Chitosan from crabs (*Scylla serrata*) Represses Hepato-renal Dysfunctions in Rats via Modulation of CD43 and p53 Expression in High Fat Diet-induced Hyperlipidemia

Regina Ngozi Ugbaja¹,³*, Kunle Ogungbemi¹,², Adewale Segun James¹, Ayodele Peter Folorunsho¹, Samuel Olanrewaju Abolade¹, Stella Ajamikoko¹, Eniola Olapeju Atayese¹, and Omowunmi Victoria Adedeji¹.

Affiliations:
1 Department of Biochemistry, College of Bioscience, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria
2 Department of Chemistry/Biochemistry, Nigerian Stored Product Research Institute, Ibadan, Oyo State, Nigeria
3 Department of Chemical Sciences (Biochemistry Program), Faculty of Science, Augustine University, Ilara-Epe, Lagos State, Nigeria

Correspondence: Regina Ngozi Ugbaja (Ph.D).
Email: ugbajarn@funaab.edu.ng; regina.ugbaja@augustineuniversity.edu.ng
Tel: +234(0)7066050043
Hepato-renal dysfunctions associated with hyperlipidemia necessitates continuous search for natural remedies. This study thus, evaluated the effect of dietary chitosan on diet-induced hyperlipidemic rats. Thirty male Wistar rats (90 ± 5.2) g were randomly allotted into six (6) groups (n=5): Normal diet, High-fat diet (HFD), Normal diet + 5% chitosan. The three other groups received HFD, supplemented with 1%, 3%, and 5% of chitosan. The feeding lasted for 8 weeks, after which the rats were sacrificed. Liver and kidneys were harvested for analyses. Hepatic alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) activity, and renal biomarkers (ALT, AST, urea, and creatinine) were assayed spectrophotometrically. Additionally, expression of hepatic and renal CD43 and p53 was estimated immunohistochemically. Hyperlipidemia caused a significant (p<0.05) decrease in the hepatic (AST, ALT, and ALP) and renal (AST and ALT) activities, while renal urea and creatinine increased. Furthermore, the HFD group showed an elevated level of hepatic and renal CD43 while p53 expression decreased. However, groups supplemented with chitosan showed improved hepatic and renal biomarkers, as well as corrected the aberrations in the expressions of p53 and CD43. Conclusively, dietary chitosan could effectively improve kidney and liver functionality via abatement of inflammatory responses.

**Keywords**: Chitosan, hyperlipidemia, high-fat diet, p53 and CD43 genes, functional indices.
1.0 Introduction

Hyperlipidemia (HLP) is a family of disorders that are characterized by abnormally high levels of lipids in the blood. It is the primary risk factor contributing to the formation and progression of atherosclerosis (AS) and cardiovascular diseases (CVD) (Navar-Boggan et al., 2015; Ahmad and Beg, 2013). The ever-increasing prevalence of HLP might be responsible for continuous upsurge in the demographic data of obesity and attendant organ dysfunction worldwide (Ugbaja et al., 2020). Indeed, chronic renal failure parallels with the occurrence of premature atherosclerosis and cardiovascular comorbidities and mortalities (Vaziri, 2006). Clinical evidence also suggests that atherosclerosis caused due to HLP predates renal failure (Kaysen, 1994). Furthermore, HLP contributes to the onset of fatty liver disease and is prodromal to type-2 diabetes (T2D) -induced hepatocellular damage and mortalities (Al-Jameil et al., 2014). The hallmarks of liver disease includes deranged liver function biomarkers (such as ALT, AST, ALP and Bilirubin), culminating in non-alcoholic fatty liver (NAFLD), cirrhosis, and acute liver failure (Al-Jameil et al., 2014). Clearly, inflammatory responses mediated by the activated immune cells such as macrophages, monocytes, neutrophils, and T-cells represent connects HLP and hepato-renal dysfunctions (Ugbaja et al., 2020). Therefore, the mechanism underlying HLP-induced liver and kidney damage might be linked to inflammatory pathways and oxidative stress (Napoli and Flores, 2017).

