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Glomerular Haematopoietic Prostaglandin D Synthase-Prostaglandin D2 Axis Contributes to the Periodontitis-Related Exacerbation of Diabetic Nephropathy in KK-A^y Mice

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Keywords: diabetic nephropathy | glomeruli | haematopoietic prostaglandin D synthase | periodontal medicine | periodontitis | prostaglandin D2

ABSTRACT

Aims: Recent clinical studies have proposed a potential association between chronic kidney diseases, including diabetic nephropathy (DN) and periodontitis. Nevertheless, the causal relationship of periodontitis with DN and the underlying molecular mechanisms remain unclear.

Materials and Methods: Ligature-induced experimental periodontitis (LIP) was induced in KK-A^y mice to investigate the molecular mechanisms underlying the LIP-mediated aggravation of glomerular pathology in DN. Outpatients with type 2 diabetes (T2D) at Kyushu University Hospital were recruited to confirm the association of renal dysfunction and periodontitis with a urinary factor identified by RNA sequencing (RNA-seq) in the mouse glomeruli.

Results: LIP aggravated renal dysfunction and glomerular pathologies in KK-A^y mice. RNA-seq in the glomeruli revealed haematopoietic prostaglandin D synthase (HPGDS) as the possible factor bridging periodontitis with DN progression. Glomerular PGD2 levels in KK-A^y mice were significantly elevated by LIP. Oral administration of an HPGDS inhibitor, HQL-79, successfully prevented LIP-mediated DN progression in KK-A^y mice by lowering glomerular PGD2 levels. Urinary HPGDS-to-creatinine ratio could be associated with renal dysfunction and periodontitis in outpatients with T2D.

Conclusion: Periodontitis may contribute to DN progression via the glomerular HPGDS–PGD2 axis. These results suggest a novel mechanism in periodontitis-related DN progression.

Kohei Sato and Takanori Shinjo contributed equally to this work.

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1 | Introduction

The global prevalence of chronic kidney diseases (CKD) is steadily escalating, and current estimates indicate over 850 million patients (Jager et al. 2019). Progressive CKD may culminate in end-stage renal disease, requiring dialysis, and is associated with an increased susceptibility to cardiovascular diseases (CVD), known as cardiorenal syndrome (Behrens et al. 2023; House 2012).

Diabetic nephropathy (DN), a type of diabetes-borne CKD, accounts for the highest incidence (approximately 30%–40%) of CKD (Yokoyama et al. 2007). Patients with DN are highly likely to develop CVD, as diabetes is frequently accompanied by metabolic comorbidities and chronic systemic inflammation, which may accelerate CVD and nephropathy (Reutens and Atkins 2011). DN is primarily remediated via medication-induced lowering of blood glucose and lipid levels and blood pressure (Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group 2024), and factors other than hyperglycaemia, hyperlipidaemia and hypertension contribute to DN progression (Naaman and Bakris 2023; Yu et al. 2024), necessitating the exploration of DN-promoting factors and novel therapeutic strategies.

The risk of periodontitis progression is elevated in people with diabetes compared to those without (Nelson et al. 1990; Emrich et al. 1991; Khader et al. 2006). Advanced periodontitis exerts adverse effects on distant organs, including the liver and kidneys (Kshirsagar et al. 2005; Kitamura et al. 2019; Fujii et al. 2023). Therefore, periodontitis may contribute to the aggravation of diabetic complications. Advanced periodontitis can cause renal dysfunction independent of poor glycaemic control (Kuroe et al. 2004; Chang et al. 2017), suggesting its CKD-promoting ability. However, mechanisms underlying periodontitis-related CKD exacerbation remain unclear.

We analysed pathological modifications in KK-A^y mouse models of ligature-induced experimental periodontitis to identify factors contributing to DN progression in the glomeruli and verified these in patients with type 2 diabetes (T2D).

2 | Material and Methods

2.1 | Mouse Studies

KK-A^y/TaJcl (KK-A^y) and C57BL/6Ncr (C57BL/6) mice, the original background of the KK-A^y mice, at 4- to 5-week-old were obtained from CLEA Japan Inc. (Tokyo, Japan). Mice were housed under climatic conditions with a 12-h light/dark cycle with *ad libitum* access to water and a regular diet. Body weight (BW) and blood glucose levels were monitored weekly from 6 weeks of age to experimental endpoints. Blood glucose levels were measured using a glucometer Medisafe (MS-FR201B; TERUMO, Tokyo, Japan). Blood glucose levels >250 mg/dL were considered to indicate diabetes. All mice were anesthetized via intraperitoneal injection of 4 mg/kg midazolam (LF6476; SANDOZ, Tokyo, Japan), 0.3 mg/kg Dorbene (9021; Kyoritsu Pharmaceutical, Tokyo, Japan) and 5 mg/kg Vetorphale (3270952; Meiji Seika Pharma, Tokyo, Japan) at the induction

of ligation or euthanasia. The latest ARRIVE 2.0 guidelines for animal experiments were followed. All animal experiments and euthanasia protocols were approved by the Institutional Animal Care and Use Committee of Kyushu University (A22-080-2 and A20-226-1).

2.2 | Induction of Ligature-Induced Periodontitis

LIP was induced in mice as previously described (Shinjo et al. 2023; Zeze et al. 2023). Briefly, a ligation was created using a 6–0 silk thread (Akiyama Medical MFG, Tokyo, Japan) around maxillary bilateral second molars of 13-week-old mice under anaesthesia. The non-ligated group was only anesthetized. The mice were monitored to check whether the silk thread was intact. For the recovery study, silk ligations were removed using scalpels from KK-A^y mice at 1.5 weeks (10.5 days) after ligation. Assessment of alveolar bone loss is described in the [Supplementary Methods](#).

2.3 | Assessment of Glomerular Dysfunction and Pathologies in DN

Urinary albumin and creatinine concentrations were determined using Albuwell M Albumin ELISA kit (1011; Ethos Biosciences, Philadelphia, PA, USA) and LabAssay Creatinine (290-65901; Fujifilm Wako, Osaka, Japan), respectively. Absorbance was measured using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Urinary albumin-to-creatinine ratio (UACR) in each mouse was then calculated. DN-related metabolic profiles and histological analyses are described in [Supplementary Methods](#).

2.4 | Isolation of Glomeruli and Tubules From Kidneys

Glomeruli were isolated from kidneys of perfused mice as previously described (Fu et al. 2022). Dissected kidneys (1 and 1/2 kidneys) from each mouse were minced and digested in 1 mg/mL of collagenase A (10103578001; Sigma-Aldrich, St-Louis, MO, USA) and 100 U/mL DNase I in Hank's Balanced Salt Solution (HBSS; 14175095; Thermo Fisher Scientific, Waltham, MA, USA) at 37°C for 30 min. Then, the digested mixture was passed through a 100-µm cell strainer (542000; Greiner, Kremsmünster, Austria) and placed on a magnet for purification. After washing with HBSS twice, glomeruli were collected, and >95% purity was confirmed by microscopy. The remaining digested mixture after glomeruli purification was passed through a 70-µm cell strainer (542070; Greiner). Then, the strainer was inverted, and tubular fractions were collected in HBSS. After washing with HBSS twice, tubules were collected.

2.5 | RNA Extraction and Quantitative Real-Time PCR

RNA extraction, quantitative real-time PCR, and primers are described in [Supplementary Methods](#) and Table S1.

2.6 | Immunofluorescent Staining

Immunofluorescent staining methods are described in [Supplementary Methods](#).

2.7 | RNA Sequence and Data Analysis

RNA sequencing was performed on the Illumina NovaSeq 6000, PE 150 platform (Novogene, Beijing, China) using glomerular RNA from mice ($n=3/\text{group}$). Details are given in [Supplementary Methods](#).

2.8 | Western Blotting

The methodologies and antibodies used are described in [Table S2](#).

2.9 | Interventional Experiments Using Haematopoietic Prostaglandin D Synthase (HPGDS) Inhibitor

Under the same conditions as described above, KK-A^y mice with or without ligatures were orally administered the HPGDS inhibitor, HQL-79 (AOB1729; AOBIOUS, INC, Gloucester, MA) or an equal amount of vehicle (PBS) at 30 mg/kg using an oral dosing needle (KN-348; Nazme Seisakusho Co. Ltd., Tokyo, Japan) once daily from the day after ligation till the endpoint.

2.10 | Prostaglandin D2 (PGD2) ELISA

Glomerular PGD2 levels were measured using the Prostaglandin D2 ELISA kit (512031; Cayman Chemical Company, Ann Arbor, MI).

