The impact of partial hepatectomy on oxidative state in the liver remnant – An in vivo swine model

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Abstract

Background: Previous studies on oxidative state after partial hepatectomy (PHx) report conflicting data on levels of glutathione (GSH) and are mainly presented in rodent models by methodology less sensitive than the present technologies. The current swine model presents GSH levels and the following genetic response post-PHx, utilizing an analytical platform more sensitive and precise than earlier available.

Method: Twelve pigs were randomized to a PHx- and a control group (n=6 in each). The PHx group had a 60% hepatectomy. Serial in vivo liver biopsies during 12 h of anaesthesia post-PHx were analyzed for GSH by liquid chromatography mass spectrometry (LC-MS/MS). Transcriptional alterations of genes (GS, GCLM, GCLC, GR, HGF, NFE2L2, TGFβ1) regulating GSH synthesis were measured by real-time PCR.

Results: No difference was detected between the GSH levels in the PHx- and the control group during the first seven hours post-PHx. The results show that the liver has an extended capacity to maintain GSH homeostasis during major stress and parenchymal loss, even at the early onset of such trauma. This observation was not explained by increased expression of key genes in GSH pathways. Consequently, the results indicate an inherent compensatory capacity to maintain GSH homeostasis in the reduced organ.

Conclusion: The results show that partial hepatectomy (PHx) influences the oxidative state in normal and pathologic liver by increased GSH synthesis after PHx [2]. These studies report a reperfusion injury in the heart and liver disease [3–5,7,8,24,25,29,30].

The hepatic GSH homeostasis plays a central role in the defence against oxidative stress [18,19]. Under physiological conditions, the liver is protected from oxidative stress by the capacity of hepatocytes to synthesize GSH [9]. Pathophysiological consequences of hepatic oxidative damage include dysregulation of lipid metabolism (steatosis), impaired liver function (hepatocyte degeneration and death) and activation of the immune response (inflammation and fibrosis/cirrhosis) [22]. Alteration in liver GSH homeostasis may become manifest in liver diseases such as non-alcoholic fatty liver disease [23], alcoholic liver disease [1] and drug-induced liver injury [21]. Yet, the precise mechanism by which depletion of GSH promotes pathophysiological changes in the liver has not been clearly defined.

Previous studies have shown that partial hepatectomy (PHx) influences the oxidative state in normal and pathologic liver by increased GSH synthesis after PHx [2]. These studies report a

1. Introduction

Glutathione (γ-glutamylcysteinylglycine; GSH) is present as an ubiquitous compound in virtually all mammalian tissues, particularly in the liver [15,17]. GSH plays a major role in the defence against oxidative stress by protecting cells from fatal damage of reactive oxygen species (ROS). GSH has emerged as the determinant of oxidative state in mammalian cells [19] and is regarded as a critical intracellular antioxidant [13]. There is growing awareness that GSH has a protective role by scavenging oxidative stress in clinical conditions such as malignant diseases, diabetes, atherosclerosis, chronic inflammation, viral infection, ischemia-

To distinguish between genes and their proteins, gene names are typed in italics.
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diversity of conclusions regarding levels of GSH in the liver remnant \[7,14,16,28\]. Further, the studies are based on less sensitive analytical platforms and the majority has focused on rodent models. In contrast, the capacity of the liver to maintain GSH homeostasis after PHx in large animal models, like swine, has received little attention despite the fact that porcine liver is more anatomically and physiologically comparable with human liver.

We recently presented a newly established method of quantifying GSH in swine hepatocytes using liquid chromatography tandem mass spectrometry (LC-MS/MS), including reference levels of GSH in swine hepatocytes as supplement to the methodological approach in future liver GSH research \[11\]. Moreover, application of LC-MS/MS in quantifying GSH is limited, and as far as we know, not yet established as a quantitative method of GSH analysis after PHx in a porcine model. Additionally, characterizing the gene expression of GSH related enzymes using specifically designed reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR), has not previously been presented in a swine model.

Accordingly, the primary aim of this study was to investigate whether a PHx in a swine model would alter the GSH levels in the liver remnant, using the precision and sensitivity of LC-MS/MS technique. The second aim was to elucidate possible consequences in the regulation of key genes in GSH metabolism using RT-qPCR.