CD43, (Sailophorin or Leukosialin) is one of the most common leukocyte transmembrane sialoglycoprotein that is expressed by inflammatory cells such as monocytes, neutrophils, macrophages, and T-lymphocytes with distinct physiologic functions such as differentiation,
proliferation, and apoptosis. But its overexpression has also been implicated in different tumors of non-hematopoietic cells and immune-deficiency (Balikova et al., 2012). Apart from its roles in DNA repair, cell cycle arrest, apoptosis, and onco-gene activation (Vousden, 2009), p53 has been shown to coordinate intermediary metabolism such as regulation of glycolysis and repression of lipogenesis via the inhibition of sterol regulatory binding protein-1 (SREBP-1). This way, p53 regulates lipid anabolism by compensatory activation of lipid oxidation pathways (Napoli and Flores, 2017). Moreover, alterations of p53 expression has been shown to contribute to aging and associated diseases, cardio-metabolic disorders, atherosclerosis, and cellular hyper-proliferation (Minamino et al., 2009; Napoli and Flores, 2017).

Chitosan (CTS), a de-acetylated derivative of chitin, is a natural polymer of glucosamine derived from the cell walls of some fungi, and the exoskeleton of crustaceans including shrimps, crabs, lobsters, and prawns (Zhang et al., 2012; Ugbaja et al., 2020). The hypolipidemic effect of chitosan has been extensively explored by many scientists (Zhang et al., 2011; 2013; Selvasudha and Koumaravelou, 2017). Further, chitosan has attracted much awareness as a biomedical material of importance, due to its reported expansive biological activities, including, but not limited to antitumor (Kumar et al., 2004), immune-stimulating (Jeon et al., 2001), anti-allergic (Vo and Kim, 2014), cholesterol-lowering, and anti-inflammatory activities (Chung et al., 2012), and free radical scavenging activities (Ugbaja et al., 2020). Despite the extensive studies carried out on the bioactivities of chitosan, there is a dearth of information on the effect of chitosan with respect to HLP-induced hepato- and reno-cellular dysfunctions. In addition, the effect of chitosan on hyperlipidemia-invoked dysregulation of p53 and CD43 expression has not been explored. We therefore, examine the effects of chitosan on the hepatic and renal biomarkers as well as the expressions of CD43 and p53 in hyperlipidemic rats in vivo.
2.0 Materials and methods

2.1 Materials

2.1.1 Sample collection and Preparation of raw materials
Crab (*Scylla serrata*) shell wastes (carapace) containing majorly the exoskeleton was collected from Epe market in Lagos, South-West, Nigeria. The crab shells were air-dried and grinded to powder. The powdered crab shell was then transferred to the Chemistry laboratory of Nigerian Stored Product Research Institute (NSPRI), Onireke, Ibadan, Oyo State, Nigeria for the extraction of chitosan.

2.1.2 Extraction of Chitosan
Extraction of chitosan from the powdered carapace samples were done, following the methods of Burrows *et al.* (2007) as succinctly described previously (Ogungbemi *et al.*, 2020).

2.1.3 Experimental Animals
Thirty (30) male Wistar rats, weighing between (90 ± 5.2) g were procured from the Department of Physiology, College of Veterinary Medicine, Federal University of Agriculture, Abeokuta (FUNAAB), South-west Nigeria, and used for the study. The rats were allowed to acclimatize the experimental location for two weeks before the commencement of the experiment in the Animal House of the Department of Biochemistry, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria. The study was approved by the Departmental Ethical Committee (FUNAABBCH1819-1). The animals were kept in well-ventilated plastic cages at ambient conditions and handled humanely as conformed to the guidelines of the National Research Council (NRC, 2010) on the use of experimental animals.

2.1.4 Experimental diet composition
The High-fat diet containing 50% maize, 10% soya, 15% groundnut cake, 10% fish meal, 8% Palm kernel cake, 5% soya oil and 0.5% of methionine, lysine, grower premix and salt was mixed with the dietary chitosan using a blender to ensure uniform mixing of the components. Diets were compounded to include 1%, 3%, and 5% chitosan, pelletized and feed to the animals for 8 weeks (Ogungbemi et al., 2020). The animals’ body weight was monitored throughout the experimental period.