2.11 | Assessment of Patients With T2D

Outpatients with T2D at the Diabetology department of the Kyushu University Hospital were recruited from March 2021 to August 2024 ($N=12$). Outpatients under 20 years of age, those who had undergone dialysis or anti-cancer treatment, those diagnosed with severe systemic diseases such as liver or kidney, or heart failure, those medicated with immunosuppressive reagents, pregnant women and those who were on antibiotics within 3 months before recruitment were excluded. After informed consent was obtained, blood was collected from the participants to measure HbA1c levels, and urine samples were also collected. Blood test results and information from medical records were anonymized. eGFR was calculated as follows: $194 \times \text{creatinine (mg/dL)}^{-1.094} \times \text{age}^{-0.287}$ for men, and $194 \times \text{creatinine (mg/dL)}^{-1.094} \times \text{age}^{-0.287} \times 0.739$ for women. Excess urine was collected at routine urine tests to measure UACR. The samples were centrifuged and stored at -80°C until measurement of urinary HPGDS levels. Participant characteristics are shown in [Table 1](#). The clinical study was approved by Kyushu University (#2020-741) and followed the ethical standards of the Helsinki Declaration and conforms to the STROBE guidelines.

TABLE 1 | The demographic data and periodontal parameters of the participants.

	<i>N</i> (%), mean $\pm$ SD, median (Q1–Q3)
Sex (male)	7 (58.3)
Age	70.9 $\pm$ 7.1
BMI (kg/m ²)	23.8 $\pm$ 3.7
eGFR (mL/min/1.73 m ²)	63.1 $\pm$ 21.0
HbA1c (%)	7.85 $\pm$ 1.12
UACR (mg/gCr)	46.6 (15.4–378.4)
Severity of periodontitis	
No	0 (0)
Mild	4 (33.3)
Moderate	7 (58.3)
Severe	1 (8.3)
PPD (mm)	2.83 $\pm$ 0.37
Clinical AL (mm)	2.19 $\pm$ 0.76

Abbreviations: AL, attachment loss; BMI, body mass index; eGFR, estimated glomerular filtration rate; HbA1c, haemoglobin A1c; PPD, periodontal pocket depth; UACR, urinary albumin-to-creatinine ratio.

For periodontal case definition, the pocket depth and clinical attachment loss were assessed as previously described (Shinjo et al. 2019). Periodontitis severity was assessed according to the Centers for Control and Prevention and the American Academy of Periodontology (CDC/AAP) case definitions (Eke et al. 2015). Detailed methodologies are described in the [Supplemental Methods](#).

2.12 | Statistical Analysis

All data are presented as mean $\pm$ standard deviation (SD) or median with 1st and 3rd quartile. Comparisons between two groups were performed using unpaired Student's *t*-test. One-way and two-way analysis of variance (ANOVA), followed by post hoc tests, Spearman rank correlation analysis and Mann–Whitney *U* tests were performed to compare multiple groups using GraphPad Prism8 software (GraphPad Software, San Diego, CA). Statistical significance was set at $p < 0.05$.

3 | Results

3.1 | Experimental Periodontitis Exacerbates DN in KK-A^y Mice

Regardless of ligature, KK-A^y mice exhibited considerably elevated BW and blood glucose levels than C57BL/6 mice ([Figure 1A–C](#)). KK-A^y mice exhibited a marked exacerbation of LIP-mediated alveolar bone loss compared with C57BL/6 mice at 3 weeks post-ligation ([Figure 1D–F](#)). Food intake ([Figure S1A](#)), daily water consumption and urine volume were higher in KK-A^y mice than in C57BL/6 mice ([Figure S1B,C](#)). Three-week ligation increased the heart rate, systolic blood

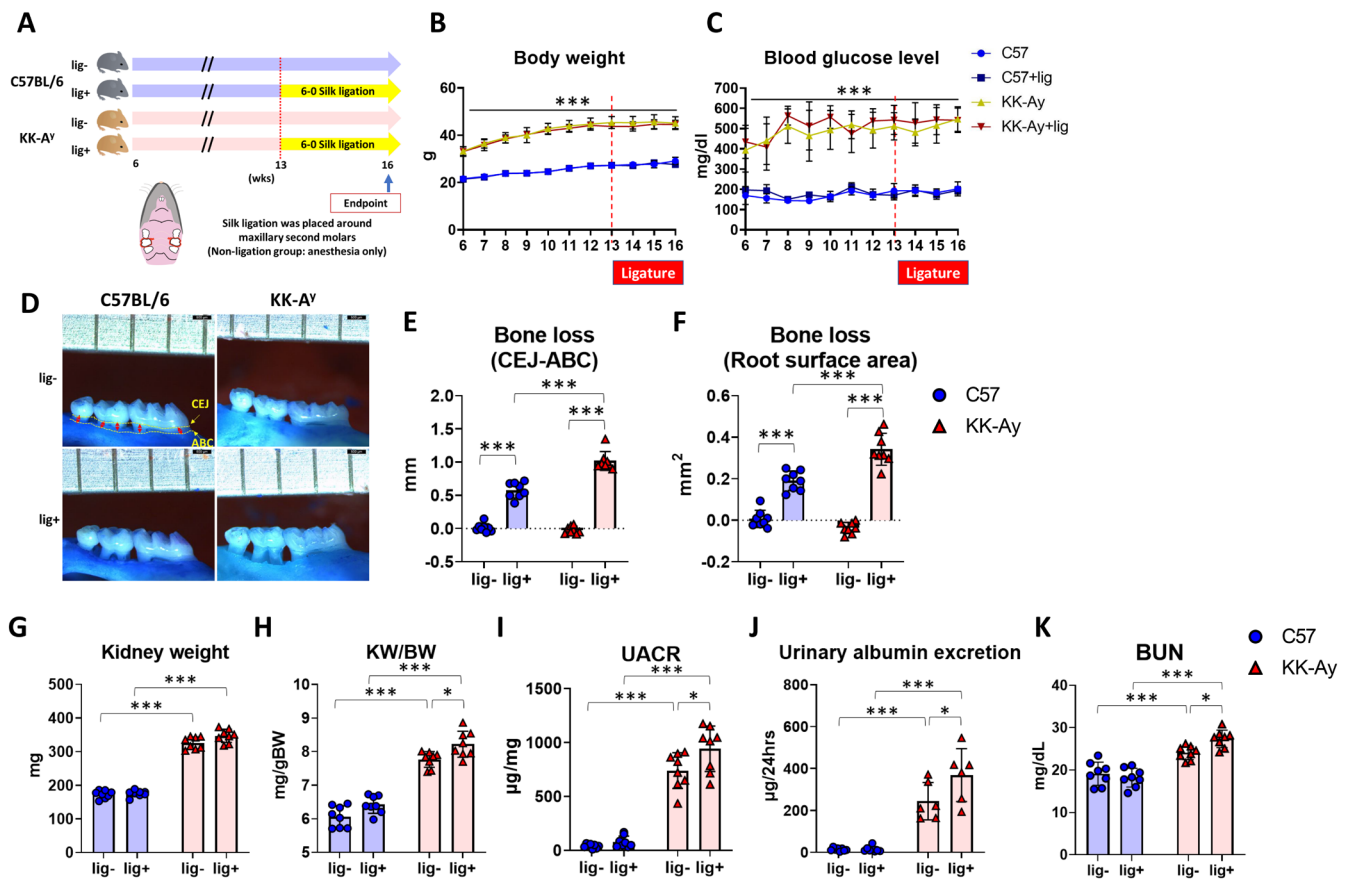


FIGURE 1 | Experimental periodontitis exacerbates diabetic nephropathy in KK-Ay mice. (A) A schema of experiments. lig-: Without ligation; lig+: With ligation. (B) Body weight changes in each group during the experiments ($n=8$). (C) Random blood glucose levels in each group during the experiments ($n=8$). (D) Representative photos of the alveolar bone of each group at the endpoint. (E) Quantified buccal alveolar bone loss based on the distances between cement–enamel junction (CEJ) and alveolar bone crest (ABC) of each mouse ($n=8$). (F) Quantified alveolar bone loss based on buccal root surface area of maxillary molars in each mouse ($n=8$). (G) Kidney weight (KW) and (H) KW-to-body weight (BW) ratio of each mouse at endpoint ($n=8$). (I) Urinary albumin-to-creatinine ratio (UACR) of each mouse at endpoint ($n=8$). (J) Urinary albumin excretion for 24 h of each mouse at endpoint ($n=6$). (K) Blood urea nitrogen (BUN) concentration of each mouse ($n=8$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. All individual data are shown in a scatter plot (E–K). Data are presented as mean $\pm$ SD. p -values were determined by two-way ANOVA followed by post hoc tests.

pressure and diastolic blood pressure in both C57BL/6 and KK-Ay mice; however, the increase was not significant (Figure S1D–F).

