1   **Characterisation of the Renal Cortical Transcriptome Following**

2   **Roux-en-Y Gastric Bypass Surgery in Experimental Diabetic Kidney Disease.**

3

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25   **Running Title:** RNA-Seq analysis of the effect of Gastric Bypass on the Renal Transcriptome

26   **Keywords**: kidney, transcriptome, diabetes, obesity, surgery, fibrosis

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1

2   **ABSTRACT**

3   **Introduction-**Roux-en-Y Gastric Bypass Surgery (RYGB) reduces albuminuria and the long-

4   term incidence of end-stage renal disease in patients with obesity and diabetes. Preclinical

5   modelling in experimental diabetic kidney disease (DKD) demonstrates that improvements

6   in glomerular structure likely underpin these findings.

7   **Research Design & Methods-**In adult male Zucker Diabetic Fatty (ZDF) rats, we profiled the

8   effect of RYGB on weight and metabolic control as well biochemical, structural and

9   ultrastructural indices of diabetic renal injury. Furthermore, we sequenced the renal cortical

10   transcriptome in these rats and used bioinformatic pathway analyses to characterise the

11   transcriptional alterations governing the renal reparative response to RYGB

12   **Results-**In parallel with improvements in weight and metabolic control, RYGB reduced

13   albuminuria, glomerulomegaly, podocyte stress, and podocyte foot process effacement.

14   Pathway analysis of RYGB-induced transcriptomic changes in the renal cortex highlighted

15   correction of disease-associated alterations in fibrosis, inflammation and biological

16   oxidation pathways. RYGB reversed disease-associated changes in the expression of TGF-β

17   superfamily genes that strongly correlated with improvements in structural measures of

18   glomerulopathy.

19   **Conclusions-**Improved glomerular structure in ZDF rats following RYGB is underpinned by

20   pathway level changes, including interruption of the TGF-β driven pro-fibrotic programme.

21   Our data provide an important layer of experimental support for clinical evidence

22   demonstrating that RYGB arrests renal damage in patients with obesity and Type 2 Diabetes.

23

1 **KEY MESSAGES**

2 *1. What is already known about this subject?*

3 • RYGB is an effective treatment for obesity and type 2 diabetes and longitudinal

4 cohort studies have demonstrated it's albuminuria lowering effect and evidence of

5 longer term reno-protection.

6 • Studies in pre-clinical models of diabetic kidney disease have described favourable

7 changes in measures of renal structure and ultrastructure following RYGB.

8

9 *2. What are the new findings?*

10 • The present study directly correlates structural and ultrastructural improvements in

11 the ZDF rat kidney following RYGB with corrective shifts in the global renal

12 transcriptome.

13 • Chronic renal remodelling responses in experimental DKD that are governed by TGF-

14 β signalling are interrupted and reversed by RYGB.

15

16 *3. How might these results change the focus of research or clinical practice?*

17 • These data will support further interrogation of RYGB specific shifts in the renal

18 transcriptome with a view to identifying tractable targets for treatment response

19 biomarkers and bariatric mimetic based diet and pharmacotherapy based

20 interventions.

21

1

2   **INTRODUCTION**

3   In a 2017 analysis of the global burden of Chronic Kidney Disease (CKD), over 30·7% of

4   associated disability-adjusted life years occurred in patients with background of diabetes

5   mellitus, this being the largest contribution from any single cause. (1) Analysis of 2013-2016

6   U.S data from the National Health and Nutrition Examination Survey (NHANES) illustrate this

7   point; with a microalbuminuria prevalence rate of over 40% in patients with an HbA1c

8   >64mmol/mol (DCCT >8%). (2) Approximately 25% of patients in this category have an

9   estimated glomerular filtration rate (eGFR) of less than or equal to 60ml/min/1.73m^2. (2)

10   Longitudinal analysis of NHANES III data reveals that patients developing progressive

11   albuminuria and eGFR decline have an estimated 10-year all-cause mortality incidence rate

12   of 47%, 4-times higher than the rate observed in patients with diabetes and no evidence of

13   renal impairment. (3)

14   The United Kingdom Prospective Diabetes Study (UKPDS) demonstrated the independent

15   and additive impact of targeting hyperglycaemia and hypertension on microvascular

16   outcomes of type 2 diabetes mellitus (T2DM) at medium-long term follow-up. (4)

17   Multimodal lifestyle and pharmacology based protocols such as those deployed in the

18   STENO-2 trial emphasise the benefits of comprehensive risk-factor control. (5) Recently

19   developed glucose-lowering therapies, including glucagon-like peptide 1 receptor agonists

20   (GLP1RAs) and sodium glucose co-transporter 2 inhibitors (SGLT2is), improve cardiovascular

21   and renal outcomes in people with diabetic kidney disease (DKD). (6-9) Nevertheless, DKD

22   remains a progressive disease.

1   Bariatric surgery is an effective intervention with long-term weight-loss and glucose-

2   lowering efficacy in patients with obesity and type 2 diabetes. (10-13).