2.2 Methods

2.2.1 Experimental design

The experimental animals were divided into six (6) groups, each group with five animals. Group 1: Control animals fed with Normal diet, group 2: High-fat diet (HFD) control received HFD only group 3: Normal diet supplemented with 5% chitosan. Groups 4, 5, and 6 were fed with the HFD, supplemented with 1%, 3%, and 5% chitosan respectively.

2.2.2 Sacrifice and collection of samples

After the stipulated weeks of treatment, the rats were weighed and sacrificed after an overnight fast under light anaesthesia. The liver and kidney were excised, washed in cold physiological saline and frozen until needed for analyses. The organs were fixed in 4% phosphate buffered formalin for immuno-histochemical analyses.

2.2.3 Biochemical Analyses

Alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine transaminase (ALT) activities urea and creatinine levels were estimated by standard procedures according to the manuals from Randox Diagnostic Kits (Crumlin, England, United Kingdom).

2.2.4 Immuno-histochemical analyses for CD43 and p53

Paraffin-embedded sections of the liver and kidney tissues were de-paraffinized and rehydrated using graded alcohol concentration (Krishna et al., 2014). Antigen retrieval was achieved by
boiling in 10 mmol/L sodium citrate buffer for 10 min and then steadily cooling to room temperature. Subsequently, the sections were blocked using 3% H$_2$O$_2$ in methanol for 15 min to inhibit endogenous peroxidase activity. Following washing in phosphate buffer saline (PBS), the sections were incubated overnight at 4°C with monoclonal rabbit anti-p53 and anti-CD43 primary antibodies at a dilution of 1:40. The sections were incubated with peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) at the same dilution of 1:500 for 2 h at 37°C. The sections were washed in PBS, developed in prepared DAB chromogen solution, lightly counterstained with haematoxylin, dehydrated, mounted and visualized under light microscope.

2.3 Statistical Analysis

Data are expressed as mean ± standard error of mean. Analyses was done using statistical package for social sciences (SPSS) version 20, the level of homogeneity among the results test groups, was done using one-way analysis of variance (ANOVA), with p<0.05 considered significant. Where heterogeneity occurred, the groups were separated using Duncan Multiple Range Test (DMRT). Graphs were plotted using GraphPad Prism (Version 5). Immunohistochemistry quantification was done using ImageJ in triplicates.

3.0 Results

3.1 Cumulative weight gain of the experimental animals

There was significant (p<0.05) increase in the body weight of the animals fed with HFD only when compared with those fed with the normal diet (Table 1). Nevertheless, there was progressive reduction in the body weight of the animals fed with HFD containing varying level of chitosan (1, 3, and 5% respectively). The reduction in the body weight appeared to be lowest in the group fed with HFD + 1% chitosan.
Table 1: Effect of chitosan supplementation on cumulative weight gain of rats fed with High-fat Diet (HFD)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cumulative weight gain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal diet</td>
<td>42.33±1.76&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>High fat diet (HFD)</td>
<td>60.05±3.44&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Normal diet and 5% Chitosan</td>
<td>27.52±1.09&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HFD and 1% Chitosan</td>
<td>26.61±1.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HFD and 3% Chitosan</td>
<td>46.42±1.59&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HFD and 5% Chitosan</td>
<td>40.78±1.61&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM (n=5). Values with different superscript (a, b, c) down the column are significantly different (p<0.05).

3.2 Effects of chitosan supplementation on renal biomarkers

The specific activities of AST and ALT, as well as the levels of urea and creatinine of rats maintained on HFD and/or chitosan is depicted in figure 1. There were significant (p<0.05) decrements in the activities of AST and ALT in the group fed with HFD alone when compared with the normal diet group. However, groups supplemented showed gradual improvement in the activities of these enzymes. Interestingly, there appeared to be a hormetic response in the chitosan-treated group, as the 1% chitosan- supplemented group showed more improved activities of AST and ALT. Further, there was no marked difference in the AST and ALT activities between the normal diet and normal diets ± 5% chitosan. Urea level increased significantly (p<0.05) in the untreated HFD group when compared with the normal diet group.
Similarly, the renal creatinine level increased in the HFD only group relative to the control. Nevertheless, the group fed with HFD + 5% chitosan showed a lowered urea and creatinine level when compared with the HFD control group. The normal diet + 5% chitosan group did not differ from the normal diet group for both the urea and creatinine levels.