The kidney weight (KW) and KW-to-BW ratio were higher in KK-Ay than in C57BL/6 mice (Figure 1G,H). The KW/BW ratio was notably higher in ligatured than in non-ligatured KK-Ay mice (Figure 1H). Ligatured KK-Ay mice demonstrated a considerable escalation in the UACR, daily urinary albumin excretion and plasma blood urea nitrogen (BUN) levels compared with non-ligatured KK-Ay mice (Figure 1K–M). Neither glucose tolerance nor systemic insulin resistance was altered by the 3-week-long ligation (Figure S1G–J).

3.2 | LIP Promoted Glomerular Pathologies in KK-Ay Mice

Glomeruli were histologically evaluated as UACR elevation may reflect glomerular dysfunction. KK-Ay mice possessed discernibly larger glomeruli, mesangial expansion and fibrotic areas than C57BL/6 mice (Figure 2A–G). LIP induced a striking

progression of these pathological changes in KK-Ay but not in C57BL/6 mice (Figure 2A–G). Fibrosis-related and inflammatory gene expression was upregulated in KK-Ay mice compared with C57BL/6 mice (Figure 2H) and was markedly elevated by LIP in KK-Ay but not in C57BL/6 mice (Figure 2H). CD68-positive cell infiltration in the glomeruli was promoted in KK-Ay mice, and LIP visibly enhanced this effect (Figure 2I–K). Consistently, the expression of tight junction–related genes ZO-1 and Occludin was significantly lower in KK-Ay mice than in C57BL/6 mice, and LIP further downregulated their expression in KK-Ay mice (Figure 2L).

3.3 | LIP-Induced Upregulation of HPGDS Expression in the Glomeruli of KK-Ay Mice

RNA sequence analysis (Figure 3A) revealed that glomerular *Hpgds* expression was potentially induced by diabetes and further enhanced by LIP (Figures 3B and S2A), consistent with the finding from the Gene Ontology (GO) analysis that prostaglandin (PG) metabolic and biosynthesis processes were remarkably enriched in ligatured KK-Ay mice compared to non-ligatured ones (Figure 3C).

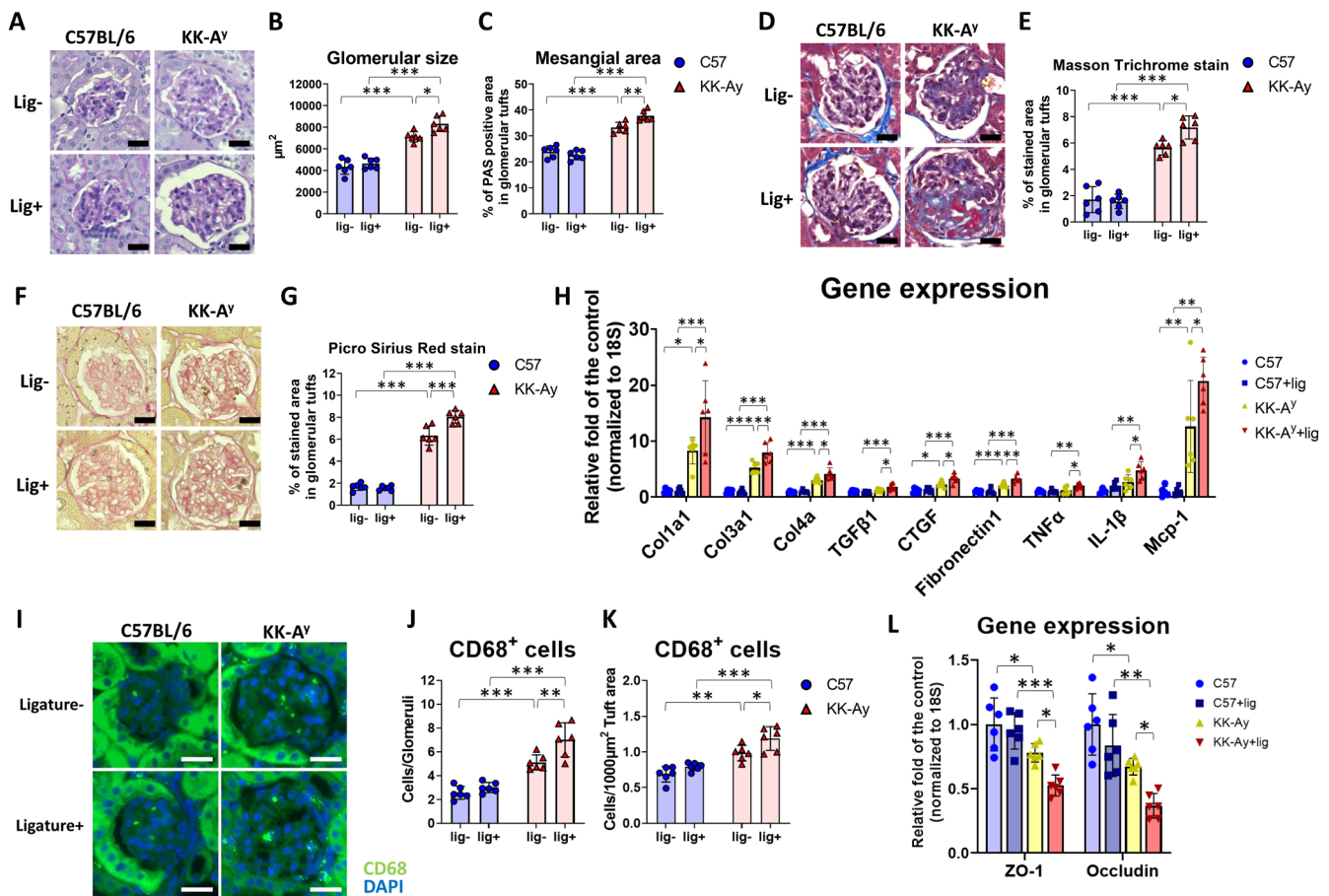


FIGURE 2 | Exacerbation of glomerular pathologies by experimental periodontitis in KK-A^y mice. (A) Representative microscopic images of Periodic acid-Schiff stain (PAS) stain in each group. (B) Glomerular size and (C) percentage of mesangial area (PAS-positive area) in the glomerular tuft area of each group ($n = 6$, each). (D) Representative microscopic images of Masson Trichrome stain in each group. (E) Percentage of Masson Trichrome-stained area in the glomerular tuft area ($n = 6$). (F) Representative images of Picro Sirius Red stain in each group. (G) Percentage of Picro Sirius Red-stained area in the glomerular tuft area in each group ($n = 6$). (H) qPCR data of fibrotic and pro-inflammatory genes in the glomeruli of each mouse ($n = 6$). (I) Representative images of immunofluorescent stain with CD68 (green) and DAPI (blue) in the glomeruli of each group. (J) CD68-positive cells in the glomeruli and (K) CD68-positive cells per 1000 μm^2 of glomerular tuft area in each mouse ($n = 6$, each). (L) qPCR data of tight junction-related genes (ZO-1 and occludin) in the glomeruli of each mouse ($n = 6$). Scale bar = 25 μm . * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. All individual data are shown in a scatter plot. Data are presented as mean $\pm$ SD. p -values were determined by two-way ANOVA followed by post hoc tests.

Glomerular *Hpgds* expression was approximately 200-fold up-regulated in KK-A^y mice than in C57BL/6 mice and was further elevated by approximately 1.8-fold by LIP in KK-A^y mice (Figures 3B and S2A). Lipocalin-type PGD synthase (*Lpgds*), another PGD synthase, was approximately 1.8-fold up-regulated in KK-A^y mice, and LIP enhanced its expression by 1.5-fold (Figure 3D). Regarding PGD2 receptor expression, *PTGDR* (*DP1*) was discernibly suppressed, whereas *PTGDR2* (*DP2*) was up-regulated in the glomeruli of KK-A^y mice compared with that in C57BL/6 mice (Figure 3D).

Glomerular HPGDS levels were substantially higher in KK-A^y than in C57BL/6 mice, and LIP enhanced them by 1.8-fold in the KK-A^y mice (Figure 3E,F). KK-A^y mice subjected to ligature for 1.5 weeks and subsequent removal for 1.5 weeks revealed glomerular HPGDS levels comparable with those in KK-A^y mice without ligature for 3 weeks (Figure 3G,H), supporting the fact that LIP-induced glomerular HPGDS expression in KK-A^y mice.