2. Method

2.1. Animal welfare and preparation

The protocols were approved by the committee of the Norwegian Experimental Animal Board, and all experiments were conducted in compliance with the institutional animal care guidelines and the National Institute of Health’s Guide for the Care and Use of Laboratory Animals [DHHS Publication No. (NIH) 85-23, revised 1985]. A total of 12 female 3 months old Yorkshire/Landrace hybrid pigs, average weight of 38.2 kg, were stalled and acclimatized in the animal research facilities one week before experiments. A standardized diet, light-dark cycle and water access was maintained. All animals fasted overnight with free access to water before experiments were started between 7 and 8 a.m. One pig had gastroenteritis the days before surgery, but was included in the experiment.

2.2. Anaesthesia and monitoring

Intramuscular ketamine 15 mg/kg (Narketan; Véloquinol, Ittigen, Switzerland), 1 mg of atropine (Atropin; Nycomed Pharma, Asker, Norway) and midazolam 1 mg/kg (Midazolam B. Braun, Melsungen, Germany) were used as premedication before the animal was cleaned and weighed. Mask inhalation of 4% isoflurane (Isofluran Baxter;Baxter, Irvine, CA) in 100% O₂ was given before intubation. Gas anaesthesia throughout the experiment was maintained with isoflurane and an alveolar concentration of 0.8–1.2% mixed with 45–65% oxygen. Deep anaesthesia was induced by an IV bolus of 0.1 mg/kg fentanyl (50 μg/ml Fentanyl-Hameln: Hameln Pharmaceuticals Gmbh, Hameln, Germany) and maintained with IV infusion of 0.02 mg/kg/h fentanyl and 0.3 mg/kg/h midazolam. Respiratory rate was adjusted to achieve an Et CO₂ between 3.5 and 6 KPa, monitored by a Capnomac Ultima (Datex, Helsinki, Finland). Mean arterial pressure and heart rate was monitored through a 20-gauge arterial catheter (BD Arterial Cannula with FloSwitch; Ohmeda, Swindon, UK) placed in the superficial femoral artery. Body temperature was maintained at 38.5 °C by a heating blanket. The urine production was monitored by a cystotomy and a 20 Ch Foley catheter.

2.3. Infusions

An 18-gauge IV catheter (Optiva 2 18 G; Medex Medical, Halsingden, UK) was placed in the ear vein for infusion of 0.9% sodium chloride (B. Braun), fentanyl and midazolam. A 1000 ml initial IV of 0.9% sodium chloride was given during the first 45 min of anaesthesia. The infusion was continued at 20 ml/kg/h for the rest of the anaesthesia, supplemented by 25 ml glucose (Glucose 50 mg/ml, Fresenius Kabi, Halden, Norway).

2.4. Experimental set up

Twelve pigs were randomized into the PHx group and the control group by drawing lots (n = 6 for each, see Fig. 1). The experimental setup consisted of an initial phase in both groups with establishment of the anaesthesia protocol, placement of all catheters and infusions as described above. The liver surgery was performed in the PHx group as described under operative procedure. In the control group only a laparotomy was performed. Both groups were observed 12 h after the partial hepatectomy (PHx group) and the laparotomy (control group) including serial liver biopsies as described in Fig. 1. Finally, all animals were sacrificed after 12 h by an overdose of IV Pentobarbital (Ås Produksjonslab AS, Ås, Norway).

2.5. Operative procedure

A midline laparotomy was established in the control group. In the PHx group a major liver resection was performed. The hepatic artery supplying segments II and III (left lateral lobe) together with the portal branch of these segments were located, isolated and ligated using an absorbable polyfilament suture on a large needle. The parenchyma of segment II and III was crushed by finger fracture technique (Lin TY 1958) under inflow occlusion to isolate vessels and bile ducts for ligation. A 0.5-cm wide cotton ribbon around the base of segments II and III was used after digital

![Fig. 1. Experimental setup. Experimental setup including time-points for each analysis. Time-points in hours after laparotomy. GSH •– liver biopsies for determining glutathione (GSH) values in LC-MS/MS. qPCR ●– liver biopsies for gene expression analysis. Before baseline both groups established the anaesthesia protocol. At baseline both group had a laparotomy before the first liver biopsy was collected. Then only the PHx had a 60% liver resection. Both groups were observed 12 h after baseline with biopsies included.](image-url)
compression to keep occlusion during the resection. The segments were removed with blood loss between 5–15 ml. The segments were weighed. Segments IV, V, and VIII (right lateral lobe) were removed in a similar manner leaving segments VI, VII, and I in place as a liver remnant. The procedure represented a total liver resection of approximately 60%, hence a liver remnant in the PHx group corresponding to approximately 40%. The partial hepatectomy was completed after approximately 30 min.

