



Effects of dietary supplementation with docosahexaenoic acid (DHA) on hippocampal gene expression in streptozotocin induced diabetic C57Bl/6 mice[☆]

Jency Thomas^{a, c, d}, Manohar Lal Garg^{a, c, *, 1}, Doug William Smith^{b, c, 1}

^a Nutraceuticals Research Group, University of Newcastle, Callaghan, NSW 2308, Australia

^b Priority Research Centre for Translational Neuroscience and Mental Health, University of Newcastle, Callaghan, NSW 2308, Australia

^c School of Biomedical Sciences & Pharmacy, University of Newcastle, Callaghan, NSW 2308, Australia

^d Human Biosciences, Faculty of Health, La Trobe University, Victoria 3083, Australia

ARTICLE INFO

Article history:

Received 30 October 2014

Received in revised form

27 March 2015

Accepted 6 April 2015

Available online 27 May 2015

Keywords:

Diabetes

Hippocampus

DHA

Cognitive function

Synaptic plasticity

Inflammation

ABSTRACT

A body of evidence has accumulated indicating diabetes is associated with cognitive impairments. Effective strategies are therefore needed that will delay or prevent the onset of these diabetes-related deficits. In this regard, dietary modification with the naturally occurring compound, docosahexaenoic acid (DHA), holds significant promise as it has been shown to have anti-inflammatory, anti-oxidant, and anti-apoptotic properties. The hippocampus, a limbic structure involved in cognitive functions such as memory formation, is particularly vulnerable to the neurotoxic effects related to diabetes, and we have previously shown that streptozotocin-induced diabetes alters hippocampal gene expression, including genes involved in synaptic plasticity and neurogenesis. In the present study, we explored the effects of dietary supplementation with DHA on hippocampal gene expression in C57Bl/6 diabetic mice. Diabetes was established using streptozotocin (STZ) and once stable, the dietary intervention group received AIN93G diet supplemented with DHA (50 mg/kg/day) for 6 weeks. Microarray based genome-wide expression analysis was carried out on the hippocampus of DHA supplemented diabetic mice and confirmed by real time polymerase chain reaction (RT-qPCR). Genome-wide analysis identified 353 differentially expressed genes compared to non-supplemented diabetic mice. For example, six weeks of dietary DHA supplementation resulted in increased hippocampal expression of *Igf II* and *Sirt1* and decreased expression of *Tnf-α*, *Il6*, *Mapkapk2* and *ApoE*, compared to non-supplemented diabetic mice. Overall, DHA supplementation appears to alter hippocampal gene expression in a way that is consistent with it being neuroprotective in the context of the metabolic and inflammatory insults associated with diabetes.

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Introduction

While a decline in cognitive function and memory is considered to be a normal consequence of ageing [1], disease conditions such as diabetes also lead to the development of cognitive dysfunction and memory impairments [2–5]. Both type I and II diabetes are associated with memory deficits, and the hippocampus is

[☆] The present study was supported by a grant from the Priority Research Centre for Physical Activity and Nutrition, University of Newcastle, Australia.

* Corresponding author. School of Biomedical Sciences & Pharmacy, University of Newcastle, Callaghan, NSW 2308, Australia.

E-mail address: manohar.garg@newcastle.edu.au (M.L. Garg).

¹ Denotes co-senior authors.

particularly vulnerable, with structural and functional alterations being caused by the disease processes [6–8]. For example, morphological changes, such as dendrite atrophy (reduced dendritic length and number of branch points), have been observed in experimental models of diabetes [9,10]. Diabetes also leads to abnormal neurotransmitter release in the hippocampus [11]. Lastly, neurogenesis, the processes through which new neurons are generated and integrated into existing circuits, and that is thought to be crucial for normal cognitive functions, is impaired in animal models of diabetes [12]. Indeed, impaired hippocampal synaptic plasticity and neurogenesis appear to be major factors in the cognitive deficits associated with diabetes [13]. While the underlying cellular mechanisms for these impairments remain to be fully elucidated, we have started to characterise these mechanisms and

previously reported diabetes causes alterations in the hippocampal expression of genes that are important for both synaptic plasticity and neurogenesis [14].