3.4 | HQL-79 Administration Suppresses LIP-Borne DN Exacerbation in KK-A^y Mice

KK-A^y mice with or without ligature for 3 weeks were orally administered the HPGDS inhibitor, HQL-79 or vehicle (Figure 4A). No notable alterations were observed in BW or blood glucose levels or LIP-induced alveolar bone loss in KK-A^y mice after HQL-79 treatment (Figure 4B–D). UACR and BUN were considerably decreased in ligatured KK-A^y mice administered HQL-79 compared with those administered vehicle (Figure 4E,F). qPCR analysis demonstrated that HQL-79 administration notably downregulated the expression of glomerular fibrosis-related and inflammatory genes in ligatured KK-A^y mice as well as *Col1a1*, *Col3a1* and *Mcp-1* in non-ligatured KK-A^y mice (Figure 4G). In contrast, HQL-79 treatment significantly increased ZO-1 and Occludin mRNA levels in both ligatured and non-ligatured KK-A^y mice (Figure 4H).

HQL-79 administration slightly decreased KW and the KW-to-BW ratio in ligatured KK-A^y mice (Figure 4I,J). Notably, glomerular

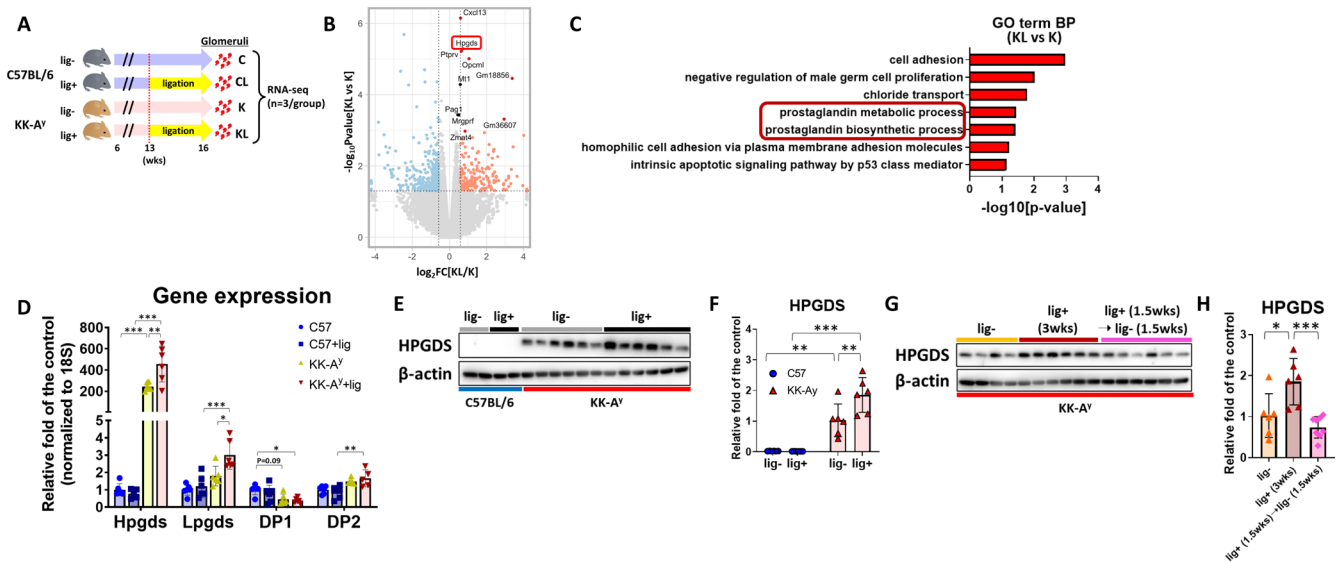


FIGURE 3 | Haematopoietic prostaglandin D synthase (HPGDS) expression was upregulated by experimental periodontitis in the glomeruli of KK-Ay mice. (A) A schematic of RNA-seq using the glomeruli of each mouse. C: C57BL/6, CL: C57BL/6 With ligatures, K: KK-Ay, KL: KK-Ay with ligatures. (B) The volcano plot of the comparison between K and KL. (C) The pathway analysis of the Gene Ontology (GO) term 'Biological Process' based on the comparison of genes between K and KL. Genes significantly ($p < 0.05$) increased by > 1.5 fold in KL compared with K were analysed. (D) qPCR data of prostaglandin D synthase-related genes in the glomeruli of each mouse ($n = 6$). (E) The representative blots of HPGDS and β -Actin in the glomeruli of each mouse. (F) The quantitative HPGDS expression in the glomeruli of each mouse ($n = 6$). (G) The representative blots of HPGDS and β -Actin in the glomeruli of non-ligatured KK-Ay mice, ligatured KK-Ay mice and KK-Ay mice with 1.5 weeks of ligation and subsequent 1.5 weeks of non-ligation. (H) The quantitative HPGDS expression in the glomeruli of each mouse ($n = 6$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. All individual data are shown in a scatter plot. Data are presented as mean $\pm$ SD. p -values were determined by two-way ANOVA (D, F) and one-way ANOVA (H) followed by post hoc tests.

PGD2 levels were increased by diabetes and further elevated by LIP, and HQL-79 administration successfully suppressed PGD2 in the glomeruli of KK-Ay mice to levels similar to those in C57BL/6 mice (Figure 4K). Periodontitis-mediated progression of mesangial expansion, fibrosis and CD68-positive cell infiltration in the glomeruli of ligatured KK-Ay mice administered HQL-79 was remarkably suppressed to levels comparable to those in non-ligatured KK-Ay mice administered the vehicle (Figure 4L–Q).

3.5 | Urinary HPGDS Levels Correlated With Renal Dysfunction and Periodontal Pocket Depth in People With T2D

We then confirmed whether urinary HPGDS levels correlated with renal dysfunction and periodontitis in people with T2D ($N = 12$), possibly resulting from HPGDS leakage from the glomeruli owing to its dysfunction. There were 7 male and 5 female participants, and the mean age, BMI, eGFR and HbA1c were 70.9, 23.8, 63.1 and 7.85 respectively (Table 1). Median UACR in the cohort was 46.6 (Table 1). Periodontal examination indicated the mean periodontal pocket depth (PPD) and clinical attachment loss were 2.83 and 2.19 mm, respectively, and there were 4 people with mild periodontitis, 7 people with moderate periodontitis, and 1 person with severe periodontitis (Table 1).

Urinary HPGDS-to-creatinine ratio (U-HPGDS/Cr) showed a tendency of positive correlation with UACR ($r = 0.539$, $p = 0.075$) and HbA1c ($r = 0.557$, $p = 0.064$), and did not show a tendency of negative correlation with eGFR ($r = 0.406$, $p = 0.1928$)

(Figure 5A–C). Mean probing pocket depth showed a significant positive correlation with U-HPGDS/Cr ($r = 0.657$, $p = 0.028$) and UACR ($r = 0.678$, $p = 0.019$) (Figure 5D,E). Participants with moderate and severe periodontitis tended to have lower eGFR than those with mild periodontitis (Figure 5F) and did not show a significant elevation of UACR and U-HPGDS/Cr (Figure 5G,H).

4 | Discussion

The progression rate of DN can be determined based on the involvement of the primary cause, hyperglycaemia and other factors, such as hypertension, hyperlipidaemia, chronic inflammation and endotoxins (Siddiqui et al. 2022). There should be contributing factors other than hyperglycaemia, as extensive glucose control could not completely prevent DN progression in some patients (Naaman and Bakris 2023; Oshima et al. 2021). Epidemiological studies have suggested an association between periodontitis and CKD, including DN (Kshirsagar et al. 2005; Kitamura et al. 2019; Chang et al. 2017). However, the mechanism by which periodontitis contributes to DN exacerbation is not understood.

We used KK-Ay mice as a DN model, frequently used earlier (Mizuno et al. 2019; Oda et al. 2023), and applied them to LIP, which induces dysbiotic periodontal inflammation and displays pathologies similar to those in human periodontitis (Marchesan et al. 2018). We confirmed that ligatured KK-Ay mice exhibited a marked increase in UACR and urinary albumin excretion