Figure 1: Effects of chitosan supplementation on renal biomarkers of High-fat diet (HFD) - fed rats. Values are expressed as mean ± SEM (n=5). Bars with distinct letters are statistically different. AST- Aspartate aminotransferase; ALT – Alanine aminotransferase; CTS - chitosan

3.3 Effects of chitosan supplementation on hepatic biomarkers

In figure 2, the hepatic biomarkers (i.e. AST, ALT, and ALP) are shown. Consistently, AST, ALT, and ALP activities were significantly (p<0.05) lowered in the HFD-treated group when matched with the normal diet-fed group. However, the AST activity improved more in the
HFD+1% chitosan group while no significant difference exists between the 3- and 5% chitosan-supplemented diet group. Further, there was dose-dependent increment in the ALP activity of the HFD groups supplemented with chitosan. There was no significant difference in the ALT activity of the rats in the HFD + 3- and 5% groups; otherwise, the increment of the activity is dose-dependent.
Figure 2: Effects of chitosan supplementation on hepatic biomarkers of High-fat diet (HFD) - fed rats. Values are expressed as mean ± SEM (n=5). Bars with distinct letters are statistically different. AST- Aspartate aminotransferase; ALT – Alanine aminotransferase; ALP- alkaline phosphatase; CTS - chitosan
3.4 Effects of chitosan supplementation on hepatic CD43 and p53 expressions

The percentage positivity of hepatic CD43 is shown in figure 3. The expression was markedly (p<0.05) in the HFD group relative to the normal diet group. Nonetheless, the protein expression were significantly lower in the chitosan-supplemented HFD groups. Interestingly, the group supplemented with 1% chitosan appeared to be the most effective in lowering the CD43 expression. Contrastingly, the expression of p53 decreased significantly in the HFD-fed group when compared with the normal diet group. However, a dose-dependent increase was observed in the chitosan supplemented group. Indeed, the 5% chitosan normalized, completely the expression of p53 comparable to the normal diet group (figure 4).

Figure 3: Photomicrograph of immune-histochemical expression of hepatic CD43. Values are expressed as mean ± SEM (n=3). Bars with distinct letters are statistically different.
3.5 Effects of chitosan supplementation on renal CD43 and p53 expressions

A significant (p<0.05) increment was observed of renal CD43 expression in rats fed with HFD in relation to the normal diet group (figure 6). But, the HFD + chitosan groups showed considerable decrements in the CD43 positivity. The lowering of CD43 by the different chitosan inclusion appeared to be similar as there was no statistical difference between the groups. The renal p53 was considerably decreased in the HFD group when compared with the normal diet-fed group (figure 7). Interestingly, the group fed with HFD + 3% chitosan showed an increased level of p53. Noteworthy, the HFD + 5% chitosan group showed a comparable p53 expression to the normal diet group.
Figure 5: Photomicrograph of immune-histochemical expression of renal CD43. Values are expressed as mean ± SEM (n=3). Bars with distinct letters are statistically different.

Figure 6: Photomicrograph of immune-histochemical expression of renal p53. Values are expressed as mean ± SEM (n=3). Bars with distinct letters are statistically different.
4.0 Discussion

