal injection was performed as described (Jimenez *et al.* 2011) with minor adjustments. Mice were anesthetized with isoflurane. The duodenum was isolated with the common bile duct attached. A microclamp was placed on the bile duct caudal to the liver. Using a 27G needle, the duodenum was punctured after which the needle was inserted to advance retrograde through the sphincter of Oddi into the common bile duct. The needle was secured in place using a ligation and 100 µL PBS containing 1.4×10^{11} viral genome particles was injected into the duct over approximately 1 min. At 1 min post injection the microclamps and needle were removed. The puncture in the duodenum was closed using tissue glue. Mice received buprenorphine (0.05 mg/kg s.c.) directly and 8 h after surgery. Mice were allowed to recover for 2 weeks on chow diet (RMH-B, Hope Farms, Woerden, the Netherlands), after which mice received chow or high-fat diet (60% kcal% fat diet, #D12492, Research Diets Inc., New Brunswick, USA) for 4 weeks. All experiments were performed with the approval of the Ethical Committee for Animal Experiments of the University of Groningen.

Primary mouse islet isolation, cell culture and *in vitro* insulin secretion assay

Islets were isolated by collagenase digestion as previously described (Salvalaggio *et al.* 2002). Islets were rinsed and handpicked in RPMI media containing 10% FBS after which islets were frozen immediately for RNA isolation

Table 1 Primer sequences.

Primer name	Sequence	Gene
scAAV-GFP-fw	tactacgaattcaccatgtgtgagcaagggcgag	GFP
scAAV-GFP-rv	tactacaagctttcactgttacagctcgcca	GFP
scAAV-RIP-fw	gtggagtcgtgtaccgggcc	RIP
scAAV-RIP-rv	gttgccaggtcagtgccatgcctgc	RIP
Mmu-mir132-fw	gcgaaacctgcaggtccctgcgcgctgtccgcg	Mmu-mir132
Mmu-mir132-rv	gcgaaatccggatgccacctcgcagacacat	Mmu-mir132

or cultured overnight. The following day, static insulin secretion assay was performed on size-matched islets as previously described (Brunham *et al.* 2007). Insulin was measured by ELISA (Mouse-Insulin Ultra-Sensitive ELISA or the Rat Insulin ELISA, Alpco, Salem, NH, USA). Rat insulin-producing INS-1E cells (provided by Dr P Maechler, Centre Médical Universitaire, Geneva, Switzerland) were cultured as previously described (Merglen *et al.* 2004). INS-1E cells of passage numbers 50–60 were used in our experiments. For transfection, INS-1E cells were transfected using Lipofectamine 2000 (Invitrogen) with miRNA mimics (Ambion, Life Technologies). At 2 days after transfection, glucose-stimulated insulin secretion was measured. To determine whether hyperglycemia would impact miR-132 expression levels, primary mouse islets were cultured for 24 h in RPMI containing 2 mM, 8 mM or 16 mM glucose with 1% BSA with or without 0.4 mM palmitate (Sigma-Aldrich).

Glucose tolerance test and BrdU labeling

Oral glucose tolerance tests were performed on 8-h fasted mice administered with 2 g glucose per kg of body weight. Blood was taken from the saphenous vein and blood glucose levels were measured using a glucometer and test strips (Life Scan). For plasma insulin levels, blood was collected using EDTA-coated capillary tubes and insulin levels were measured by ELISA (Mouse-Insulin Ultra-Sensitive ELISA, Alpco, Salem, NH, USA). For beta cell proliferation measurements, mice were daily injected i.p. with 1 mg/animal BrdU (Sigma-Aldrich) in PBS for 4 days before killing.