3

4   A substantial evidence base now exists in the literature pointing to improved long-term

5   cardiovascular and renal outcomes following bariatric surgery. (14). A recent metanalysis

6   including data from 17,532 patients drawn from 10 high quality clinical studies reported that

7   bariatric/metabolic surgery was associated with significant improvements in DKD versus

8   medical treatment (odds ratio 15·41, 95% CI 1·28 to 185·46; P =0·03). (15) Much of the

9   benefit observed was premised upon reductions in urinary albumin excretion, supporting

10   previous focussed meta-analysis of the beneficial impact of bariatric/metabolic surgery on

11   this parameter in patients with DKD. (16) Two further key reports have emerged in 2020,

12   which add significant weight to the clinical evidence base. Firstly, in a balanced comparison

13   of the impact of metabolic surgery (Teen-LABS study NCT00474318) versus active lifestyle

14   and medical therapy (TODAY study NCT00081328) on DKD in adolescents with obesity and

15   T2D, marked increases in odds ratios for hyperfiltration (OR 17.2, 95% CI 2.6–114.5; P=0.03)

16   and albuminuria (OR 27.3, 95% CI 5.2–146.2: P<0.0001) were noted over 5 year follow-up in

17   the TODAY participants. (17) This reflected opposing trajectories in these parameters over

18   follow-up. Secondly, we have recently collaborated in the *Clinical Study on Metabolic*

19   *Surgery Compared to the Best Clinical Treatment in Patients with Type 2 Diabetes Mellitus*

20   *(MOMS)* RCT (NCT01821508). (18). This study demonstrated that at 2 year follow-up in

21   patients with T2DM, Class I obesity (BMI 30-35kg/m^2) and mild-moderate DKD (albumin-to-

22   creatinine ratio [uACR] 3-30mg/mmol and eGFR >30mL/min/1.73m^2) microalbuminuria

1 remission occurred in 82% (95% CI=72-93%) of patients after RYGB versus 54.6% (95%

2 CI=39-70%) of patients after best medical treatment (P=0.006). (18)

3 The potent glucose lowering effects of Roux-en-Y gastric bypass surgery (RYGB) have been

4 demonstrated to be complimented by improvements in blood pressure which may be

5 mechanistically linked in part to a unique natriuretic effect of the procedure. (19-20) We

6 have previously reported that RYGB effectively reduces albuminuria in the Zucker Diabetic

7 Fatty (ZDF) rat in conjunction with evidence of improvements in glomerular histology and

8 ultrastructure. (21, 22)

9 Herein we present an in-depth analysis of global changes in the renal cortical transcriptomic

10 occurring in response to RYGB in ZDF rats. We report that improvements in renal injury

11 following RYGB in the ZDF are underpinned by significant pathway levels shifts in the renal

12 cortical transcriptome that correlate with structural improvements, cross-reference to

13 human DKD and highlight that RYGB interrupts the pro-inflammatory and TGF-β driven

14 fibrotic programme characteristic of progressive CKD.

15

1   **METHODS**

2

3   *Animal Studies*- Experiments were undertaken under governmental project license (Health

4   Products Regulatory Authority– AE18692-P084). ZDF (fa/fa) and fa/+ rats (Charles River

5   Laboratories (France and U.K.)) were purchased at 6 weeks of age and maintained on Purina

6   5008 chow *ad libitum*. Experimental groups consisted of fa/+ rats (n=6), SHAM-operated

7   fa/fa rats (n=8), and RYGB-operated fa/fa rats (n=9). Surgery was performed at 12 weeks of

8   age and animals were humanely killed at the end of the eighth post-operative week. Body

9   weight and glycaemia (ACCU-CHEK®-Roche) were assessed weekly.

10

11   *Surgical Protocols*- Insulin degludec (Novo Nordisk, Denmark) was used for 7-days prior to

12   surgery to achieve morning glucose readings of <12 mmol/L. Under isoflurane anaesthesia, a

13   midline laparotomy and mobilisation of the gut was performed as SHAM surgery. According

14   to standard protocol (21, 22), in RYGB-operated rats the proximal jejunum was transected

15   12cm from the pylorus and a side-to-side jejuno-jejunal anastomosis created leaving a

16   common channel of 30cm. A small gastric pouch was created and end-to-end anastomosed

17   to the alimentary limb.

18

19   *Biochemical Analyses*- Albumin-Creatinine Ratio (ACR)- Urinary albumin and creatinine were

20   assessed in urine samples collected over 16 hours in metabolic cage. Metabolic cage studies

21   were undertaken the week prior to surgery and 8 weeks later. Analytes were measured

22   using an autoanalyzer (Roche/Hitachi Cobas c502 Modular analyser) and resulting values

23   used to derive the ACR (mg/g). Colorimetric assay was used to measure mg/dL

1   concentrations of plasma triglyceride (Cayman Chemical 10010303) and total cholesterol

2   (WAKO 294-65801). Urinary osteopontin concentrations (ng/ml) were assessed by ELISA

3   (R&D DOST00), normalised by urinary creatinine and reported as ng/mg.

4

5   Glucose Tolerance Test

6   RYGB operated ZDF rats and fa/+ animals underwent glucose tolerance tests at 19 weeks of

7   age, 8 weeks after the ZDF rats had underwent RYGB. Animals were fasted for 16 hours

8   before receiving a bolus of glucose (1g/kg), intra-peritoneally or orally. Blood glucose

9   measurements were taken at time 0, 15, 30, 45, 60, 90 and 120 minutes following bolus

10   administration. Area under the curve was calculated using the trapezoidal rule.

11

12   *Histological & Immunohistochemical Analyses*- Immunohistochemical studies were carried

13   out using 4μm thick sections of formalin fixed paraffin embedded kidney or pancreas.

14   Picrosirius Red staining of kidneys was carried out on 10μm thick sections using a kit (

15   Polysciences 24901). Immunohistochemistry of Wilms' Tumour (WT-1), (antibody C-19, sc-

16   192 Santa Cruz Biotechnology, 1:250 dilution) and desmin (Antibody Clone Dako.Cytomation

17   1:100 dilution) and insulin (antibody ab181547 Abcam, 1:64,000 dilution) were carried out

18   using a Dako Autostainer Link 48 system. Signal amplification was achieved using the

19   horseradish peroxidase-based FLEX system with signal development using 3,3'-

20   Diaminobenzidine (DAB)+ substrate. IgG1 isotype controls and no antibody controls were

21   used to confirm specificity of staining.