2.6. Sampling and preparation of liver biopsies

The liver biopsies were collected as presented in Fig. 1 using a 14-gauge biopsy needle (GTA Medical Devices). Biopsies for GSH analysis by LC-MS/MS and for the qPCR analysis were taken from the liver remnant in the PHx group and from the liver in the control group. The sample processing before the LC-MS/MS analysis started immediately as reported earlier [11]. The biopsies for the qPCR analysis were immersed directly in RNAlater (Ambion Inc) and frozen at −70 °C before the RNA isolation took place. The RNA isolation was performed by using the DNA/RNA Miniprep Kit from Qiagen on an automated Qiacube processor (Qiagen AB, Sweden) according to the manufacturer’s instructions.

3. Analysis

3.1. Liquid chromatography tandem mass spectrometry (LC-MS/MS)

The sample processing of the liver biopsies was performed as earlier described [11]. Samples were analyzed by using a Waters Acquity™ I-class system (Waters, Milford, MA) equipped with a Waters Acquity HSS T3 column (2.1 × 100 mm, 1.8 μM) coupled to a Waters Xevo TQ-S tandem quadrupole mass spectrometer (Waters, Manchester, UK) as earlier described [11,20,26].

3.2. Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

The reverse transcription was performed using iScript (BioRad) in all experiments/preparations and experimental accuracy was improved by running samples in duplicate [27]. All TaqMan assays were designed in Beacon Designer 8 (Premier Biosoft, Palo Alto, CA, USA) using porcine mRNA and DNA sequences retrieved from GenBank. Probes were placed across exon splicing points to avoid detection of genomic DNA. Where available, exon splicing points were retrieved from GenBank, alternatively deducted by manual alignment between mRNA and genomic sequences. Three candidate housekeeping genes were tested; hypoxanthine phosphoribosyltransferase 1 (HPRT1), beta actin (ACTB) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (YWHAZ). Assays were run using standard reaction conditions (annealing/extension at 60 °C; denaturation at 95 °C; Mg2+ at 5 mM; Sso Advanced probe mastermix from BioRad) on a BioRad CFX Connect platform. All assays were evaluated by measuring the slope on a “standard curve” plot of a dilution series of a selected sample. Primer sequences are listed in Table 1.

3.3. Statistical analysis

The GSH and gene expression data were analyzed using repeated measures ANOVA by presenting differences over time within the group or comparing groups over time (time by group) (IBM SPSS Statistics 21 for Windows; SPSS Inc., Chicago, IL, USA). All experiments were performed with a balanced design; thus type I sum of squares was used. When deviation from the assumption of sphericity was observed, adjustment of DF by Huynh-Feldt or Greenhouse-Geisser was applied as appropriate. A simple contrast was applied with the baseline measurement as reference. Furthermore, post-hoc pairwise comparisons were run using Bonferroni correction. For gene expression data, ΔCT values (CTTarget – CTRefERENCE) were calculated. As housekeeping (reference) gene a construct variable of HPRT1, ACTB and YWHAZ was validated to be stable across time-points and groups using repeated measures ANOVA. This constructed variable represented the CTRefERENCE in calculating the ΔCT values. In case of non-Gaussian distributed raw data, log-transformation was applied. Differences were considered statistically significant at P < 0.05.