Immunostaining and islet morphology analysis

Formalin-fixed pancreatic tissues were embedded in paraffin using standard techniques. Four micrometer sections were deparaffinized, rehydrated and incubated with blocking solution. Sections were incubated overnight at 4°C with antibodies against insulin and glucagon (Dako), GFP (Life Technologies) and/or BrdU (Abcam), followed by secondary antibodies conjugated to FITC or Cy3 (Life Technologies). DAPI-containing mounting media (Vector Laboratories, Burlingame, CA, USA) was added to coverslips. Proliferating beta cells were identified by co-staining for BrdU and insulin. All islets in two pancreatic sections of 200 µm apart were analyzed, resulting in the counting of at least 573 beta cells/mouse with $n=4$ mice per group. For beta cell area measurements, the percentage of insulin-positive area was

determined using ImageScope (Aperio) from 5 to 6 evenly spaced sections per pancreas.

Measurement of miRNA and mRNA expression

Total RNA from isolated islets was isolated using the mirVana kit (Life Technologies) according to instructions. RNA quality and concentration was measured using the Biorad Experion Bioanalyzer and miRNA microarrays were run with miRNA microarrays (MirBase release 17.0) of Agilent (Santa Clara). Array images were analyzed using Agilent feature extraction software (10.7.3.1) and GeneSpring GX (Agilent). After quantile normalization, statistical significance was tested with an unpaired *t* test followed by Benjamini–Hochberg multiple testing correction (false discovery rate: 0.01 and a fold change of at least 2). cDNA for miRNA expression measurements was synthesized using TaqMan miRNA reverse transcription kit (Life Technologies). For mature miRNA transcript expression, we used TaqMan miRNA Assays (Life Technologies). cDNA for mRNA expression measurement was synthesized using Superscript II (Life Technologies). SYBR Green PCR Master Mix (Life Technologies) was used for RT-PCR in an ABI Prism 7700 Sequence Detection System. Expression values were normalized to *Gapdh* for mRNA and small nucleolar (sno) RNA202 for miRNA qRT-PCR.

Protein analysis

Isolated islets were lysed in M-PER lysis buffer (Thermo Fisher Scientific). Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked using TBST with 2% milk and 0.5% BSA for 1 h at room temperature followed by incubation with the primary antibodies in TBST with 0.5% BSA for 2 h. Primary antibodies used were anti-retinoblastoma (Santa Cruz Biotechnology), anti-CACT (Novus Biologicals), anti-actin (Sigma-Aldrich) and anti-GAPDH (Millipore). After probing with the primary antibodies, the membrane was incubated with HRP-conjugated secondary antibodies (rabbit-anti-mouse HRP-conjugated, or goat-anti-rabbit HRP-conjugated, Dako). Chemiluminescence was determined using SuperSignal West Dura Extended Duration Substrate buffer (Thermo Fisher Scientific) and Chemidoc (Biorad).

Statistical analysis

Graphpad Prism 6.0 was used for statistical analysis. Data are presented as Tukey's Box-and-Whiskers plot

using median and 25th and 75th percentile intervals (P_{25} – P_{75}). Differences between groups were calculated by Mann-Whitney test with a P value of 0.05 considered significant. A two-way ANOVA, followed by Bonferroni *post hoc* tests, was used to evaluate the glucose tolerance tests.

Results

Leptin deficiency resulted in profound differences in miRNA profiles of ob/ob islets, with 36 miRNAs increased and 36 miRNAs decreased by >two-fold compared to control islets ($n=6$, FDR <1%). miR-132 was one of the most induced miRNAs in ob/ob islets with a fold change of 6.2 (Fig. 1A). Using RT-PCR, we showed increased expression of miR-132 in islets of ob/ob mice, high-fat-fed mice and 14-month aged mice (Fig. 1B). As all the diabetic/insulin-resistant models tested had elevated fasting blood

glucose levels, we determined whether hyperglycemia itself regulated expression of miR-132. Indeed, miR-132 expression levels were upregulated by culturing primary mouse islets in 16 mM glucose for 24 h. This increase was augmented by the addition of 2 mM palmitate (Fig. 1C). In agreement with previous data (Nesca *et al.* 2013, Soni *et al.* 2014), overexpression of miR-132 in INS1E cells resulted in increased glucose-stimulated insulin secretion (Fig. 1D). Together, these results confirm previous data showing a role for miR-132 in glucose-regulated insulin secretion in beta cells *in vitro*.