The restoration of normal hippocampal synaptic plasticity and neurogenesis is therefore an important goal for the prevention of, or recovery from, diabetes associated cognitive decline. Of the various substances that have been shown to promote hippocampal and overall cognitive function, docosahexaenoic acid (DHA) is showing considerable promise [15]. DHA is a long chain omega-3 polyunsaturated fatty acid (LnC-PUFA) and an integral component of neural membrane phospholipids. DHA influences membrane fluidity, modulates signal transduction and also receptor affinity, and thereby affects multiple brain functions [16]. Several epidemiological studies have shown a close relationship between DHA and cognition, with low plasma DHA levels associated with cognitive deficits in healthy elderly subjects and also in patients suffering from neurodegenerative disorders such as Alzheimer's disease (AD) [17–20]. Additionally, preclinical studies have shown that DHA enhances performance in learning and memory tasks in aged animals [21], and it also improves cerebral blood flow [22].

DHA is highly concentrated in the brain and has been shown to have anti-oxidant, anti-inflammatory, and anti-apoptotic properties [23]. Importantly, a high dietary intake of DHA leads to accumulation of DHA in brain membrane phospholipids [21]. The potential beneficial effects of DHA to cognitive function have been reported [24–27], however, there are no studies exploring the effects of DHA on hippocampal gene expression in diabetes. This type of study could provide insight into the underlying molecular mechanism by which DHA could confer its beneficial effects. In the present study we employ streptozotocin (STZ) induced diabetic animals to study the effect of dietary supplementation with DHA on hippocampal gene expression.

Materials and methods

Subjects

Seven week-old, male C57BL/6 mice (University of Newcastle; weighing 20–25 g upon arrival) were individually housed in a temperature and humidity controlled environment under a 12 h light/dark cycle, with food and water available *ad libitum*. All procedures were performed in strict accordance with protocols approved by University of Newcastle Animal Care and Ethics Committee, the New South Wales Animal Research Act and Regulations, and the Australian code of practice for care and use of animals for scientific purposes.

Methods and treatment

After one week of acclimatization, animals received daily injections of streptozotocin (STZ; Sigma; 50 mg/kg i.p.) freshly dissolved in 100 μ l of 0.5 M sodium citrate buffer for five consecutive days as previously described [14]. Blood glucose levels were determined from saphenous vein blood using an Accu-Check Integra (Roche Diagnostics Mannheim, Germany). After three weeks of STZ administration, animals exhibiting blood glucose levels ≥ 15 mM were considered to be diabetic. Following confirmation of a diabetic state, animals were put on a semi-pure diet (AIN93G; Specialty Feeds Western Australia), which satisfies all nutritional requirements. For DHA supplementation (EPAX, 1050TG), the animals received 50 mg/kg/day of DHA mixed with AIN93G diet every day for a period of 6 weeks. Blood glucose levels and body weight were monitored weekly for six weeks. Food intake was monitored and recorded every week, and all animals had *ad libitum* access to drinking water.

Animal sacrifice and tissue harvesting

At the end of the six-week period of stable diabetes and dietary supplementation, animals were overdosed with pentobarbitone and the brains were rapidly removed, immediately cooled in ice-cold PBS and frozen in dry-ice chilled isopentane. Tissue was stored at -80°C until required. Ten μ m thick, coronal hippocampal sections were obtained by cryosection, mounted on nuclease-free glass microscope slides and stored at -80°C until further use. Blood was drawn from saphenous vein and processed for gas chromatography to analyse erythrocyte fatty acid analysis (see Table 1).

Erythrocyte fatty acid analyses

The fatty acid composition of erythrocytes was determined according to modified Lepage and Roy (1986) method where acetyl chloride methylation procedure is appropriated. Fatty acid methyl esters were quantified using gas chromatography. The identity of each fatty acid peak was ascertained by comparison of the peak's retention time with the retention times of synthetic standards of known fatty acid composition (Nu Check Prep, Elysian, MN, USA). The relative amount of each fatty acid was quantified by integrating the area under the peak and dividing the result by the total area for all fatty acids. Fatty acid results are reported as percentage of total fatty acids.

RNA extraction

Sections were rinsed in ice-cold PBS (DEPC treated) and then fixed in ice-cold ethanol (100%) for 3 min. Whole hippocampi were dissected from sections at approximately 250 μ m intervals through the rostrocaudal extent of the hippocampus. Total RNA was extracted from hippocampal tissue pooled for each animal. Contaminating genomic DNA (gDNA) removed in solution by DNase-I digestion using Qiagen's RNeasy mini kit and DNase reagents, according to the manufacturer's instructions. The concentration of DNase treated RNA was determined by UV absorbance spectrophotometry (Thermo Scientific Nanodrop 1000).