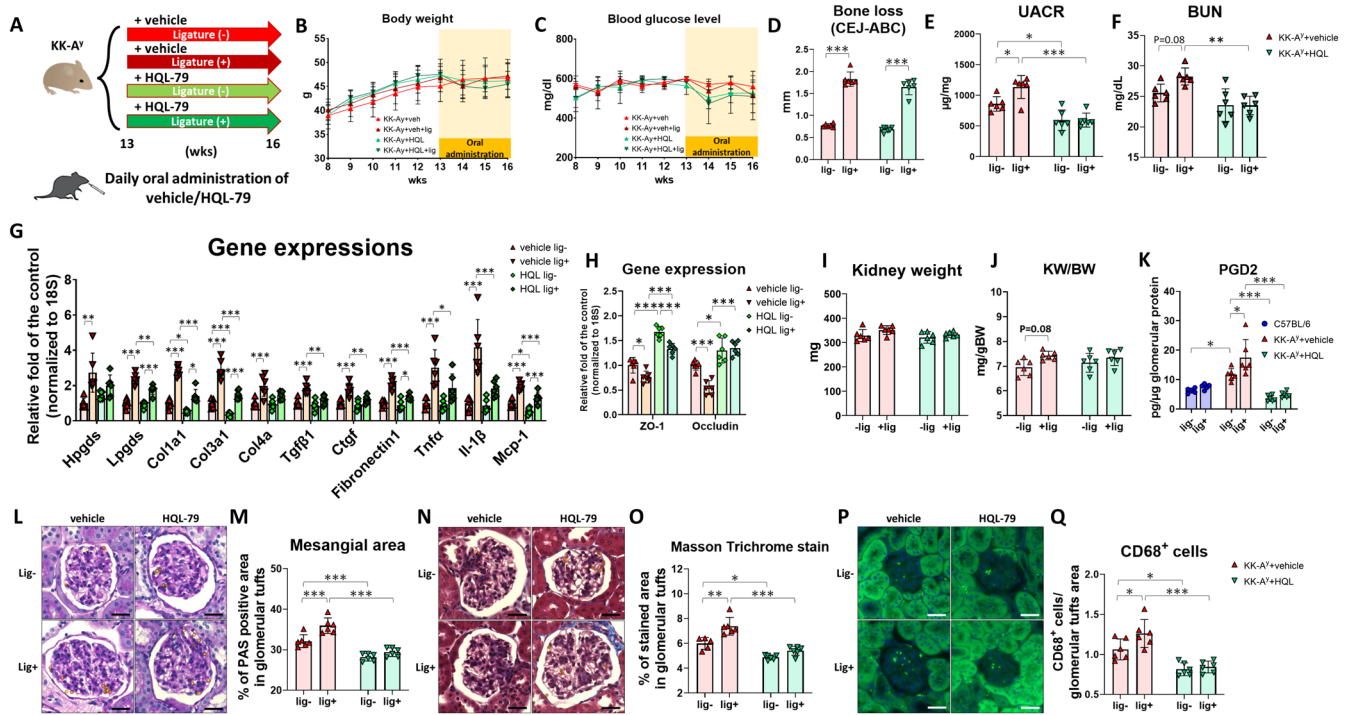


FIGURE 4 | Effect oral administration of haematopoietic prostaglandin D synthase (HPGDS) inhibitor HQL-79 on KK-*Ay* mice with or without ligatures. (A) A schema of the experiment. (B) Weekly body weight (BW) changes and (C) random blood glucose levels of each group during the experiment ($n=6$). (D) Quantified alveolar bone loss of each mouse administered HQL-79 (30 mg/kg) or vehicle ($n=6$). (E) Urinary albumin-to-creatinine ratio (UACR) of each mouse ($n=6$). (F) BUN levels in each mouse ($n=6$). The mRNA expressions of (G) *Hpgds*, *Lpgds*, fibrotic, inflammatory and (H) tight junction-related genes in the glomeruli of each group ($n=6$). (I) Kidney weight (KW), (J) KW-to-body weight (BW) ratio of each mouse ($n=6$). (K) Prostaglandin D2 (PGD2) levels in the glomeruli of C57BL/6 and KK-*Ay* mice administered vehicle or HQL-79 in the presence or absence of ligature ($n=6$). (L) Representative microscopy images of PAS stains in each group. (M) Percentage of mesangial area (PAS-positive area) in the glomerular tuft area of each group ($n=6$, each). (N) Representative microscopic images of Masson Trichrome stain in each group. (O) Percentage of Masson Trichrome-stained area in the glomerular tuft area ($n=6$). (P) CD68-positive cells in the glomeruli and (Q) CD68-positive cells per 1000 μm^2 of the glomerular tuft area in each mouse ($n=6$, each). Scale bar = 25 μm . * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. All individual data except for (B) and (C) are shown in a scatter plot. Data are presented as mean $\pm$ SD. p -values were determined by two-way ANOVA followed by post hoc tests.

(Figure 1I,J) compared with non-ligated KK-*Ay* mice, suggesting that LIP promoted glomerular dysfunction that increased albumin leakage to urine (Alicic et al. 2017).

Representative glomerular pathologies in DN—increased size, mesangial and fibrotic areas and inflammatory cell infiltration (Kikkawa et al. 2003; Najafian et al. 2011)—were strikingly escalated by LIP in KK-*Ay* mice (Figure 2A–G,I–K). Chronic inflammation is a key factor in the progression of glomerular pathologies (Wilkening et al. 2020) and is closely related to fibrosis in distant organs under diabetic complications and comorbidities (Meli et al. 2014; Sanganalmath et al. 2023; Li et al. 2022). Ligatured KK-*Ay* mice exhibited enhanced inflammatory response and downregulation of tight junctions in the glomeruli (Figure 2H,L), consistent with increased CD68-positive cell infiltration (Figure 2I–K). The interaction between mesangial cells and macrophages could contribute to CKD progression (Hashikata et al. 2014). Overall, LIP may contribute to the upregulation of inflammation in the glomeruli, resulting in glomerular dysfunction via fibrosis progression.

RNA sequencing identified that glomerular *Hpgds* could bridge the progression of DN with LIP in KK-*Ay* mice (Figures 3B and S2A). Glomerular *Hpgds* levels in KK-*Ay* mice were over 200-fold higher than those in C57BL/6 mice, and LIP further

enhanced glomerular *Hpgds* expression by 1.8-fold (Figures 3D and S2A), whereas glomerular *Lpgds* levels in KK-*Ay* mice were not notably upregulated (Figure 3D). Consistently, glomerular PGD2 levels were significantly upregulated by ligatures in diabetic KK-*Ay* mice (Figure 4K). Notably, the upregulation of glomerular *Hpgds* expression in KK-*Ay* mice was LIP dependent (Figure 3G,H). Studies have shown that *Lpgds* but not *Hpgds* is involved in tubulointerstitial fibrosis after acute kidney injury (Ito et al. 2012). In KK-*Ay* mice, tubular *Hpgds* and *Lpgds* expressions were higher and lower than those in controls, respectively (Figure S2B), suggesting that glomerular *Hpgds* upregulation is a hallmark of DN.

HPGDS is responsible for 90% of PGD2 production in the body (Song et al. 2018). Considering our finding that glomerular HPGDS expression is upregulated in diabetes and LIP, we predicted that PGD2 action in glomerular cells could be related to pathological changes shown in Figure 2.

Increased glucose influx elicits cAMP production and subsequent protein kinase A activation, leading to increased p65 and cAMP response element binding protein (CREB) phosphorylation, resulting in upregulation of profibrotic factors such as Tgfb1/3 and Ctgf in mesangial cells (Deb et al. 2017).

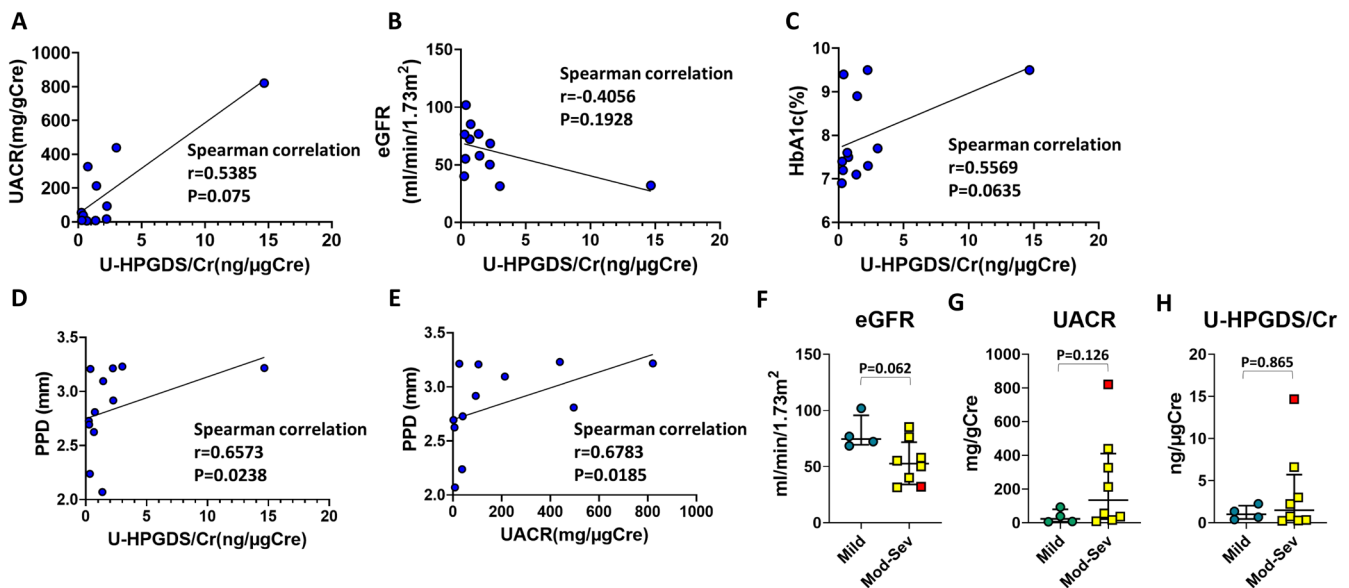


FIGURE 5 | The association of urinary haematopoietic prostaglandin D synthase (HPGDS)-to-creatinine ratio with renal dysfunction and periodontitis in people with type 2 diabetes. Plots of the correlation of urinary HPGDS-to-creatinine ratio (U-HPGDS/Cr) with (A) urinary albumin to creatinine ratio (UACR), (B) estimated glomerular filtration rate (eGFR), (C) haemoglobin A1c (HbA1c) and (D) mean periodontal pocket depth (PPD) in people with type 2 diabetes ($N=12$). (E) A plot of the correlation of UACR with PPD. The non-parametric unpaired two-group comparison of (F) eGFR, (G) UACR and (H) U-HPGDS/Cr between people with mild (Mild: Green circles), moderate (Mod: Yellow squares) and severe (Sev: Red squares) periodontitis. Data are presented in a scatter plot (A–E) and median with first and third quartile. p -values were determined using Spearman rank correlation test (A–E) and Mann–Whitney U test (F–H).