Increased miR-132 expression result in improved insulin secretion during high-fat feeding

To determine whether increased expression of miR-132 would increase insulin secretion *in vivo*, we created double-stranded adeno-associated virus serotype 8 (dsAAV8)

Figure 1

Hyperglycemia and obesity-induced miR-132 expression resulting in increased insulin secretion. (A) Several miRNAs were differently expressed in pancreatic islets of 12-week-old ob/ob mice ($n=6$). (B) Increased miR-132 expression in islets isolated from ob/ob ($n=4$), 10-week high-fat diet-fed C57Bl6 mice ($n=4$) or 14-month-old C57Bl6 mice ($n=7-8$). (C) Culture of primary islet for 24 h in high glucose media or media containing 2 mM palmitate-induced miR-132 expression ($n=4$). (D) Overexpression of miR-132 in INS1E cells resulted in increased glucose-stimulated insulin secretion ($n=4$).

vectors containing mouse miR-132 and GFP cDNA or GFP cDNA driven by the rat insulin promoter (AAV8-RIP-miR132 and AAV8-RIP-GFP). Injection of the constructs in the pancreatic duct resulted in GFP expression specifically in β -cells in the islet (Fig. 2A). Based on the GFP, the proportion of islets with GFP-positive cells was relatively high; $77 \pm 15\%$ for control and $88 \pm 5\%$ for AAV-RIP-miR132-treated mice. Increased expression of miR-132 was detected in isolated islets of AAV-RIP-miR132-injected animals, confirming miR-132 overexpression in β -cells *in vivo* (Fig. 2B). Histological examination of the pancreata

of the mice showed no evidence of pancreatitis or fibrosis. Islets of both groups displayed normal morphology with the β -cells in the core and the α -cells at the rim of the islet (Fig. 2D).

Therapeutic overexpression of miRNAs could potentially modify the processing of other cellular miRNAs transcripts due to the same processing pathways (Grimm *et al.* 2006). Therefore, we tested the expression of miR-375, a microRNA highly expressed in beta cells and involved in regulation of insulin secretion (Poy *et al.* 2004) and miR-184, a modulator of compensatory beta

Figure 2

AAV8-mediated gene transfer resulted in miR-132 overexpression in beta cells. (A) Representative image of immunofluorescent staining against GFP (green) and insulin (red) in control AAV-RIP-GFP or AAV8-RIP-miR132-treated mice. (B) Increased expression of miR-132 in islets isolated from AAV8-RIP-miR132-treated mice compared to islets isolated from control mice ($n = 4-6$). (C) Expression of miR-375, miR-184 and miR-212 in islets was comparable between AAV8-RIP-miR132-treated and control mice ($n = 4-6$). (D) Representative image of islet morphology based immunofluorescent staining against insulin (green) and glucagon (red).

cell expansion during insulin resistance (Tattikota *et al.* 2014). To exclude a possible negative feedback loop due to miR-132 overexpression on the miR-132/miR-212 cluster, miR-212 levels were determined in the islets of control and miR-132-treated mice. Overexpression of miR-132 did not impact expression of miR-212, miR-375 or miR-184 (Fig. 2C), indicating that overexpression of miR-132 did not interfere with the processing of other miRNAs.

After initial weight loss in the first week after the injection, all mice gained weight 1 month after the injection (Fig. 3A) after which glucose homeostasis was analyzed. Surprisingly, overexpression of miR-132 did not impact fasted glucose or insulin plasma levels. In addition, glucose tolerance testing showed no difference in glucose

control between the animals (Fig. 3B and C). To increase the demand on beta cells, mice were put on a high-fat diet for 4 weeks. In the control AAV-RIP-GFP-treated mice, high-fat diet increased fasted glucose levels and impaired glucose tolerance (Fig. 3D). Overexpression of miR-132 specifically in beta cells, however, resulted in improved glucose tolerance compared to control mice. This was due to increased glucose-stimulated insulin secretion as measured during the glucose tolerance test (Fig. 3E) and *ex vivo* in isolated islets (Fig. 3F). Insulin content of isolated islets was similar in control and AAV-RIP-miR132-treated mice ($0.37 \pm 0.04 \mu\text{g}/\text{islet}$ in control islets vs $0.33 \pm 0.04 \mu\text{g}/\text{islet}$ in miR-132 islets; $n=6$). In addition, gene expressions of genes related to insulin secretion