Microarray profiling

Gene expression profiling was carried out by the Australian Genome Research Facility (AGRF, Australia). Approximately 200 ng of total RNA was prepared for hybridization to Illumina Mouse WG-6 v2.1 Expression microarrays. Expression data was first analysed using Illumina's Genome studio 2010.2 software and incorporated

Table 1

Fatty acid levels in the erythrocytes of DHA supplemented diabetic group and non-supplemented diabetic group.

Common name	Lipid name	Diabetic (n = 10)	DHA (n = 10)	P value*
Palmitic acid	C16:0	23.8 $\pm$ 0.9	32.9 $\pm$ 2.7	<0.0001
Palmitoleic acid	C16:1n-7	0.9 $\pm$ 0.3	1.6 $\pm$ 0.8	0.0366
Stearic acid	C18:0	12.1 $\pm$ 1.2	12.2 $\pm$ 1.8	0.846
Oleic acid	C18:1n-9	21.9 $\pm$ 1.9	20.6 $\pm$ 1.9	0.346
cis-vaccenic acid	C18:1n-7	2.4 $\pm$ 0.3	3 $\pm$ 0.5	0.531
Linoleic acid	C18:2n-6	8.4 $\pm$ 0.8	5.8 $\pm$ 0.3	0.0024
Arachidonic acid	C20:4n-6	21.3 $\pm$ 0.9	6.2 $\pm$ 0.9	<0.0001
Eicosapentaenoic acid	C20:5n-3	0.124 $\pm$ 0.02	4.4 $\pm$ 0.76	<0.001
Docosahexaenoic acid	C22:6n-3	5.8 $\pm$ 0.4	9 $\pm$ 1.7	0.0264
Lignoceric acid	C24:0	1.1 $\pm$ 0.1	1.7 $\pm$ 0.6	0.112
Nervonic acid	C24:1n-9	2.1 $\pm$ 0.2	1.6 $\pm$ 0.6	0.048

*P values were obtained using independent samples t-test for diabetic and DHA supplemented diabetic group.

normalisation by averaging. Fold-change in either direction of ≥ 1.2 , average signal intensity of ≥ 100 , and statistical significance as determined by ANOVA, were criteria used to identify genes of interest.

Reverse transcription and q RT-PCR

Reverse transcription

Reverse transcription was performed using Superscript III (Invitrogen, Australia), according to manufacturer's instructions. Briefly, 200 ng of total RNA, 1 μ l oligo(dT)₁₈ primer, 1 μ l of 10 mM dNTP, and molecular grade water to 13 μ l, were mixed and heated for 5 min at 65 °C for 5 min, the chilled on ice for 1 min. Next 4 μ l 5 $\times$ first strand buffer, 1 μ l of 0.1M DTT, 1 μ l RNaseOUT (40 U/ μ l) and 1 μ l of superscript, were added and the mixture incubated for 60 min at 50 °C, followed by a 70 °C enzyme inactivation step for 15 min. Reverse transcription, without reverse transcriptase (RT–), was also performed to assess gDNA contamination.

Real-time qPCR

All gene transcript information was obtained from Ensembl (www.ensembl.org). qPCR primers (Table 4) were designed using NCBI's primer design tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). qPCR volumes were 12 μ l, contained 1 ng cDNA, and were run in triplicate on an ABI 7500 real time PCR system (Applied Biosystems, USA). qPCR data was analysed using Applied Biosystems 7500 sequence Detection software (version 1.4). For each gene, all samples, including water controls, were run on a single qPCR plate. For each animal, the delta Ct (Δ Ct, threshold cycle) was determined for the gene of interest relative to the average of two house-keeping genes, succinate dehydrogenase (SDH) and beta actin. For each gene, the $\Delta\Delta$ Ct method was used to compare diabetic and diabetic plus DHA group [28].

Statistics

All statistical analyses were performed for qPCR were performed using PASW statistics software (version 18; SPSS Inc., Chicago, IL USA). Statistical significance was assessed using one-way analysis of variance (ANOVA). Results were considered statistically significant if the observed significance level was ≤ 0.05 . As we have previously reported findings for the control and diabetic conditions [14], comparisons in the present study are between the diabetes plus DHA group and the previous diabetes group [14].