Consistently, ligatured KK-AY mice showed elevated glomerular Tgf β 1 and Ctgf (Figure 2H) accompanied by increased peripheral PGD2 levels (Figure 4K). Besides, DP1 signalling mediates cAMP increase, and DP2 signalling suppresses it (Jang et al. 2020), implying that DP1 signalling contributes to extracellular matrix (ECM) production mediated by PGD2. qPCR demonstrated downregulation of Dp1 and upregulation of Dp2 in the glomeruli of KK-AY mice (Figure 3D), possibly resulting from negative feedback of PGD2–DP1 signalling. The PGD2–DPs axis might be involved in tissue fibrosis via the up-regulation of inflammatory cell infiltration and ECM production (Ito et al. 2012; Sarashina et al. 2014; Jandl et al. 2016), whereas PGD2 could play an anti-inflammatory role in several acute and allergic inflammatory diseases (Murata et al. 2013; Maehara et al. 2019). PGD2 has anti-inflammatory and pro-inflammatory roles via endothelial cells in multiple disorders (Murata et al. 2013; Sarashina et al. 2014; Li et al. 2017). In particular, PGD2 exerts anti-inflammatory effects in the early phase but a pro-inflammatory effect in the late phase of chronic inflammation owing to the promotion of inflammatory cell infiltration via the leaky endothelial junction (Sarashina et al. 2014). Further studies should investigate molecular mechanisms focusing on DP1 and DP2 signalling in glomerular cells to understand the pathological role of each receptor in DN.

Oral HQL-79 administration suppressed LIP-mediated pathological changes in the glomeruli of KK-AY mice via suppression of glomerular PGD2 levels (Figure 4E–Q) without any effect on BW, blood glucose levels or alveolar bone loss induced by ligation (Figure 4B–D). HQL-79 treatment partially improved but failed to normalize these pathological changes in KK-AY mice with or without ligation (Figure 4I,J,L–Q), consistent with the

finding that hyperglycaemia was not improved by systemic HPGDS inhibition (Figure 4C). Therefore, glomerular HPGDS might play an important role in the acceleration of DN mediated by LIP in KK-AY mice and may be partially involved in DN progression under hyperglycaemia.

Lastly, we confirmed the association of U-HPGDS/Cr with UACR ($r=0.539$, $p=0.075$) and HbA1c ($r=0.557$, $p=0.064$) in people with T2D (Figure 5A,C). Although this was a small cohort, the results suggest that excess glomerular HPGDS could have leaked through the endothelium into the urine under DN. We also found that U-HPGDS/Cr was significantly correlated with deeper PPD ($r=0.657$, $p=0.024$) in the T2D cohort (Figure 5D). There was no correlation between eGFR and urinary U-HPGDS/Cr ($r=0.4056$, $p=0.193$) (Figure 5B), which was possibly owing to the compensational increase of eGFR that was frequently observed in the early stage of DN and calculation using the formula based on age and blood creatinine levels. Additionally, a significant correlation of UACR with PPD was noted in the T2D cohort (Figure 5E). While urinary LPGA has been reported to be one of the key factors involved in the control of tubular injury in people with CKD (White et al. 2015), this is the first study to report the possible correlation of HPGDS with DN.

We also found that eGFR in people with moderate and severe periodontitis was lesser than in those with mild periodontitis ($p=0.062$) (Figure 5F) while UACR and U-HPGDS/Cr were not significantly different, possibly owing to a wide range distribution of both parameters (Figure 5G,H). Of note, a participant with severe periodontitis showed substantially low eGFR and the highest UACR and U-HPGDS/Cr (Figure 5F–H). Further investigation should be conducted using larger cohorts to perform

robust statistical analysis, such as logistic regression analysis, regarding the association of the severity of periodontitis with renal dysfunction markers and U-HPGDS/Cr and to confirm HPGDS upregulation in the glomeruli of people with T2D and severe periodontitis.

This study has some limitations. First, the investigation involved only male mice. Male patients have been reported to be at a greater risk of developing DN than female patients, although some studies did not show male predominance (Maric 2009; Harjutsalo and Groop 2014). Further replication studies using female DN mice are required. Second, we used only KK-A^y mice as the DN model. KK-A^y mice possess a mutation in the agouti gene and have suppressed MCR4-mediated signalling, resulting in bulimia (Ebihara et al. 1999; Inamori et al. 2018). Similar experiments using other T2D model mice such as db/db mice should be conducted. Third, we only confirmed the pharmacological inhibition of HPGDS in DN model mice. A validation study should be performed using conventional or conditional *Hpgds* knockout mice with DN and LIP. Finally, the T2D cohort in the current study was small. Additional research with a larger cohort is needed to study the association between urinary HPGDS levels and the severity of periodontitis in people with T2D in detail.

5 | Conclusion

Activation of the glomerular HPGDS-PGD2 axis in response to periodontitis may contribute to DN exacerbation via an increase in mesangial ECM production and inflammatory cell infiltration in the glomeruli. Therefore, targeting HPGDS overexpression could elucidate the molecular basis underlying the association between periodontitis and DN and serve as a novel therapeutic approach against periodontitis-related DN progression. Detailed investigations regarding mechanisms in glomerular cells should be undertaken in future with genetically modified *Hpgds* and PGD2 receptors.

Author Contributions

K.S. conducted the experiments, and T. Shinjo conceived the ideas and designed and conducted the experiments. K.S., T. Shinjo, T.Z., A.A., H.O., H.Y., N.S., M. Imagawa, Y.N., N.R., A.Y., T.F., T. Sanui, M. Iwashita and F.N., were involved in data analyses and interpretation. H.Y. and N.S. recruited outpatients with type 2 diabetes. K.S. and T. Shinjo acquired the funding. K.S. and T. Shinjo wrote the manuscript with feedback from all of the authors.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data published in the current article are available upon reasonable request. The RNA-sequencing dataset was uploaded to the NCBI GEO repository (GSE261386).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting information

Supplementary Methods

Mouse studies

Assessment of periodontitis-induced alveolar bone loss in mice

The measurements of alveolar bone loss were performed as previously reported [1,2]. In brief, the defleshed maxillae were stained with 0.05% toluidine blue. Photographs were taken using a Leica MZ10 F microscope (Leica-Microsystems, Wetzlar, Germany). The distance of the apex of the alveolar bone crest (ABC) from the cemento-enamel junction (CEJ) of the center of the third molar, the proximal and distal roots of the second and first molars were measured using ImageJ ver. 1.53i software (National Institute of Health, Bethesda, MD, USA). Alveolar bone loss was quantified by subtracting the sum of ABC–CEJ distance at five points of the buccal side in each group from that of the control group.

Assessment of DN-related metabolic profiles in mice

Daily water consumption, urine volume, urinary albumin excretion, and food intake were measured by 24-hour metabolic caging with devices (CM-10S; CLEA Japan, Inc.) as previously reported [3]. For urinary albumin-to-creatinine (UACR) measurement, spot urine was collected from each mouse at euthanasia (16-weeks old). Heart rate and systolic and diastolic blood pressure (SBP and DBP) were measured at endpoints using a tail-cuff Blood Pressure Monitor (MK-2000ST; Muromachi Kikai, Tokyo, Japan). Blood urea nitrogen (BUN) levels in plasma were measured using L-type Wako UN • V (469-73701; Fujifilm Wako, Osaka, Japan) according to manufacturer's instructions.