Figure 3

Impact of miR-132 overexpression in beta cells on glucose homeostasis. (A) Body weights of AAV8-RIP-GFP control and AAV8-RIP-miR132-treated mice ($n=6$) during chow and high-fat diet (HFD) feeding. (B) AAV8-RIP-miR132-treated mice showed similar glucose levels during fasting or after oral glucose bolus as control mice fed a chow diet ($n=6$). (C) Insulin levels at 0 and 15 min after oral glucose bolus were similar between the two groups ($n=6$). (D) Oral glucose tolerance testing showed improved glucose tolerance in AAV8-RIP-miR-132-treated mice after 4 weeks of high-fat diet ($n=6$). (E) Analysis of insulin levels at 0 and 15 min after oral glucose bolus showed increased insulin secretion in mice overexpressing miR-132 after the glucose bolus ($n=6$). (F) *Ex vivo* analysis of glucose-stimulated insulin secretion showed increased insulin secretion at 16.7 mM glucose in miR-132-overexpressing islets ($n=6$). (G) Isolated islets of AAV8-RIP-miR132-treated mice showed normal gene expression of beta cell-related genes ($n=4-6$). (H) Increased expression of miR-132 coincided with reduced expression of CACT mRNA and protein levels ($n=4-6$).

or beta cell function, such as *insulin*, *glucose transporter 2* (*Glut2*), *prohormone convertase 2* (*Psc2*), *MAF bZIP transcription factor A* (*Mafa*) or *pancreatic and duodenal homeobox 1* (*Pdx1*) were similar between groups (Fig. 3G). Gene expression and protein levels of the miR-132 target carnitine acyl-carnitine translocase (CACT) (Soni *et al.* 2014) was decreased in islets of AAV-RIP-miR132-injected animals (Fig. 3H).

Increased beta cell proliferation in mice overexpressing mir-132

Overexpression of miR-132 in dispersed rat islet cells has been shown to increase beta cell proliferation *in vitro* (Nesca *et al.* 2013). In order to determine whether overexpression of miR-132 *in vivo* also lead to increased beta cell proliferation, beta cell proliferation was studied in the AAV-RIP-miR132 and control mice after 2 weeks of high-fat diet feeding using BrdU incorporation. Overexpression of miR-132 leads to 2.4-fold increase in BrdU⁺ beta cell (Fig. 4A and B). In agreement with this, we found increased

expression levels of the proliferation marker *Ki67* in isolated islets from AAV-RIP-miR132 mice fed a high-fat diet for 4 weeks (Fig. 4C). Although gene expression levels of the previously identified miR-132 target *retinoblastoma protein* (*Rb*) (Park *et al.* 2011) were similar in both groups (1.0 ± 0.5 relative expression in control islets vs 1.2 ± 0.3 relative expression in miR-132 islets; $P=0.48$), protein analysis revealed decreased RB protein levels in islets of miR-132 treated mice (Fig. 4D, E). To determine whether the increased proliferation resulted in increased beta cell mass, beta cell area was analyzed. However, the beta cell area did not significantly differ between the groups ($0.84 \pm 0.29\%$ beta cell area in control islets vs $1.18 \pm 0.38\%$ beta cell area in miR-132 islets; $n=4$; $P=0.2$).

Discussion

Our data show that AAV8-mediated gene transfer of miR-132 to beta cells improves beta cell function in mice in response to a high-fat diet. We found significantly increased

Figure 4

Signs of increased proliferation in beta cells of AAV8-RIP-miR132-treated mice. (A) Pancreatic sections of control or AAV8-RIP-miR-132-treated mice stained using immunofluorescence for insulin (green) and BrdU (red). (B) Percentage of BrdU-positive beta cells in pancreata of control and AAV8-RIP-miR-132 mice ($n=4$). (C) Isolated islets of AAV8-RIP-miR132-treated mice showed increased *ki67* gene expression ($n=4-6$). (D) Decreased RB protein levels in isolated islets of AAV8-RIP-miR132-treated mice, of which the quantification is shown in (E) ($n=6$).

glucose-stimulated insulin secretion and enhanced beta cell proliferation in mice treated with the dsAAV8-RIP-miR132 construct. These data indicate that miR-132 is a potential target to improve beta cell dysfunction during obesity.