Results

Blood glucose and body weight

The diabetic plus DHA group showed an increase in blood glucose levels three weeks after STZ administration. All animals in

the group that exhibited blood glucose levels ≥ 15 mM were considered diabetic. The average ($\pm$ SEM) blood glucose levels in the diabetes plus DHA group was 18.7 ± 1.47 mM. There was not a significant difference in blood glucose levels between the diabetes and the diabetes plus DHA group ($P = 0.609$). Also, there was no significant difference in body weight between the diabetes and the DHA supplemented diabetic group ($P = 0.800$).

Erythrocyte fatty acid

In response to DHA supplementation, erythrocyte fatty acid analysis showed significant increase in DHA ($P = 0.0264$) and EPA ($P < 0.001$) in the DHA supplemented group. A significant reduction in arachidonic acid was seen in the DHA supplemented group as opposed to the non-supplemented diabetic group ($P < 0.0001$) (see Table 1).

Genome wide gene expression analysis

Using the criteria described above, 353 genes were differentially expressed in the hippocampus of the DHA supplemented diabetic group compared to the non-supplemented diabetic group. Twenty-one genes had increased expression and 332 (Supplementary table) had decreased expression. Genes that were significantly differentially expressed in the hippocampus of diabetic animals compared to non-diabetic controls, and that had this diabetes-induced change in expression either normalized or exacerbated by DHA, are shown in Table 2.

Pathway analysis

Pathways associated with diet induced gene expression changes

To garner a better understanding of diet-induced changes in hippocampal gene expression, and to assess potential biological function, the list of differentially expressed genes obtained from microarray analysis was subjected to pathways analysis using Database for Annotation, Visualization, and Integrated Discovery (DAVID) [29]. DAVID analyses gene lists to statistically determine whether there is enrichment for genes that belong to *a priori*-defined gene sets. This analysis found 5 significantly (<0.05) enriched pathways (see Table 3), using Kyoto Encyclopedia of Genes and Genomes (KEGG). The most significantly enriched pathways in the DHA supplemented group were the Mapk and Jak/Stat signalling pathways.

Confirmation by RT-qPCR

A subset of genes was chosen for RT-qPCR confirmation. Significant increases in *Igf II* (3.31 fold) and *Sirt1* (1.81fold) and decreases in expression of *ApoE* (–1.38 fold), and *Tnf- α* (–1.76 fold) were confirmed (Fig. 1).

Table 2

List of genes whose expression was affected by diabetes relative to non-diabetic controls, and that were either partly or wholly normalised or exacerbated, by DHA supplementation.

Gene names	Symbol	Fld change DHA/Diab
Apolipoprotein E	ApoE	↓1.2
Insulin like growth factor 2	Igf2	↑1.6
Sirtuin 1	Sirt1	↑1.5
Tumour necrosis factor- alpha inducing protein 1	Tnfaip1	↓1.6
Mitogen activated protein kinase activating protein kinase 2	Mapkapk2	↓1.6
Interleukin 1 beta	Il1b	↓1.8
Interleukin 20	Il20	↓1.5
Interleukin 6 signal transducer	Il6st	↓1.6
Phosphoinositide-3-kinase, regulatory subunit 2	Pi3kr2	↑1.6

Table 3

List of pathways identified by KEGG pathway analysis, showing those pathways that were enriched in the hippocampus of the DHA supplemented diabetic group, relative to the non-supplemented diabetic group.

Pathways	P-value*	Fold enrichment	Benjamani	Genes
MAPK signalling pathway	0.0011	4.1	0.084	Mapkapk2, Dusp9, fgf2, Grb2, Il1b, Mef2c, Ntrk1, Ptpn7, Max
Jak-STAT signalling pathway	0.0012	5.6	0.049	Csf2rb2, Grb2, Ifnb1, Il13ra2, Il20, Il6st, Stat3
Pathways in cancer	0.013	3	0.31	asp9, Fgf2, Grb2, Ntrk1, Ptgs2, Max, Stat3, Wnt5a
Alzheimer's disease	0.015	4	0.26	ApoE, Bad, Casp9, Il1, Cx1, Cx1V
Apoptosis	0.032	5.6	0.42	asp9, Csf2rb2, Il1b, Ntrk1

Table 4

List of qPCR primers.