22

1   Glomerular morphometry was performed using Image J 1.48v software analysis of scanned

2   20x images of WT-1 stained sections (Hamamatsu Photonics, Digital Slide Scanner). Thirty

3   glomeruli per sample were analysed. Glomerular volume (μM^3) was estimated using the

4   Weibel and Gomez formula. (23) Quantification of desmin staining was carried out in 25

5   individual glomeruli per sample. Desmin and insulin staining was quantified using an

6   algorithm with macros specific to DAB staining derived from the Aperio Cytoplasmic V2

7   Algorithm (Leica).

8

9   *Transmission Electron Microscopy (TEM)*- Glutaraldehyde fixed and 1% osmium tetroxide

10   post-fixed renal tissue was dehydrated and infiltrated with EPON™ Epoxy Resin. Ultra-thin

11   sections were prepared and imaged by transmission electron microscopy (Technai 12).

12   Glomerular basement membrane (GBM) thickness was measured according to the Haas

13   method at 22500x (24). Twenty GBM thickness measurements were recorded per specimen,

14   from a minimum of five separate capillary loops and representative of at least three

15   separate glomeruli per sample. Podocyte Foot Process Frequency (PFPF) was estimated at

16   9900x by determining the number of podocyte foot processes per unit length (8μM) of

17   peripheral GBM. Podocyte Foot Process diameter (PFPD) was also measured as a reciprocal.

18   Six measurements were recorded per sample.

19

20   *Transcriptomic and Bioinformatic Analyses*- RNA isolation was carried out using RNeasy mini

21   kits (Qiagen). RNA concentration and quality were measured using NanoDrop 2000 (Thermo

22   Fischer) and bioanalyzer analyses (Agilent 2100) respectively. An RNA integrity number (RIN)

23   of >7 was set as the quality threshold for subsequent RNA sequencing. RNA Library

1   preparation was carried out using the TruSeq® Stranded mRNA NeoPrep™ Kit (Illumina NP-

2   202-1001). Libraries were pooled and diluted to 1.8pM then sequenced (NextSeq-Illumina

3   FC-404-2002). Raw data processing and differential expression analysis was carried out with

4   the support of The Bioinformatics Core Facility, University of Gothenburg, Sweden followed

5   by pathway enrichment analyses using the R programming language (Supplementary File

6   S1). Sequencing data files have been deposited in GEO (GSE117380)

7

8   *qPCR and Western Blotting*- Validation of selected genes was conducted at the mRNA level

9   by TaqMan quantitative reverse transcriptase PCR (Thermo-Fischer). Amplification was

10   performed on QuantStudio 7 Flex® (Applied Biosystems). Beta-actin was used as an

11   endogenous control for normalisation of target genes. Results were analysed based on

12   ΔΔCt quantitation. method of analysis. For Western blot analysis, normalized protein

13   extract was resolved by SDS-PAGE. Proteins were then transferred onto PVDF membranes

14   (Millipore), blocked with TBS-T supplemented with 5% (w/v) non-fat dried milk or BSA and

15   then incubated with the following antibodies at 4°C overnight: Alpha-tubulin (1:10,000 in

16   5% milk; Abcam Ab4074), Fibronectin (1:5,000 in 5% milk; Abcam Ab2413), and Vimentin

17   (1:1,000 in 5% BSA; Cell Signalling Rabbit mAb #5741). Membranes were subsequently

18   incubated with horseradish peroxidase-linked secondary antibodies for 1 hour at room

19   temperature (Cell Signalling) and developed using chemiluminescence reagents

20   (WesternBright™).

21

22   *Descriptive and Inferential Statistics*- Analyses were conducted using GraphPad Prism V.6

23   and IBM SPSS V.24. Individual data points for each group are presented in boxplots with

1   annotation of the group median and inter-quartile range (IQR). Longitudinal repeated

2   measures data was assessed by Wilcoxon signed-rank test. Cross-sectional data was

3   assessed by Kruskal-Wallis testing, correcting for multiple comparisons using Dunn's test.

4   Statistical significance was set at $p < 0.05$.

5

1   **RESULTS**

2   **Weight Loss and Improvements in Metabolic Control Following RYGB (Figure 1).**

3   Despite maintaining a higher bodyweight for the majority of the study period, by study

4   close, there was no significant difference in body weights between SHAM-operated ZDF rats

5   and fa/+ lean controls 363.5g (332.5-383.3) vs. 386.5g (374.8-413.3); p=0.22, Figure 1A).

6   Bodyweight in the RYGB group was significantly lower than in the SHAM group 327g (316-

7   340) vs. 386.5g (374.8-413.3); p<0.001) and not significantly different from weights in the

8   fa/+ group (p=0.31, Figure 1A). Over the course of the intervention period, median AUC

9   calculated from the sum of morning plasma glucose readings in the SHAM group was 4-fold

10   greater than that recorded in the fa/+ group (283.0 (237.8-338.4) vs. 71.7 (69.36-72.81);

11   p<0.001, Figure 1B). Median AUC plasma glucose in the RYGB group, although elevated

12   relative to the fa/+ group, was significantly lower than in the SHAM group (164.3 (137.7-

13   184.4) vs. 283.0 (237.8-338.4) p=0.03). Morphological examination of the pancreatic islets

14   was carried out alongside immunohistochemical detection of pancreatic insulin expression

15   (representative images in Figure 1C). Islets in in the fa/+ group were of a round, uniform

16   morphology. In contrast, islets in the SHAM group were less uniformly round, and often

17   had a disrupted appearance. Islets in the RYGB group were more uniform. Insulin staining in

18   the islets of SHAM operated animals demonstrated a trend to be lower relative to the fa/+

19   group with the median number of insulin positive cells/mm^2 in the SHAM group being 2231

20   (1413-2634) and in the fa/+ group 3304 (3135-4354) (p=0.053). The median number of

21   insulin positive cells per mm^2 in the RYGB group was 4480 [IQR 3548-4833] and hence

22   insulin staining was significantly increased in the RYGB islets relative to the SHAM group

23   (p=0.008). Although there was no difference in insulin staining between the RYGB and fa/+

24   groups (p>0.999) and morning glucose levels were largely normalised after RYGB (Figure

1   1B), evidence of ongoing beta-cell dysfunction in RYGB operated animals relative to fa/+

2   was observed by conducting oral and intra-peritoneal glucose tolerance tests at 8-week

3   follow-up (Figure 1D).