Sample collection

Mice were euthanized by blood collection from hearts under anesthesia. Plasma was obtained from blood by centrifugation at 400×g for 20 minutes. Then, 20 mL of PBS with 50 µL of Dynabeads M-450 Tosylactivated (14013; Invitrogen, Waltham, MA, USA) was perfused into the mouse aorta as previously described [3].

Intraperitoneal glucose and insulin tolerance tests

Intraperitoneal glucose and insulin tolerance tests (IPGTTs and IPITTs, respectively) were conducted as previously described [1,2]. For GTTs, mice were intraperitoneally injected with 2 g/kg glucose after 16 hours of fasting at endpoints (3 weeks after ligation). For ITTs, mice were intraperitoneally injected with 0.75 U/kg insulin after 6 hours of fasting at endpoints. Blood was collected from the tail vein at 0, 15, 30, 45, 60, 90, and 120 minutes after injection, and glucose levels were measured using a glucometer. The area under the curve was calculated from the plot of each mouse.

Histological analysis in mice kidney sections

The glomerular pathologies in mice were assessed with 5- μ m-thick sections obtained from paraffin-embedded mouse kidneys fixed with 10% formalin in 24 hours. Sections were stained with Periodic acid-Schiff stain (PAS), Masson Trichrome, and Picro-Sirius Red (PSR1; ScyTek Laboratories, Logan, UT, USA) for the assessment of glomerular size, mesangial expansion area, and glomerular fibrosis, respectively. Stained images were visualized using a BZ-X800 fluorescence microscope (Keyence, Tokyo, Japan). Image J software was used for analysis.

RNA extraction and quantitative real-time PCR

Glomerular RNA was isolated using PureLink RNA Mini Kit (12183018A; Invitrogen) according to the manufacturer's instructions. RNA from cultured cells was isolated using Isogen II RNA purification reagent (311-07361; Nippon Gene, Tokyo, Japan). RNA concentration was determined using a NanoDrop Lite Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). cDNA was synthesized using PrimeScript RT Master Mix (RR036A; Takara Bio, Shiga, Japan) using a total of 500 μ g RNA. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using Luna Universal qPCR Master Mix (M3003; New England BioLabs, Ipswich, MA, USA) and StepOne Plus™ Real-Time System (Applied Biosystems, Carlsbad, CA, USA) under the following conditions: 95 °C for 3 min, 40 cycles of 95 °C for 3 s and 60 °C for 30 s. The results were analyzed using StepOne™ software V2.2.2 (Applied Biosystems) utilizing the auto-calculated threshold cycle. The $\Delta\Delta$ CT method was used to calculate the relative expression levels of the individual genes. 18s rRNA was used as an internal control. The primer sequences used in the current study are listed in Supplementary Table 1.

Immunofluorescent staining

Paraffin-embedded mouse kidneys were deparaffinized with xylene and rehydrated with ethanol. Antigen activation was performed at 95 °C for 10 min using citrate buffer (pH 6). Sections were washed with PBS for 5 minutes and then incubated with 1% BSA for 30 minutes at room temperature for blocking. Sections were incubated with primary antibodies at 4 °C overnight in the shade. The sections were then washed with PBS and incubated with a secondary antibody for 2 hours at room temperature in the shade. Nuclei were stained with SlowFade Diamond Antifade Mountant with DAPI (S36964; Invitrogen). Visualization was performed using a Keyence BZ-X800 fluorescence microscope. Positively stained cells in the glomeruli were counted and quantified using ImageJ software. Detailed information regarding the antibodies used in this are given in Supplementary Materials. anti-mouse CD68 antibody (#97778; Cell Signaling Technology, Danvers, CO, USA).

RNA sequencing and data analysis

A total of 1 µg of each RNA sample was used for RNA sample preparation. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina (E7530; NEB, MA, USA) following the manufacturer's recommendations and index codes were added to attribute sequences to each sample. Fragments per kilobase of transcript sequence per millions (FPKM) of each gene were calculated based on the length of the gene and read count mapped to this gene. We used the edgeR program to detect differentially expressed genes (DEGs). Log fold-changes (logFC) and p-values were obtained from the gene-level raw counts. Then, we established criteria for DEGs: p-value ≤ 0.05 and ratio ≥ 1.5 -fold (up-regulated genes) or 0.67-fold (down-regulated genes). Gene Ontology pathway analysis was performed using Functional Annotation Bioinformatics Microarray Analysis (DAVID) software (<https://david.ncifcrf.gov/tools.jsp>).

Western blotting

Glomeruli isolated from each mouse and cultured cells were homogenized in ice-cold RIPA lysis buffer (16488-34; Nacalai Tesque) supplemented with a protease inhibitor mixture (1:100 dilution; 25955-11, Nacalai Tesque) to collect protein. Protein concentrations were determined using the Pierce™ BCA Protein Assay Kit (A55864; Thermo Fisher Scientific). Tissue or cell lysates with a fixed amount of protein were subjected to SDS/PAGE, and the separated proteins on the gels were transferred to PVDF membranes (IPVH00010; Merck, Rahway, NJ, USA). After blocking with 5% skim milk, the membrane was incubated with primary antibodies at 4 °C overnight followed by incubation with appropriate secondary antibodies for 1 hour at room temperature. Membranes were developed using Chemi-Lumi One Super (02230-30; Nacalai Tesque). Blots were scanned using Image Quant LAS800 (GE Healthcare, Chicago, IL) and analyzed using ImageJ software. Antibody concentrations for western blotting are listed in the Supplemental Table2.

Assessment of periodontitis in patients with T2D

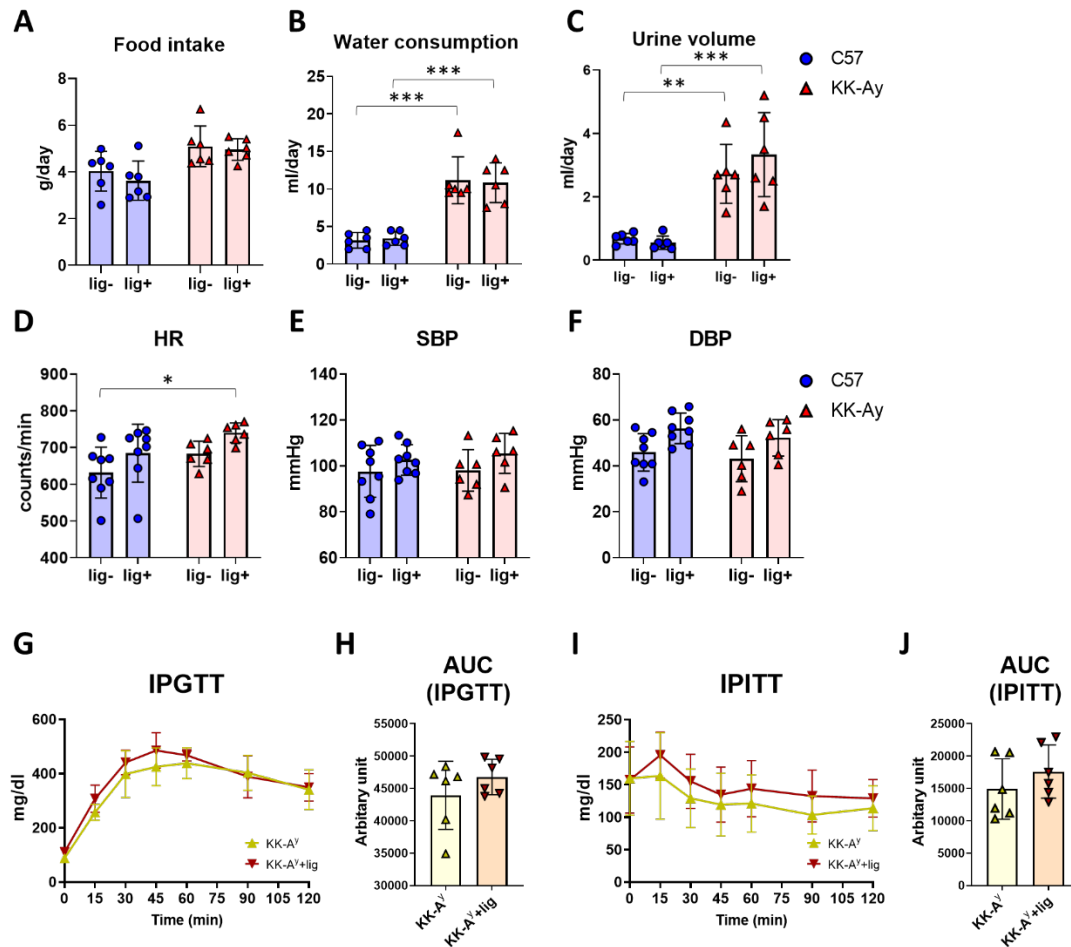
Briefly, pocket depth was measured using Perio Probe® (YDM Corp., Tokyo, Japan) at six sites per tooth (disto-buccal angled, mild-buccal, mesio-buccal angled, disto-lingual angled, and mid-lingual and mesio-lingual angled). The distance from the gingival margin (GM) to the cemento-enamel junction (CEJ) was measured using Perio Probe® (YDM Corp., Tokyo, Japan) at the six sites. All teeth present in a given arch were measured, excluding those with large restoration or a full crown invading the CEJ. Then, clinical attachment loss was calculated from the following formula: PD – distance from GM to CEJ (mm). The criteria were as follows: No periodontitis: No evidence of mild, moderate, or severe periodontitis. Mild periodontitis: ≥ 2 interproximal sites with clinical AL ≥ 3 mm, and ≥ 2 interproximal sites with PD ≥ 4 mm (not on same tooth) or one site with PD ≥ 5 mm. Moderate periodontitis: ≥ 2 interproximal sites with clinical AL ≥ 4 mm (not same tooth), or ≥ 2 interproximal