Table 1: TaqMan assays, primer sequences.

| Gene ID | Primers Forward reverse | Probe | Accession no | E |
|---------|--------------------------|-------|--------------|---|
| GS      | GCAGAAGAATGCAACGTGAC    | AGAATTGTCTGCTACGACCTGCC | NM_001244625 | 2.07 |
| GR      | TCGACAATTCCACATA        | CCGTTACCCTTACCTGTAGGCTT | XM_003483635 | 1.98 |
| GCM     | CGGCTGCAATCTAACTCA     | AACTCAAGCAAGCAGACATAAGAA | XM_001926378 | 2.11 |
| GCLC    | GCACAAACCAAACACATC     | CTCCTACGGTTCGACCC     | XM_003482164 | 2.15 |
| NFE2L2  | CCAGAAATGTGCCGAGTCTAAC | CTGAGACTAGCGAGGCCAGTCAAAG | XM_003133500 | 2.12 |
| TGFBI   | GCAGACGGGGTATAAGGC    | TGCCCACTGTTGCTGAGCA  | NM_214015 | 2.14 |
| HGF     | TCAGCAACTCTTACAGTCT    | CCTACATCCTTCTCTGCTCT | XM_003130222 | 1.99 |
| YWHAZ   | GCAGACATGCAAGCTAGGG   | CTCCTTCTTCTGCTGACGC  | XM_001927228 | 2.04 |
| ACTB    | CAGCCGGAAGGACTGCTTG   | AGACCACCTTACCGCAAC   | XM_003357928 | 1.93 |
| HPRT1   | ACTGGAAGAATGCTGACTGGA | TTGCCAGTTGCAATATATCTCTCAACA | NM_001032376 | 2.09 |

Porcine RT-qPCR primer/probe sets were designed in Beacon Designer 8.12 using GenBank sequences as indicated. All primers and probes were subject to a BLAST search showing no sequence homologies of concern on other parts of the pig genome or exome. Sequences are listed 5’ to 3’. The probes were conjugated with fluorescein amide (6-FAM)/black hole quencher (BHQ1). Assay efficiency (E) was measured by serial dilution of cDNA prepared from total RNA extracted from pig liver.
4. Results

4.1. GSH

Raw data GSH values for the PHx- and control group are shown in Fig. 2 with mean values in bold from baseline up until 12 h. No significant difference between the PHX- and control group was observed at baseline ($p=0.2$). There was a significant effect of time adjusted for group differences (ANOVA time $p=0.005$). A simple contrast against the baseline time-point showed significant differences at time-point 1 h, 3 h and 6 h ($P=0.006$, $P=0.032$, $P=0.002$ respectively, marked with asterisk in Fig. 2). There was no significant difference between the PHX- and the control group over time (ANOVA interaction time $\times$ group $P=0.247$) and there was no difference in grand mean between groups ($P=0.451$). An outlier can be noted in the PHX group showing high GSH values from 6 h and through the experiment (Fig. 2). The RM-ANOVA was rerun excluding this animal, but it did not change the conclusions stated above.

4.2. RT-qPCR

4.2.1. Target genes related to GSH synthesis

Endogenous GSH levels are regulated by two pathways (Fig. 3), i.e. the GSH de novo synthesis and the GSH regeneration cycle [10].

4.2.2. Target genes related to the de novo synthesis of GSH and GSH regeneration cycle

To examine the expression of genes involved in the GSH synthesis, we measured the mRNA levels of target genes in the PHX- and control group using qPCR on RNA isolated from liver biopsies. Earlier reports suggest GSH synthase (GS) and glutamate cysteine ligase subunits (GCLM and GCLC) to be the essential enzymes in the de novo GSH synthesis [19]. Further, transforming growth factor-β1 (TGFβ1) and hepatocyte growth factor (HGF) are described to have modifying effect on GSH synthesis, hence TGFβ1 decreases GCLC activity and HGF stimulates both GCLM and GCLC by increasing the enzymatic steps [19]. The transcription factor nuclear factor erythroid 2 (NFE2L2) is described to be a regulator in the GSH synthesis by specifically increasing GCLC gene expression activity [19]. The GS gene expression was significantly different in the PHX group compared to the control group (ANOVA interaction time $\times$ group, $P=0.026$). Hence, the gene expression of GS in the PHX group was significantly lower the first nine hours (Contrast vs baseline: 0.5 h $P=0.009$, 1 h $P=0.012$, 1.5 h $P=0.042$, 5 h $P=0.049$, 9 h $P=0.048$), then the gene expression increased and did not differ from the control group the last three hours (Fig. 4A and B). Further, the enzyme subunits GCCL and GCLM showed no significant differences between the PHX- and control group, $P=0.058$ and $P=0.356$ respectively (interaction time $\times$ group). Still, there was a group independent significant change over time in the gene expression within these two enzyme subunits ($P<0.0005$ for both), as well as within GS ($P<0.0005$).