4

5   Median plasma triglyceride concentration was elevated in the SHAM group relative to the

6   fa/+ group 515.6mg/dL (419.5-8-532) vs.   76.8mg/dL (53.2-105.7);p=0.01, Figure 1E).

7   Triglyceride levels were not lowered relative to SHAM in the RYGB group. Median plasma

8   cholesterol was 2.7-fold higher in the SHAM group relative to the fa/+ group (201.1mg/dL

9   (145.2-241.3) vs. 75.3mg/dl (56.1=87.5); p<0.001, Figure 1E). Plasma cholesterol levels were

10   reduced in the RYGB group relative to SHAM group (110.3 (101.9-121.1) vs. 201.1mg/dL

11   (145.2-241.3) ; p=0.03 Figure 1E).

12

13   **Improvements in Albuminuria, Glomerular Structure and Ultrastructure Following RYGB**

14   **(Figure 2).**

15   Longitudinal paired analysis of reduced urinary albumin-to-creatinine ratios (uACRs) (Figure

16   2B) after RYGB showed reductions at 8 weeks post-surgery relative to baseline pre-

17   operative values (17.7µg/mg (9.5-55.7) vs. 159.4µg/mg (10-468.8); p=0.010). Median

18   glomerular volume (Figure 2C) was elevated in the SHAM group relative to the fa/+ group

19   $(1.2x10^6µM^3$ $(1.1x10^6-1.5x10^6)$ v's $8.1x10^5$ $µm^3$ $(7.61x10^5-8.6x10^5)$;   p=0.005. Median

20   glomerular volume in the RYGB group was reduced by 33% relative to the SHAM group

21   (p=0.020) and was not different to the fa/+ group (p>0.999).

22

1   Glomerular desmin staining (Figure 2D) was increased in distribution and intensity in the

2   SHAM group compared to fa/+ and RYGB. Staining was localized within the glomeruli, in the

3   mesangium, and at the periphery of the glomerular tuft. Median glomerular desmin

4   positivity (Figure 2D) was elevated 6-fold in SHAM relative to the fa/+ group

5   (512.1cells/mm^2 (350.8-893.9) vs. 84.4 cells/mm^2 (8.3-211.5); p=0.021). Glomerular desmin

6   positivity was significantly lower in RYGB relative to SHAM (168.6cells/mm^2 (4.2-289.2) vs

7   512.1cells/mm^2 (350.8-893.9) 'p=0.014). There was no difference in desmin staining

8   between the fa/+ and RYGB group (p>0.999).

9

10   Podocyte foot process frequency (PFPF, Figure 2E) was reduced by 30% in the SHAM group

11   relative to the fa/+ group (13.5 (12.5-15.5) vs. 19.0 (18.2-21.0); p=0.002). Median PFPF in

12   the RYGB group was higher than in the SHAM groups (17. (15.71-19.54) vs 13.5 (12.5-15.5);

13   p=0.026) Accordingly median podocyte foot process diameter (PFPD, Figure 2F) was 40%

14   higher in the SHAM group relative to the fa/+ group (0.46μM (0.43-0.47) vs. 0.33μM (0.30-

15   0.36); 0.33±0.04μM; p=0.002, with measures in the RYGB group significantly lower than in

16   SHAM (p=0.015). Increases in glomerular basement membrane (GBM) thickness (Figure 2G)

17   were observed in the SHAM group relative to the fa/+ group (228.4nm (220.3-238.2) vs.

18   211.3nm (203.9-220.8); p=0.044). Median GBM thickness in the RYGB group was

19   intermediate to those of fa/+ and SHAM at 220.0nm (212.5-227.9).

20

1   **RYGB Corrects Disease-Associated Pathway Level Changes in The Renal Cortical**
2   **Transcriptome (Figure 3).**

3

4   Figure 3A shows dimension reduction of variance in the sequencing data along the first 2

5   principal components. Separate clustering of fa/+ and RYGB derived samples is evident

6   against a background of relatively increased variance in the SHAM group. A total of 379

7   genes were identified as being differentially expressed in the ZDF rat kidney versus the fa/+

8   (212 downregulated, 167 upregulated, absolute fold-change ≥1.3 and adjusted p-

9   value<0.05-Supplementary Table S2), corresponding to a change in 2.1% (379/18,423) of the

10   common renal transcriptome. Genes showing marked changes in expression in the kidneys

11   of diabetic animals included cardinal indicators of tubular injury and inflammatory and

12   fibrotic change such as such as *Umod* (uromodulin), *Havcr1* (Kidney-Injury Molecule 1),

13   *Vim1* (vimentin), *Fn1* (fibronectin), *Spp1* (osteopontin) and I*L24* (interleukin-24)

14

15   The Comparison of the transcriptome of SHAM- and RYGB-operated ZDF rats 8 weeks after

16   surgery identifies a total of 942 genes as being identified differentially expressed (529

17   downregulated, 413 upregulated, absolute fold-change ≥1.3 and adjusted p-value<0.05

18   Supplementary Table S3). This corresponds to 5.1% (942/18,423) of the common renal

19   transcriptome. Reciprocal counter-regulation between SHAM versus fa/+ and RYGB versus

20   SHAM of the expression of recognised disease associated genes such as those mentioned

21   above is evidenced by pairwise comparison of Volcano plots (Figures 3B and 3C). RYGB was

22   also associated with augmented expression of several genes that likely reflect adaptive

23   responses to sub-optimal micronutrient assimilation following RYGB, including *Epo* and

24   *Cyp27b1*, indicative of impaired iron and vitamin D homeostasis, respectively.