This study provides a potential mechanism underlining the protective effect of crab chitosan on liver and kidneys of hyperlipidemic rats \textit{in vivo}. The role of hyperlipidemia in the induction of oxidative stress has been linked to excessive production of ROS (Ugbaja \textit{et al.}, 2020). Induction of oxidative represents a putative link between the onsets of organ dysfunctions and clinical manifestations (Gyuraszova \textit{et al.}, 2020). Hyperlipidemia has been shown to cause liver diseases such as non-alcoholic fatty liver disease (NAFLD), cirrhosis, among others while chronic kidney disease has been linked to hyperlipidemia; both organs dysfunction are inflammation–linked (Vaziri, 2006; Napoli and Flores, 2017). Inflammation is a multi-pathways cellular process involving the transcription factors such as NF-κB, AP-1, cytokines such as IL-6, and TNF-α, and protein such as p53. Much has been known of p53 as the ‘guardian of the genome’ via regulation of cell cycle, cell remodeling, apoptosis among others (Timofeev \textit{et al.}, 2013). Other metabolic activity of p53 has to do with its regulatory function on lipid metabolism and homeostasis. This protein (p53), controls the expression of lipid complex breakdown and absorption, limits lipogenesis, and inhibits the mevalonate pathway (Lacroix \textit{et al.}, 2020). Recently, the inherent pro-inflammatory role of CD43; a co-stimulatory molecule of the T- cells was identified. It was shown that, CD43 possesses the intrinsic ability to activate the NF-κB, and cAMP response element binding protein (CREB) pathways (Bravo-Adame \textit{et al.}, 2017). Furthermore, CD43 has been suggested to coordinate the expression of multiple cytokine genes and act as a chemotactic factor with a pro-adhesive role (Fierro \textit{et al.}, 2006; Bravo-Adame \textit{et al.}, 2017). This study for the first time, investigated the possible modulatory effects of chitosan on the expression of hepatic and renal p53 and CD43 as well as hepato-renal functionality of hyperlipidemic rats.

Expectedly, the increased cumulative weight gain (table 1) observed in the HFD untreated group is consistent with other study (Xu \textit{et al.}, 2008). This might be associated with increased
food intake (Ogungbemi et al., 2020) as the excessive calorie is accumulated by the animal in this group culminating in excessive weight gain and the attendant hyperlipidemia. However, following supplementation with the chitosan, the cumulative weight was normalized suggesting the ability of the animals to regulate their feed intake. It could also be due to feeling of satiation and satiety usually associated with fibre-rich diet (Dreher, 2015).

ALT and AST are cytosolic enzymes whose activities has been used as an index of hepatic and renal functions. Dysregulation of both enzymes are prodromal to chronic kidney and non-alcoholic liver diseases (Ochiai et al., 2020). These enzymes leaks out of the cytoplasm as a direct response to cellular damage into the blood stream thereby elevating their plasma activities while the activities reduces in the leaking organ (s) (Mansourian et al., 2019). Our experimental data showed a tremendous reductions in the renal activities of AST and ALT with a concomitant increments in the urea and creatinine levels in the HFD-untreated group relative to the control animals. This might not be surprising; HFD might cause alteration to renal lipid metabolism by creating an imbalance between lipogenesis and oxidation thereby, inducing a systemic cellular abnormality that might culminate to renal injury and consequent leakage of these enzymes (Deji et al., 2009; Ochiai et al., 2020). Nevertheless, treatment with chitosan however, augmented the activities of these enzymes while reducing the urea and creatinine levels to normal (figure 1). To the best of our knowledge, we are reporting the ameliorative effects of chitosan on hyperlipidemia-invoked kidney injury. Akin to AST and ALT, ALP is a marker enzyme for hepatobiliary damage and hepato-necrotic injury and might leak out from the damaged hepatic cells (Elsonbaty et al., 2019). Accordingly, hepatic AST, ALT, and ALP (figure 2) decreased in the HFD-untreated group in this study. This might be associated with hyperlipidema-induced hepatic damage (Ochiai et al., 2020). Regardless, a dose-dependent amelioration was observed in the hepatic ALP activity, while the AST and ALT activities were equally improved in the groups supplemented with chitosan. This observation is consistent with
other studies which suggested hepato-protective role of chitosan (Elsonbaty et al., 2019; Anna et al., 2020). The in vivo antioxidant and hepato-protective effects of chitosan was suggested to be due to the possession of positively-charged amine group which limits lipid absorptions in the intestine and also attracts the negatively charged bile components thereby limiting or inhibiting the accumulation of lipids into the organs (Barrea et al., 2019; Ugbaja et al., 2020).