The second pathway regulating the GSH level is reduction of GSSG by glutathione reductase (GR) [10] (Fig. 3). The GR gene expression (Figs. 4A and 4B) did not show any significant differences between the PHX- and the control group (interaction time $\times$ group, $P=0.481$). A group independent significant change in gene expression over time was detected ($P=0.015$). A simple contrast against the baseline showed significant differences at the time-points 1.5 h, 3 h, 5 h, 7 h, with $P=0.045$, $P=0.013$, $P=0.023$, $P=0.006$ respectively.

Gene expression of the three regulators in the GSH synthesis (NFE2L2, TGFβ1, HGF in Fig. 5A and B) showed that the time-course in all three regulators were significantly different in the PHX group compared to the control group (interaction time $\times$ group $P=0.014$, $P=0.029$, $P=0.011$ respectively). In more details, the gene expression of NFE2L2 was significantly lower the first nine hours in the PHX group (0.5 h $P=0.001$, 1 h $P=0.003$, 1.5 h $P=0.014$, 3 h $P=0.007$, 7 h $P=0.001$, 9 h $P=0.045$ (Fig. 5A)). Further, the expression of TGFβ1 was also significantly lower the first seven hours in the PHX group (0.5 h $P=0.033$, 1 h $P=0.044$, 3 h $P=0.011$, 5 h $P=0.046$, 7 h $P=0.009$) (Fig. 5A). The gene expression of HGF was significantly lower in the PHX group compared to the control group only at time-point 7 h ($P=0.000$).

5. Discussion

The current study presents a novel contribution in understanding in vivo GSH homeostasis after partial hepatectomy (PHx) in a large animal experimental design utilizing the precision and sensitivity of LC-MS/MS. In addition, we present transcriptional variations in the genes regulating GSH synthesis as detected by RT-qPCR specifically designed for the swine genome. The results show that the liver has an extended capacity to maintain GSH metabolism during major stress and parenchymal loss, even at the early onset of such trauma.

Knowledge regarding in vivo GSH synthesis is limited [30], consequently also in vivo reports on GSH synthesis after PHx are lacking, especially in large animal models. Previous murine studies have reported conflicting conclusions regarding GSH levels in the liver remnant after PHx [14,16,28] however based on less sensitive technologies than presented in the current report.

The GSH values in Fig. 2 presents the alteration of mean GSH values in the PHX- and the control group during the immediate 12 h after a 60% PHx. There were no significant differences in GSH values between the two groups at any time during the experiment, suggesting that even though the PHx group lost a major amount of liver tissue and was exposed to complementary stress by surgery, this did not alter the levels of hepatic GSH compared to the control group. This is in agreement with earlier findings in murine studies [28]. The first six hours mean GSH decreased significantly in both groups. This reduction may be explained by the scavenging of GSH due to the stress induced by anaesthesia/laparotomy [7], however extended stress and liver volume loss did not alter the GSH levels.

Fig. 2. Glutathione levels: presented as individual levels and as mean. Raw data of GSH (glutathione) from start (baseline) until 12 h, individual presented for all 12 pigs. The estimated marginal means of GSH values in the PHX- and GSH group is also presented in bold lines. N=6 pigs in both groups. Baseline is mean GSH of three biopsies in the PHX group and mean of two biopsies in the control group. Asterisk indicates significant deviation from baseline for all 12 observations as a group.
significantly in the PHx group. At time-point 7 h and further out, the GSH values in the PHx group tended to increase though not significantly, a tendency also seen in murine studies at 12 h after PHx [16]. The overall interpretations of GSH levels after PHx in the current study, may indicate that the early compensatory response of GSH synthesis can contain the extra functional load triggered by the surgical procedure per se even after organ reduction; this concurs with earlier findings in rat liver[6,12]. As our data is based on a 60% PHx, further studies with more radical PHx may reveal the limits of this proposed compensatory mechanism; thus our data cannot be directly extrapolated to more extensive PHx or small-for-size models. However, the extent of resection model utilized in this model is clinically relevant because the major part of liver resections for malignancy today are 60% or less when performing right- or left sided hemi-hepatectomy.