1   Disease-associated enrichment of multiple pathways in the Reactome database was

2   detected (Figure 3D), with notable emergence of pathways linked to the innate immune

3   activation and extracellular matrix remodelling. Enrichment of changes in extracellular

4   remodelling, inflammation, cell cycle and biological oxidation related pathways were highly

5   prominent in the RYGB versus SHAM comparison (Figure 3E). Reciprocal changes at pathway

6   level between SHAM versus fa/+ and RYGB versus SHAM comparisons were evident (Figure

7   3F), suggesting arrest of progressive inflammation and fibrosis as well as restoration of

8   tubular biotransformation function following RYGB. Interestingly, enrichment in cell cycle-

9   related pathways after RYGB appeared as a discrete phenomenon not directly reflecting a

10   correction away from the disease-associated profile of SHAM-operated kidneys. Congruent

11   with pathway analysis, animals that underwent RYGB surgery were predicted to have a

12   relative reduction in immune cells and fibroblasts versus SHAM-operated animals using

13   MCP-counter (Figure 3G). Picrosirius Red (Figure 3H) demonstrates that SHAM-operated

14   animals showed characteristic signs of early fibrosis including capsular fibrosis and patchy

15   collagen deposition in the tubulointerstitium at sites of strong inflammatory infiltration

16   adjacent to damaged tubules. Such foci were not uniformly observed in the kidneys of

17   animal that underwent RYGB.

18

19   **RYGB Normalises the Expression of Genes that Correlate with Markers of Renal Injury and**
20   **Cross-reference to the Human DKD Transcriptome (Figure 3).**

21   We subsequently focussed on the extent to which RYGB corrected characteristic disease-

22   associated gene expression patterns in the SHAM-operated ZDF rat. The Venn Diagram in

23   Figure 4A depicts 144 differentially expressed genes common to SHAM versus fa/+ and

24   RYGB versus SHAM comparisons. The directionality of change following RYGB was opposite

1   to that occurring between health and disease in all but one case (RANTES), highlighting that

2   38% of the major corrective impact of RYGB on the renal cortical transcriptome (Figure 4B).

3   We cross-referenced the list of 144 transcripts to the the Woroniecka human DKD

4   glomerular microarray dataset (10), identifying 22 transcripts which were significantly

5   differentially expressed in both human DKD and ZDF SHAM rats and which were also

6   counter-regulated following RYGB (Figure 4C). From within this subset of genes translating

7   to human disease, relative levels of mRNA and protein expression for vimentin, fibronectin

8   and Spp1/osteopontin were assessed as a validation of the RNA sequencing analysis (Figure

9   4D and 4E). When normalised gene expression counts for transcripts within the group of 22

10   genes translating to human disease were correlated with markers of glomerular injury

11   (Figure 4F), strong correlations with disease status were noted thereby associating these

12   specific transcriptional shifts following RYGB with quantitative changes in glomerular

13   structure and ultrastructure.

14

15   **Suppression of TGF-β1 Target Gene Expression in the Kidney Following RYGB Coincides**
16   **with Transcriptional Evidence of Restoration of BMP-7 Signalling (Figures 4).**

17   Figure 5A presents a heatmap of z-scores of the top 10 upstream regulators of pathway

18   enrichment for SHAM versus fa/+ and RYGB versus SHAM comparisons. In line with

19   previous observations, and indicative of the primacy of changes in extracellular matrix

20   pathway activation between fa/+, SHAM and RYGB groups, TGF-β1 signalling was identified

21   as a key predicted upstream regulator in disease which is prominently suppressed following

22   RYGB. Notably, RYGB also reversed the upstream regulator influence of several

23   inflammation related factors including CSF2, IL-1β and TNFα. In addition, the c-myc related

24   cell cycle regulating factor ZBTB17 predicted to act as an upstream regulator of

1　transcriptional change in the kidney following RYGB, an observation coherent with the

2　strong pathway level signal for changes in cell cycle pathway activation.

3

4　We visualised constituent changes in the KEGG TGF-β signalling pathway via Pathview for

5　the RYGB versus SHAM comparison (Figure 5B). RYGB reduced expression of genes involved

6　in the regulated sequestration and activation of TGF-β1 including fibrillin 1 (FBN1), Decorin

7　and Follistatin. RYGB also reduced the expression of the Bone-morphogenic protein (BMP)

8　antagonist Gremlin-2 and increased the expression of the BMP-7 responsive Id4 gene,

9　suggestive of restoration of anti-fibrotic signalling in the renal tubule. Tandem reciprocal

10　changes in Gremlin-2 and Id4 transcript levels following RYGB were validated by qPCR

11　analysis (Figure 5C)

12

13　**DISCUSSION**

14　We sought to extend insights into the clinically observed effects of RYGB in patients with

15　DKD using a reverse translational research approach. We corroborated our previous findings

16　regarding the beneficial impact of RYGB on the cardinal biochemical, structural and

17　ultrastructural features of experimental DKD in the ZDF rat. (21, 22) We sought then to

18　describe both the transcriptional landscape in the kidneys of ZDF SHAM rats, the changes to

19　that landscape arising secondary to RYGB and how these changes correlated with structural

20　and ultrastructural indicators of renal injury.