Target gene	Forward (5'–3')	Reverse (5'–3')
Succinate dehydrogenase	GCAGAGCCTGTGCCCTGAGC	CCCACACGGAACACTGCAGCA
Beta actin	TGTGCTGCTCACCGA	TGGCTACGTACATGG
Sirt1	GCAGATTAGTAAGCGCTTGAGGGT	TGGCATGTGCCACTGTCAGTGTT
Apolipoprotein E	GCTGCAGAGCTCCCAAGTCACAC	TTCTCCAGCTCCTTTTGTGAAGCC
Igf2	CGCCGGCTTCCAGGTACCAAT	ACACGGCGAAGGCCAAAGAGATG
Tnf- α	CAGAGGTGTGCTGCACCTCGAT	CGGGCCTCTGGGAATTCACCT

Discussion

The current study shows that dietary supplementation with DHA can alter the molecular profile of the hippocampus in diabetic mice in a way that is consistent with DHA being neuroprotective. Previous studies have shown that dietary supplementation with DHA has beneficial effects on cognition and memory and it also promotes neuronal survival [30,31]. In the present study, mice that were already diabetic had their diet supplemented with DHA for six weeks. This dietary intervention resulted in an increase in hippocampal insulin like growth factor II (*Igf II*) and *Sirt1* expression and a decrease in hippocampal expression of pro-inflammatory *Tnf- α* , *Il6* and *ApoE* lipoprotein, relative to non-supplemented diabetic mice.

Igf II is a mitogenic polypeptide that is relatively abundant in the hippocampus and is thought to be involved in cognitive functions. Indeed, hippocampal *Igf II* levels decline with ageing, in parallel with age-related cognitive decline [32–35]. Schmeisser et al., 2012 demonstrated the presence of *Igf II* and *Igf II* receptors in synaptic elements of hippocampal neurons, which is consistent with the notion that *Igf II* has a role in synaptic plasticity and cognitive functions [36]. Wuarin et al., 1996, showed that *Igf II* expression was decreased in the brains of both insulin dependent and independent diabetic animals [37] and consistent with this, we found by qPCR

analysis that STZ-induced diabetes caused a reduction in *Igf II* ($P = 0.025$). An increase in *Igf II* expression in the DHA supplemented diabetic group is particularly encouraging as *Igf II* has been shown to activate *Igf I* and *Igf II* receptors to mediate memory enhancement [34]. Additionally, Bracko et al., 2012 and Agis-Balboa et al., 2011 have demonstrated that *Igf II* is a regulator of adult hippocampal neurogenesis [38,39]. The neuroprotective effects of the *Igfs* are thought to be due to the involvement of *PI3k/Akt* signalling pathways [40]. In this regard, our DHA supplemented diabetic group showed a 1.6 fold increase in expression of *Pi3kr2*, also known as *P85- β* , a regulatory subunit of *PI3k* [41], which has been shown to be crucial for *PI3k*-dependent memory consolidation processes in the hippocampus [42].

The most significantly enriched pathways, as determined by KEGG analysis, in the DHA supplemented group, were the *Mapk* and *JAK/STAT* signalling pathways. These pathways are involved in pro-inflammatory responses and enhance apoptosis in several neurodegenerative conditions [24]. Notably, microarray analysis indicated a number of the pro-inflammatory genes in these pathways (*Mapkapk2*, *Il1b*, *Il20*, *Il13ra2*, & *Il6st*) were significantly down regulated in the hippocampus of DHA supplemented diabetic mice. Previously, it was shown that *Mapkapk2* deficiency reduced neurodegeneration and neuro-inflammation in a Parkinson's disease model [43], further demonstrating a role for *Mapkapk2* in neuro-inflammation. We also found a decrease in *Tnf- α* expression in the DHA supplemented diabetic group. *Tnf- α* is another pro-inflammatory factor and can directly trigger the death of neurons by inhibiting *Igf* survival responses [44]. There was also a trend for a decrease in expression of the pro-inflammatory cytokine, *Il6* (data not shown). Taken together, these data indicate DHA supplementation induces an anti-inflammatory effect in the context of diabetes. Increased expression of pro-inflammatory mechanisms in STZ-induced diabetes has been previously reported [45,46] and our findings support a case for DHA in limiting the potential damage cause by inflammation in this type of metabolic insult.