1 sites with PD $\geq$ 5 mm (not same tooth). Severe periodontitis: $\geq$ 2 interproximal sites with clinical AL
2 $\geq$ 6 mm (not same tooth) and $\geq$ 1 interproximal sites with PD $\geq$ 5 mm. Third molars were excluded from
3 the case definition.

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5 **References:**

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10 Periodontitis. Journal of Dental Research 2023;102(10): 1152-1161
11 3. Fu J, Shinjo T, Li Q, et al. Regeneration of glomerular metabolism and function by podocyte
12 pyruvate kinase M2 in diabetic nephropathy. JCI Insight 2022;7(5): e155260
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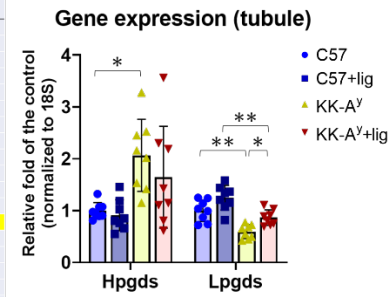
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Supplementary Fig.1 Parameters of each group of mice. (A) Daily food intake, (B) daily water consumption, (C) daily urine volume, (D) heart rate (HR) per minute, (E) systolic blood pressure (SBP), and (F) diastolic blood pressure (DBP) of each mouse at 16 weeks of age (n=6). (G) Blood glucose levels and (H) the area under the curve (AUC) during intraperitoneal glucose tolerance test (IPGTT) (n=6). (I) Blood glucose levels and (J) AUC during intraperitoneal insulin tolerance test (IPITT) (n=6). *: p<0.05, **: p<0.01, ***: p<0.001. All individual data are shown in a scatter plot (A-F,H,J). Data are presented as mean $\pm$ SD. P-values were determined by two-way ANOVA followed by post hoc tests (A-F) and unpaired Student's t-test (H,J).

A

gene_name	C Ave	CL Ave	K Ave	KL Ave	Fold	Pval	K-KL FDR	gene_description
Gm43305	6.094	11.73	5.705	15.962	2.7991	0.013		1 predicted gene 43305
Poln	1.574	1.37	1.79	3.9875	2.2265	0.014		1 DNA polymerase N
Opcml	17.84	13.44	13.96	29.225	2.0942	8E-06	0.03256	opioid binding protein/cell adhesion molecule-like
Celf4	0.981	0.888	1.457	2.8305	1.9444	0.034		1 CUGBP, Elav-like family member 4
Slc4a1	3.671	6.1	1.654	3.0457	1.8403	0.004		0.99028 solute carrier family 4 (anion exchanger), member 1
Gm43843	3.297	3.821	1.128	2.0528	1.8164	0.002		0.93055 predicted gene 43843
Zmat4	18.54	24.71	13.54	24.428	1.8051	9E-04		0.71354 zinc finger, matrix type 4
Clca3a1	1.648	2.02	2.499	4.3806	1.7535	0.015		1 chloride channel accessory 3A1
Gm36251	0.419	0.351	1.952	3.4221	1.746	0.04		1 predicted gene, 36251
Gem	32.46	19.5	17.66	30.184	1.709	0.005		1 GTP binding protein (gene overexpressed in skeletal muscle)
Pou3f1	0.39	0.242	1.4	2.3579	1.6852	0.043		1 POU domain, class 3, transcription factor 1
2010300C02Rik	7.648	8.696	1.456	2.4454	1.679	0.024		1 RIKEN cDNA 2010300C02 gene
Hpgds	0.296	0.249	46.95	78.207	1.6632	3E-04	0.02453	hematopoietic prostaglandin D synthase
Cyslr2	8.631	8.415	3.639	6.0618	1.6639	0.002		0.89858 cysteinyl leukotriene receptor 2
Carmil1	16.12	14.6	7.857	12.871	1.6374	0.012		1 capping protein regulator and myosin 1 linker 1
Scx	8.525	7.789	3.239	5.2722	1.6293	0.015		1 scleraxis
Gm38077	1.332	1.38	1.029	1.6703	1.6275	0.033		1 predicted gene, 38077
2410004P03Rik	1.143	1.173	1.632	2.6331	1.6125	0.029		1 RIKEN cDNA 2410004P03 gene
Lingo2	12.05	12.93	8.913	14.36	1.6111	0.011		1 leucine rich repeat and Ig domain containing 2
Ptgds	2.91	2.859	6.769	10.855	1.6028	0.004		0.99028 prostaglandin D2 synthase (brain)

B



Supplementary Fig.2 RNA-seq analysis data and gene expression of *Hpgds* and *Lpgds* in the tubules of each group. **A.** The dataset of average FPKM of top 20 genes in descending order of the ratio of KK-A^y mice with ligature (KL) to KK-A^y mice without ligatures (K) and with significant changes between KL and K. C: C57BL/6 mice without ligatures, CL: C57BL/6 mice with ligatures. FDR: false discovery rate. **B.** qPCR data of tubular *Hpgds* and *Lpgds* expression in each group (n=8). *: p<0.05, **: p<0.01, ***: p<0.001. All individual data are shown in a scatter plot. Data are presented as mean ± SD. P-values were determined by two-way ANOVA followed by post hoc tests.

1 **Supplementary Table.1** Real-time qPCR primers used in the study.

Target gene	Forward	Reverse
Mouse 18s rRNA	GCTTAATTTGACTCAACACGGGA	AGCTATCAATCTGTCAATCCTGTC
Mouse Col1a1	CATAAAGGGTCATCGTGGCT	TTGAGTCCGTCTTTGCCAG
Mouse Col3a1	ACAGCTGGTGAACCTGGAAG	ACCAGGAGATCCATCTCGAC
Mouse Col4a	GACAGCCAGGTTTGACAGGT	GGCAGCTCTCTCCTTTCTGA
Mouse Tgfβ1	CCTGAGTGGCTGTCTTTTGA	CGTGGAGTTTGTATCTTTGCTG
Mouse Ctgf	GTCTTCACACTGGTGCAGCC	ACTGGAAGACACATTTGGCC
Mouse Fibronectin1	CCCTATCTCTGATACCGTTGTCC	TGCCGCAACTACTGTGATTCGG
Mouse Tnfα	GACAGTGACCTGGACTGTGG	TGAGACAGAGGCAACCTGAC
Mouse Il-1β	GAAGAAGAGCCCATCCTCTG	TCATCTCGGAGCCTGTAGTG
Mouse Mcp-1	GAAGGAATGGGTCCAGACAT	ACGGGTCAACTTCACATTCA
Mouse Hpgds	GAATAGAACAAGCTGACTGGC	AGCCAAATCTGTGTTTTTGG
Mouse Lpgds	TCGCCTCCAACCTCAAGCTGGTT	CCATGATCTTGGTCTCACACTGG
Mouse Dp1	CAGTGCTCTTCACCATGTGTTCC	GACAGGAAACGCAAGGCTTGGA
Mouse Dp2	CCTGGTGGAAAACGGAATCATC	GGAAGTAGGTGAAGAAAGGCAGG
Mouse ZO-1	GTTGGTACGGTGCCCTGAAAGA	GCTGACAGGTAGGACAGACGAT
Mouse Occludin	TGGCAAGCGATCATACCCAGAG	CTGCCTGAAGTCATCCACTC

2 All primers were purchased from FASMAC (Kanagawa, Japan).

3

1 **Supplementary Table.2** Antibodies used in the present study.

Product (target)	Supplier/Catalog No.	Host	dilution
CD68	CST/#97778	Rabbit	IF 1:200
HPGDS	Cayman/10004349	Rat	WB 1:1000
β -actin	Wako/010-27841	Mouse	WB 1:15000

2 IF: immunofluorescent stain, WB: western blot.