21

22　We identified 379 mRNA transcripts that were differentially regulated in the ZDF SHAM rat

23　kidney versus fa/+ control. This number of genes is comparable in quantity and quality to

1   previously reported changes at 28 weeks of age in whole renal tissue RNA-Seq from the

2   related obese and diabetic Zucker/SHHF (ZS) rat (26); ZDF-167 genes up 212 genes down

3   and ZS rats 184 genes up, 396 genes down. The predominance of proinflammatory and pro-

4   fibrotic signals observed in ZDF SHAM animals and the implication of TGF-β1 signalling as an

5   upstream regulator is also shared with the analysis from ZS rats (26). Whilst single cell RNA-

6   Seq studies of murine and human diabetic kidney disease have recently been published (27,

7   28), the capacity for comparative extrapolation of our data to these profiles is limited, given

8   the likely predominance of tubular epithelial derived transcripts in the renal parenchymal

9   signal in our dataset. However, of the 379 differentially expressed genes in the ZDF SHAM

10   rat kidney, 77 were significantly altered in the same direction as in the Woroniecka DKD bulk

11   glomerular dataset, showing that transcriptional changes in the whole renal tissue of rats

12   showed significant homology to transcriptional profiles in human DKD. (25)

13

14   We demonstrated that RYGB has an impact on the renal cortical transcriptome in DKD,

15   correcting 38% of the disease associated changes in the transcriptome and reversing

16   activation of many of the pathway level changes occurring in disease and their upstream

17   regulators. In this respect, the pre-eminence of patterns of transcriptional change that map

18   at the pathway level to processes governing inflammation, biological oxidation and, most

19   notably, the fibrotic response is indicative of RYGB being associated with a broad range of

20   renoprotective effects. Reduced activation of the TGF-β1 signalling pathway in the kidney

21   after RYGB is a key finding and remphasises both the importance of this pathway in the

22   progression of DKD, and involvement of interruption of this pathway as a common

1   mechanism driving the anti-fibrotic effects of established therapies such as renin-

2   angiotensin-aldosterone system blockade. (29)

3

4   Importantly, those RYGB-induced improvements in the renal transcriptome that translated

5   to the human DKD transcriptome also correlated with improvements in biochemical,

6   histological and ultrastructural markers of glomerular damage. This is interesting given that

7   tubular injury related signals likely predominate in our dataset and supports the concept

8   that although glomerular damage in early DKD is readily apparent at both the biochemical

9   and histological level, these changes may be triggered by renal tubular dysfunction in early

10   DKD through tubulo-glomerular cross-talk. (30) The specific responses of the renal tubule to

11   RYGB should be the focus of future studies, particularly the impact of improved metabolic

12   control on mitochondrial bioenergetics and the metabolic switch away from fatty acid

13   oxidation that is now proposed to underpin renal tubular dedifferentiation and pro-fibrotic

14   activation in DKD. (31).

15

16   Our data show coherent downward shifts in renal mRNA expression of the *Spp1* gene and

17   the urinary excretion of its protein product osteopontin after RYGB, suggesting the potential

18   utililty of this protein as a marker of treatment response. In addition, post-operative

19   improvements in the mRNA expression of other cardinal indicators of tubular injury and

20   recovery such as *Havcr1* (KIM-1, four fold reduction in RYGB versus Sham) and Umod

21   (Uromodulin, two-fold increase in RYGB versus Sham) points to the potential utility of

22   treatment responsive biomarker assessment which could be used in parallel with

23   measurement of urinary albumin excretion as a non-invasive means of monitoring the short

1   to medium term therapeutic response to surgery. Serum uromodulin has already been

2   identified as a potentially useful marker of renal improvement post-metabolic surgery in

3   patients with T2DM and obesity (32)

4

5   The comprehensive transcriptome wide profiling approach adopted herein have permitted

6   us to that approximately 86% of changes to the renal transcriptome induced by RYGB are

7   unique to the procedure itself and do not simply reflect reversal of disease-associated gene

8   expression patterns. Some of the alterations in expression signatures particular to the RYGB

9   intervention may be related to unique beneficial effects of the procedure, and interrogation

10   of candidates in this regard will form the basis of future mechanistic studies. Conversely, a

11   proportion of these changes likely reflect adaptive responses to undesirable effects of the

12   procedure. Owing to the anatomical reconfiguration occurring in RYGB, uptake of certain

13   nutrients and minerals including iron, calcium and vitamin D which are normally absorbed in

14   the proximal small intestine can be impaired. (33-35) For example, calcium absorption

15   efficacy in the proximal jejunum is regulated by activated vitamin D via the vitamin D

16   receptor. (36) RNAseq analysis revealed that one of the top differentially expressed genes in

17   the RYGB ZDF rat kidney was Cyp27b1. Cyp27b1 encodes 25-Hydroxyvitamin D3 1-α-

18   hydroxylase, the enzyme that converts vitamin D to its active form 1,25-dihydroxyvitamin

19   D3. Upregulated expression of the enzyme in the RYGB kidney suggests that there could be

20   resistance to the effects of 1,25 dihydroxy-vitamin D after RYGB, which thus prevents

21   normal negative feedback inhibition of Cyp27b1 in the vitamin D replete state. (37) Future

22   studies directed at differentiating the beneficial from the detrimental effects of RYGB on the

1   kidney via interrogation of the RYGB-specific transcriptional changes and assessment of

2   their likely impact on the kidney using *in vitro* modelling merit consideration.