In this study, there was downregulation of renal and hepatic p53 expression following HFD feeding relative to the normal diet group. The homeostatic and regulatory role of p53 on intermediary metabolism includes inhibition of biogenesis via a convergent clamp down of de novo fatty acid synthesis and regulation of the mevalonate pathway (Lacroix et al., 2020). P53 essentially represses the transcription factor; sterol regulatory binding protein-1c (SREBP-1c) which is a master regulator of key lipogenic enzymes such as fatty acid synthase (FASN), ATP-citrate lyase, HMG-CoA reductase and so on (Aylon and Oren, 2016; Lacroix et al., 2020). Therefore, attenuation of p53 expression in the HFD-fed group might result into over-production of the cellular lipid pools which might also lead to accumulation of such lipids in the renal and hepatic tissues. As earlier noted, accumulation of lipids in the renal and hepatic cells are prodromal to hepato-renal dysfunctions (Napoli and Flores, 2017). More importantly, the downregulation of p53 might exacerbate inflammation and increases predisposition to cancer (Komarova et al., 2005). Nevertheless, our data showed that chitosan possess the ability to normalize the abatement of p53 expression due to HFD consumption. A dose-dependent increase in p53 expression was observed in the hepatic tissue while the 3% chitosan-supplemented group showed the highest expression in the kidney. The underplaying playing mechanism behind this observation remains yet enigmatic. However, Lee et al. (2016) showed that chitosan nanoparticle activated and upregulated the expression of p53 due its ability to enter the nucleus to enhance p53 transcription. This might be the underplaying mechanism.
CD43 expression was elevated in the renal and hepatic tissues of the HFD group that were not treated with chitosan. CD43 is a glycoprotein that is expressed on macrophages and other inflammatory cells with a pro-inflammatory cytokines adhesive roles. It has been implicated in every stages of atherosclerotic plaque formation (Meiler et al., 2015). Another implicative role of CD43 upregulation is the inhibition of cholesterol efflux via the ABCA1 and ABCG1 proteins suggesting its pro-hyper-cholesterolemic effect and the attendant atherosclerotic plaque formation. Indeed, hypercholesterolemia initiates and sustains atherosclerosis due to the generation of oxidized low-density lipoproteins (OxLDL) and other metabolic by-products that triggers inflammatory responses (Gruber et al., 2016). Therefore, upregulation of CD43 expressions in the liver and kidney tissues might be the molecular trigger for the organs dysfunction (i.e. liver and kidneys). Nevertheless, our data shows that inclusion of chitosan into the HFD significantly reduced the expression of the hepatic and renal CD43. This observations suggests that crab chitosan might possess anti-inflammatory role and the ability regulate the activity of CD43 and also, intrinsic ability to inhibit the formation of atherosclerotic plaque \textit{in vivo}. Indeed, mouse deficient of CD43 ($CD43^{-/-}$ BMT mice) did not present with atherosclerosis following sixteen weeks of high-fat diet consumption. Suggesting that, the upregulation of CD43 is necessary for the formation of atherosclerosis following HFD intake. Accordingly, the anti-inflammatory role of chitosan derivatives has been alluded to inhibition of the hyper-proliferation of immune cells, reduction of infiltration of neutrophils and mast cells as well as augmentation of cellular antioxidant systems (Ngo et al., 2015; Ugbaja et al., 2020). This study further strengthen the reported pro-inflammatory role of CD43 during hyperlipidemia and present chitosan as a possible therapeutic target in the management of the same. Although, this study showed a reciprocal modulation of chitosan on the expressions of p53 and CD43, the underlining mechanism remains yet elusive. This might be a focus for further studies as this will shed more light into the role of chitosan in the remediation of
hyperlipidemia. Taken together, this study shows that chitosan derived from crabs possesses hepato-renal protective effects against hyperlipidemia- invoke damages. This is mediated by modulation of kidney and liver biomarkers and down regulation of pro-inflammatory CD43 expression and upregulation of p53 in rats submitted to high-fat diets.

Authors’ Contributions

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript: RNU and OK conceptualize the study, SOA, EOA, SA, OVA carried out the project, ASJ, OK, APF supervised the project, RNU, OK, and ASJ prepared the manuscript.

Conflict of Interests

We declare no known competing interests

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