Although overexpression of miR-132 improves insulin secretion *in vitro*, mice glucose homeostasis remained unaltered in chow-fed mice overexpressing miR-132 indicating that the risk on hypoglycemia is low. Mice treated with dsAAV-RIP-miR132, however, did show improved insulin secretion during high-fat diet feeding. Recent findings identified CACT as miR-132 target in beta cells (Soni *et al.* 2014). CACT is a transporter involved in transporting long-chain acyl-carnitines into the mitochondria for β -oxidation (Wang *et al.* 2011). Downregulation of CACT results in an accumulation of fatty acyl-carnitines, which enhances insulin secretion. Addition of long-chain acyl-carnitines to beta cells stimulated insulin secretion; an effect that was enhanced by CACT downregulation (Soni *et al.* 2014). This could explain our finding that only during increased fatty acid influx, as achieved by the high-fat diet, insulin secretion was increased in the miR-132 overexpressing beta cells.

In this study, we chose to apply beta cell-specific gene therapy to evade possible side effects of miR-132 overexpression in non-beta cells. miR-132 has previously found to be involved in facilitating pathological angiogenesis in tumors (Anand *et al.* 2010) and is overexpressed in pancreatic adenocarcinomas (Park *et al.* 2011). In the pancreatic cancer cell line PAN-1, overexpression of miR-132 leads to decreased expression of the tumor suppressor RB, leading to increased proliferation (Park *et al.* 2011). No difference in expression of *Rb* mRNA in islets of dsAAV8-RIP-miR132-treated mice was found, which could be due to the relative high gene expression of *Rb* in alpha cells compared to beta cells (Kutlu *et al.* 2009). Protein levels of RB, however, were decreased in islets overexpressing miR-132. Interestingly, exendin-4, a GLP-1 agonist known to induce beta cell proliferation in mice, has been shown to decrease RB expression. Further study revealed that this decreased Rb expression is necessary for the beta cell proliferation stimulating effect of exendin-4 (Cai *et al.* 2014). GLP-1 agonists increase miR-132 expression in beta cells (Shang *et al.* 2015), suggesting that miR-132 plays a central role in the adaptive beta cell response to obesity and GLP-1. Although we found increased BrdU incorporation and increased *Ki67* expression in islets of dsAAV8-RIP-miR132-treated

mice after high-fat feeding indicating increased beta cell proliferation, beta cell area was not significantly different between the groups. The high variation in beta cell area within the groups, the small group size and the relatively short period of high-fat diet could potentially explain this discrepancy.

Unfortunately, it was not possible to identify miR-132 targets in our setting due to the difficulty to isolate pure beta cells together with the fact that miRNAs often induce only small changes in the expression of single direct targets (Guo *et al.* 2010). However, our study does show that the physiological impact of miRNAs in beta cells can be successfully studied *in vivo* using the AAV8-mediated gene transfer system. This system could potentially help to identify the physiological roles of the over 800 miRNAs, which recent ultra-high-throughput sequencing have revealed to be expressed in the endocrine pancreas (Kameswaran *et al.* 2014).

During the last years, the importance of miRNAs in the control of beta cell function, proliferation and identity has become clear. Several microRNAs, such as, miR-375 and miR-184 have been identified as crucial regulators of adaptive beta cell expansion, whereas miR-7a regulates insulin secretion. Our study shows the beneficial effects of miR-132 overexpression in the setting of obesity and identifies miR-132 or its downstream targets as therapeutic targets to improve beta cell function.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

N L M, J K and J K K designed the experiments. N L M, R H and J K K performed the experiments and analyzed the data. J K K wrote the manuscript. All authors revised the article and approved the final version.

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