3

4   There are some noteworthy limitations of our study. As a result of loss of leptin deficiency

5   and attendant reductions in sympathetic drive, the ZDF rat is not hypertensive until much

6   later in the process of renal decline and thus our data are likely more relevant to

7   normotensive or blood pressure controlled DKD. That said, RYGB effectively treats

8   hypertension (38), a phenomenon that may be attributable in part to enhanced natriuresis

9   (19-20). The ZDF rat also does not display prominent tubulointerstitial fibrosis and renal

10   functional decline until late in the course of the disease. However, pathway analysis of the

11   correction in the renal transcriptome induced by RYGB highlights interruption of the early

12   pro-fibrotic programme as a key effect of the intervention. Definitive resolution of the effect

13   of RYGB on discrete cellular subsets in the diabetic kidney was not possible in the present

14   study but future studies using single cell RNA-Seq are warranted.

15

16   This study defines key transcriptomic and pathway level changes that characterise the arrest

17   of DKD progression following RYGB. In future it may be possible to replicate these

18   transcriptomic and pathway level changes without the use of surgery. An important

19   intermediate step in attempting to achieve this would involve generating a comprehensive

20   assessment of the activation state of implicated signalling pathways involved, ideally using

21   an unsupervised phospho-proteomics bioinformatic analysis based approach which may

22   allow for proximate novel receptor level targets to be identified for rational drug design. The

23   current mechanistic evidence however supports the potential of RYGB to form a part of the

1   treatment algorithm targeted at addressing the burden of renal disease arising from

2   increases in the prevalence of obesity and type 2 diabetes.

3

1   Foundation for the Study of Diabetes /Boehringer Ingelheim European Diabetes Research

2   Programme (BI 2017_3) to ClR and NGD, Science Foundation Ireland (15/IA/3152 and

3   15/US/B3130) to CG and EPB. WPM's contribution was performed within the Irish Clinical

4   Academic Training (ICAT) Programme, supported by the Wellcome Trust and the Health

5   Research Board (Grant Number 203930/B/16/Z), the Health Service Executive National

6   Doctors Training and Planning and the Health and Social Care, Research and Development

7   Division, Northern Ireland.

8

9   NGD and ClR are co-guarantors of this work and, as such, had full access to all the data in

10   the study and take responsibility for the integrity of the data and the accuracy of the data

11   analysis.

12

13   ClR discloses personal fees outside of the submitted work from Novo Nordisk, GI Dynamics,

14   Eli Lilly, Johnson and Johnson, Sanofi, Aventis, Astra Zeneca, Janssen, Bristol-Myers Squibb

15   and Boehringer-Ingelheim

16   Ethics Approval Disclosure Statement

17   All studies detailed were approved by the University College Dublin Animal Research Ethics

18   Committee and licensed by Government of Ireland Health Products Regulatory Agency

19   (protocol #AE18692-P084)

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27

1   **FIGURE LEGENDS**

2   **Figure 1-RYGB Reduces Bodyweight and Improves Metabolic Control**

3   Trajectories of A) Body weight (g) and B) morning plasma glucose concentrations (mmol/L)

4   before and after surgery and final values at post-operative week 8. C) Representative

5   images of insulin immunohistochemistry based assessment of pancreatic islets at post-

6   operative week 8 showing improved morphology and insulin positivity post-RYGB. D) Oral

7   and intraperitoneal glucose tolerance testing in fa/+ rats and ZDF fa/fa rats at 8 weeks post-

8   RYGB. Glucose tolerance tests were not performed in SHAM operated ZDF rats due to

9   predictable futility given severe fasting hyperglycaemia which exceeded predicted maximal

10   excursions in fa/+ rats or ZDF RYGB operated animals   E) Plasma triglyceride (mg/dl) and

11   total cholesterol (mg/dl) concentrations at post-operative week 8.) $*p<0.05$ vs fa/+;

12   #p<0.05 vs. SHAM.   SHAM-sham surgery (laparotomy); RYGB-Roux-en-Y Gastric Bypass,

13   OGTT-oral glucose tolerance test, and ipGTT-intraperitoneal glucose tolerance test).

14

1

Figure 2- Impact of RYGB on Biochemical, Structural and Ultrastructural Indices of Diabetic Renal Injury

A) Representative images of glomerular desmin expression (immunohistochemistry 20x, scale bar 200µM) and glomerular ultrastructure (TEM , 9900x, scale bar 1µM) in fa/+, SHAM and RYGB rats. B) Urinary ACR (µg/mg) during the week before surgery and at post-operative week 8. C) Glomerular volume was assessed in 30 glomeruli per animal and the median glomerular volume (μM^3) calculated. D) Glomerular desmin positivity (cells/mm^2) was assessed in 25 glomeruli per animal. E) PFPF, F) PFPD and G) GBM thickness were assessed using T.E.M. For B $p<0.05$ vs.baseline. For C through H *$p<0.05$ vs fa/+; #$p<0.05$ vs. SHAM. SHAM-sham surgery (laparotomy); RYGB-Roux-en-Y Gastric Bypass, TEM-Transmission Electron Microscopy, ACR-Albumin Creatinine Ratio, PFPF-Podocyte Foot Process Frequency, PFPD-Podocyte Foot Process Diameter, GBM-Glomerular Basement Membrane).

Figure 3Reductions in Fibrotic Pathway Activation Dominate the Transcriptomic Response to RYGB

A) Principal component analysis based summarisation of the renal cortical transcriptome in fa/+, SHAM and RYGB operated rats. Volcano plot visualisation of differential expression analysis for the SHAM versus fa/+ comparison (B) and RYGB versus SHAM comparison (C). Reactome based analysis of pathway level changes in the SHAM versus fa/+ (D) and RYGB versus SHAM comparisons (E). F) Alignment of pathway level changes between SHAM versus

1   fa/+ and RYGB versus SHAM comparisons indicating directionality of change. A fold-cut off

2   change of 1.3 was applied in pathway analyses. In each graphic, adjusted p-value and gene

3   ratios for each significantly altered pathway are represented as a function of colour and size

4   of dots respectively. G) Heat-map visualisation of MCP Counter based estimates of the

5   abundance of 8 immune and 2 stromal cell populations. Data is overlaid with heat-map

6   annotation of quantitative measures of glomerular structure and ultrastructure for each

7   sample included in the transcriptomic analysis. Colour coding indicates per sample relative

8   abundance as a function of centered and scaled row specific data. Blue indicates low

9   relative abundance and orange-red increasing relative abundance H) Representative images

10   of Picrosirius-Red staining for total collagen in renal cortex. (SHAM-sham surgery

11   (laparotomy); RYGB-Roux-en-Y Gastric Bypass).