Due to the results of sustained GSH levels, we then hypothesized that the homeostasis of GSH was maintained in the PHx group compared to the control group due to increased gene expression of key genes in the liver remnant.

The gene expression involved in the GSH de novo synthesis and the GSH regeneration cycle was analyzed the first 12 h after PHx (Figs. 4 and 5). Contradictory to our hypothesis stated above, the initial hours after PHx showed significantly reduced mRNA expression in the PHx group compared to the control group the first
seven (TGFβ1) and nine (NFE2L2, GS) hours. In spite of this, the GSH level was maintained in the PHx group (Fig. 2). This observation may be counterintuitive. A possible explanation can be that the immediate GSH production takes priority over de novo production of proteins as part of an emergency response state following PHx. Thus the GSH production capacity seems to exceed the demands imposed by a 60% PHx, but at the cost of reducing other processes like de novo synthesis of key enzymes. This may be possible because the liver remnant probably contains enough mature and pro-enzymes related to GSH synthesis to buffer the early stress. However, the limits for this surplus capacity may be reached with more radical resections.

Earlier murine studies with longer time frames have shown GSH levels in the PHx group exceeding the control group by 2-fold the first 24 h [14,16] and 48 h [28], a 2-fold difference persisting for 72 h [16] and not reverting to normal GSH values until five days after PHx [28]. This trend could not be demonstrated in our data, which may be explained to the shorter time frame in the present experiment. Unfortunately, a five day experiment post-PHx in pigs is not easily performed, but future experimental setups may profit from extending the time frame in order to investigate if a parallel increase of GSH levels can be seen after PHx in swine. As for all large animal experimental models with limited total number of animals included, the results will mainly detect large differences and/or trends due to the statistic power in the study. Yet, this latter must not reduce the emphasis of utilizing in vivo large animal models comparable with human physiology and anatomy, like the pig, in order to understand in vivo hepatic GSH synthesis.

The method of quantifying GSH proved to be sensitive, by detecting significant alterations of GSH values over time within the groups, corresponding to our earlier method evaluation [11]. The observed outlier animal with increased GSH levels in Fig. 2 also demonstrates the sensitivity of the method. This pig was exposed to additional stress by gastroenteritis the week before the experiment and furthermore developed larynx spasm during the initial phase of anaesthesia, complicating the experiment.

The results in this study represent the first attempt to investigate alterations in GSH levels after PHx in an in vivo swine model using LC-MS/MS. By presenting these results with a sensitive methodological platform, the current report may contribute to future research on the impact of PHx on post-operative oxidative state. Moreover, with this knowledge of GSH levels in the liver remnant, several clinical concerns in post-PHx progress may be addressed, including GSH influence on liver regeneration in diseased liver requiring partial hepatectomy, as well as GSH protection of hepatocytes in the liver remnant when required administration of various medication or analgesia with liver elimination.

In conclusion, the levels of GSH were maintained in the liver remnant compared to the control group during the first 12 h after partial hepatectomy in anesthetized pigs. The gene expression of the key genes in the GSH synthesis were decreased in the liver remnant during the greater part of the experiment, thereby not explaining this observation. Consequently, the maintenance of GSH homeostasis in the early phase rely on other factors than de novo production of proteins, such as an extended capacity of enzymes at hand, and possible readily mobilized pro-enzymes.

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Disclosures

No conflict of interest, financial or otherwise, are declared by the author(s).

Authors’ contributions

ÅF-K wrote the study protocol and hypothesis, performed the surgical experiments, interpreted the results drafted and revised the manuscript. RG co-planned the protocol, supervised the qPCR analysis including interpretations of the biostatistical analysis and co-revised the manuscript. O-MF was responsible for the LC-MS/MS analysis and co-revised the manuscript. IEN made substantial contributions to data acquisition and co-revised the manuscript. RHP supervised the qPCR and co-revised the manuscript. AR and KEM were responsible for conceiving the study design, supervised manuscript drafting and revising its intellectual content.

All authors have read and approved the final manuscript.
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