Another gene of interest that showed a significant increase in expression in the DHA supplemented group was *Sirt1* (silent information regulator 1). *Sirt1* is a member of the sirtuin family that encodes a class III histone deacetylase [47]. *Sirt1* is expressed in hippocampal neurons and has been shown to improve learning and memory by activating the brain derived neurotrophic factor (BDNF) gene [48–50]. *Sirt1* has been previously shown to be decreased in the brains of old rodents [51], indicating a role for *Sirt1* in age

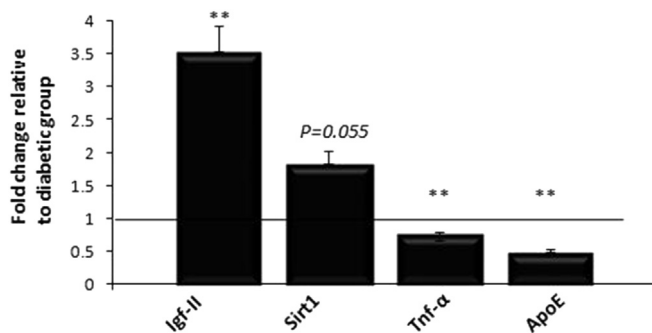


Fig. 1. Confirmation of gene expression by RT-qPCR. Increased *Igf-II* and *Sirt1*, and decreased *Tnf- α* and *ApoE* expression in the hippocampus of DHA (50 mg/kg/day) supplemented STZ-induced diabetic C57BL/6 mice. Once diabetes was established and stable, mice were fed either a control diet or one supplemented with DHA. Data are presented as means $\pm$ SEM, relative to control diet diabetic mice. ** $P \leq 0.05$ ($n = 6$ /group).

related cognitive decline. Increased expression of Sirt1 in the hippocampus of DHA supplemented diabetic group as seen in the present study may be one of the mechanisms by which DHA could lead to cognitive improvement in diabetic condition. Both Igf and Sirt1 are involved in hippocampal neurogenesis and synaptogenesis, although the mechanisms that underlie this involvement are complex and require further clarification [48,51]. Notwithstanding this lack of mechanistic detail, our finding that DHA supplementation increased the hippocampal expression of both *Igf* and *Sirt1* in diabetic but not in normal mice [27], further underscores its potential as a therapeutic agent.

Lastly, we also found a decrease in *ApoE* expression in the DHA supplemented, relative to non-supplemented, diabetic group. Previously, we reported an increase in *ApoE* expression in the hippocampus of STZ induced diabetic mice [14] and an increase in *ApoE* expression has been suggested to be indicative of ongoing neuronal dysfunction [52]. In the CNS, *ApoE* is mainly produced by astrocytes and transports cholesterol to neurons via *ApoE* receptors, a process that is crucial for synaptogenesis [53]. The decrease in *ApoE* expression could be of clinical importance as *ApoE* is involved in key immune responses, including the production of pro-inflammatory cytokines such as *Il1b*. Interestingly, *Il1b* was one of the down-regulated genes (–1.8 fold) in the DHA supplemented diabetic group. Liu et al. (2011) demonstrated that neurons exposed to *Il1b* express elevated levels of *ApoE*, a finding that is similar to that observed in neurodegenerative diseases such as AD [54]. Therefore, a significant decrease in *ApoE* expression in the hippocampus of the DHA supplemented diabetic group is yet another indicator of the potential beneficial effects of DHA and warrants further investigation.

Overall, the present study helps to elucidate the molecular changes in the hippocampus of diabetic mice following dietary supplementation with DHA. Our findings provide an important framework for future studies to further investigate the effects of nutritional intervention on the molecular mechanisms that cause diabetes related cognitive dysfunction. Furthermore, our findings underscore the potential of naturally occurring compounds such as DHA.

Authors' contribution

All authors participated in the conception and study design. JT and DWS carried out the animal work. JT performed qRT-PCR and data analysis. JT, DWS and MLG prepared the manuscript. All the authors contributed to the revisions and subsequent drafts and reviewed the final version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary material

Supplementary data related to this article can be found online at <http://dx.doi.org/10.1016/j.jnim.2015.04.001>.

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