12   **Figure 4-RYGB Normalises the Expression of Genes that Correlate with Markers of Renal**
13   **Injury and Cross-reference to the Human DKD Transcriptome.**

14   A) Venn diagram showing number of gene changes in each differential expression analysis

15   and extent of overlap. B) Heat-map of expression of 144 genes significantly changed in both

16   SHAM versus fa/+ and RYGB versus SHAM comparisons. C) Heat-map of expression of genes

17   changed in both comparisons that cross-reference to significantly altered expression profiles

18   in microarray analysis of glomerular isolates from 13 healthy living donors, and 9 diabetic

19   nephropathy patients. (Nephroseq-Woroniecka dataset). In both B and C, colour intensity

20   for each sample in the heat-maps reflects relative abundance of given transcript based on

21   normalised counts from RNA-Seq with blue indicating lower expression and orange-red

22   indicating higher expression. Heat maps are headed by annotation of quantitative measures

23   of glomerular structure and ultrastructure for each sample included in the analysis.

24   Validation of expression changes in vimentin, fibronectin and Spp1 was undertaken at the

1   mRNA level by qRT-PCR (D) and Western blotting (E). F) Correlation of expression levels for

2   human validated genes from panel C with quantitative measures of glomerular injury in

3   sections of rat renal cortex. Green indicates a positive correlation and purple a negative

4   correlation. Increasing colour intensity and spot-size signifies increasing correlation

5   coefficient. SHAM-sham surgery (laparotomy); RYGB-Roux-en-Y Gastric Bypass. *p<0.05 vs

6   fa/+; #p<0.05 vs. SHAM.For osteopontin ELISA *p<0.05 baseline versus post-operative week

7   8. (

8

9   **Figure 5- TGF-β1 Pathway activation is Reduced Following RYGB**

10   A) The upstream regulator function of Ingenuity Pathway Analysis software was used to

11   predict master transcriptional regulators underpinning disease associated shifts in the renal

12   cortical transcriptome in SHAM operated rats and changes in the disease associated

13   transcriptome arising secondary to RYGB. Colour intensity reflects weighted z-score. (B)

14   Annotation of the KEGG TGF-β signalling pathway with genes, the expression of which is

15   significantly altered by RYGB. Green signifies that expression is reduced by RYGB and red

16   indicates increased expression following RYGB. (SHAM-sham surgery (laparotomy); RYGB-

17   Roux-en-Y Gastric Bypass . C) Validation of expression changes in Grem-2 and Id4 at the

18   mRNA level was undertaken by qRT-PCR.). *p<0.05 SHAM vs fa/+ and #p<0.05 RYGB vs.

19   SHAM.

20   **Supplementary File 1: Bioinformatic and Pathway Enrichment Analyses.**

21   **Supplementary File 2:List of Differentially Expressed Genes (SHAM versus fa/+)**
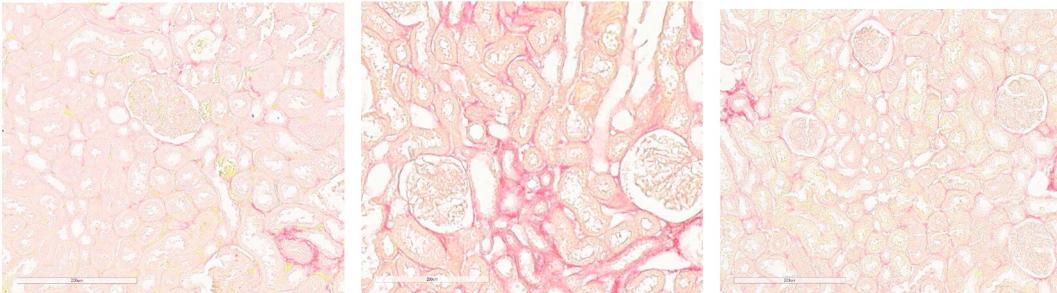
22   **Supplementary File 3:List of Differentially Expressed Genes (RYGB versus SHAM)**

1

2

A)

DESMIN

i   ii   iii

200um   200um   200um
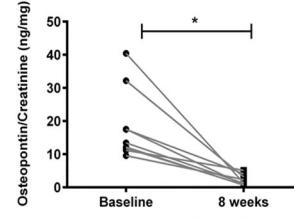
T.E.M.

iv   v   vi

fa/+   SHAM   RYGB

B) Urinary ACR (µg/mg) vs Baseline, Post-op Week 8 (*)

C) Glomerular Volume (μM^3) — fa/+, SHAM (*), RYGB (#)

D) Desmin positive cells/mm2 — fa/+, SHAM (*), RYGB (#)

E) PFPF — fa/+, SHAM (*), RYGB (#)

F) PFPD (µm) — fa/+, SHAM (*), RYGB (#)

G) GBM Thickness (nm) — fa/+, SHAM (*), RYGB

A)

B)

C)

D) SHAM vs fa/+

E) RYGB vs SHAM

F)

G)

H)

A)

B)

C)

D)

E)

F)

A) Upstream Regulators

B) TGF-BETA SIGNALING PATHWAY

C)