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Hepatocyte-derived DPP4 regulates portal GLP-1 bioactivity, glucose production and its absence alters liver disease progression

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Abstract
Elevated circulating dipeptidyl-peptidase 4 (DPP4) is a biomarker for liver disease, but its involvement in gluconeogenesis and metabolic associated fatty liver disease (MAFLD) progression remains unclear. Here we identified that DPP4 in hepatocytes but not Tie2+ endothelial cells regulates the local bioactivity of incretin hormones and gluconeogenesis. However, the complete absence of DPP4 (Dpp4−/−) in aged mice with metabolic syndrome accelerates liver fibrosis without altering dyslipidemia and steatosis. Analysis of transcripts from the livers of Dpp4−/− mice displayed enrichment for inflammasome, p53, and senescence programs compared to littermate controls. High-fat high-cholesterol (HFHC)-feeding decreased Dpp4 expression in F4/80+ cells, with only minor changes in immune signaling. Moreover, in a lean mouse model of severe non-alcoholic fatty liver disease (NAFLD), phosphatidylethanolamine N-methyltransferase (Pemt−/−) mice, we observed a 4-fold increase in circulating DPP4, disassociating its release from obesity. Lastly, we evaluated DPP4 levels in patients with hepatitis C infection with dysglycemia (HOMA-IR >2) who underwent direct antiviral treatment (±ribavirin). DPP4 protein levels decreased with viral clearance, and DPP4 activity levels were reduced at longer-term follow-up in ribavirin-treated patients, although metabolic factors did not improve. These data suggest elevations in DPP4 during HCV infection are not primarily regulated by metabolic disturbances.
Introduction

Type 2 diabetes (T2D) is a metabolic disease characterized by the development of hyperglycemia. Dysregulated islet hormone secretion and an inability to overcome peripheral insulin resistance are central components (1, 2). Given the increased appreciation of the reciprocal nature of dyslipidemia, obesity, dysglycemia and liver disease, including non-alcoholic fatty liver disease (NAFLD) and traditional risk factors, a new classification of metabolic (dysfunction) associated fatty liver disease (MAFLD) has emerged (3).

The secretion of incretin hormones from the gut, including glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), potentiates post-prandial insulin secretion, a phenomenon known as the incretin effect. GLP1- and GIP are central in coordinating nutrient intake, nutrient disposal and satiety (4-6). In patients with T2D, there is a defect in the incretin-mediated potentiation of insulin secretion (7). Additionally, individuals with NAFLD exhibit an impaired incretin effect independent of diabetes, displaying fasting hyperglucagonaemia (8) and increased hepatic gluconeogenesis (9).

The bioactivity and action of endogenous incretin hormones are limited through proteolytic cleavage and inactivation by the serine protease dipeptidyl peptidase-4 (DPP4) and renal elimination (10, 11). Enzymatically active DPP4 is present in both membrane-bound and circulating forms (12). Plasma DPP4 levels are elevated in several settings associated with metabolic dysfunction, including obesity (13, 14), chronic liver disease, including NAFLD and hepatitis C infection (HCV) (12, 15, 16) as well as Type 1 Diabetes (17) and T2D (18, 19). Increased DPP4 expression in the liver positively correlates with the degree of steatosis and NAFLD (20, 21). Studies in mice using several tissue-specific targeting strategies have confirmed that the elevation of circulating DPP4 in obesity is liver-derived (22-24), suggesting that DPP4 produced in the liver may primarily contribute to the progression of NAFLD (25).
Systemic inhibition of DPP4 decreases blood glucose by reducing hepatic glucose production (HGP) in patients with T2D (26). Given the success of incretin-based drugs in treating both diabetes and obesity, and their potential for treating NAFLD, further dissection of the regulation of hepatic metabolic pathways by DPP4 is warranted (27-33). Here, we evaluated the role of hepatic DPP4 on incretin bioactivity within the portal vein (PV), hepatic glucose metabolism, and chronic liver disease progression in mice. We additionally examined circulating DPP4 levels and the mRNA abundances of sheddases in a mouse model of severe metabolic liver disease without obesity, the phosphatidylethanolamine N-methyltransferase (Pemt−/−) mice. We also evaluated circulating DPP4 levels and substrates in a patient population undergoing treatment for hepatitis C infection.

Results

Reduced hepatic glucose production in HFHC-fed Dpp4−/− mice is due to loss of hepatocyte-derived DPP4

In patients with T2D, DPP4 inhibitors contribute to glucose homeostasis by decreasing hepatic gluconeogenesis (26, 34). To confirm this effect in mice, we performed hyperinsulinemic-euglycemic clamps in Dpp4+/− (wild-type, WT) and Dpp4−/− (global Dpp4 deletion) mice fed a high-fat high-cholesterol (HFHC) diet for 12 weeks. Glucose infusion rates (GIR, Figure 1A) and glucose disposal rates (GDR, Figure 1B) were indistinguishable between Dpp4−/− mice and their WT littermate controls. While basal levels of hepatic glucose production (HGP) were unchanged, insulin-stimulated HGP was significantly lower in HFHC-fed Dpp4−/− (Figure 1C) resulting in a significantly greater suppression of HGP by insulin in Dpp4−/− mice (Figure 1D). Body weight and fasting glucose levels were unchanged (Supplemental Figure 1A, B). While the mRNA abundance of hepatic glucokinase (Gck), the enzyme that phosphorylates glucose to produce glucose-6 phosphate, was significantly up-regulated in
Deletion of Dpp4 in hepatocytes lowers portal DPP4 activity and increases portal concentrations of bioactive GLP-1 and GIP

We evaluated glucose excursion after intraperitoneal injection of pyruvate to further evaluate the cell-specific roles of DPP4 in regulating hepatic glucose production. Consistent with our previous work (22), Dpp4hep−/− mice have significantly reduced Dpp4 mRNA expression in whole liver extracts (Supplemental Figure 2A), fasted DPP4 activity in plasma was decreased 50% in twenty-week-old Dpp4GFP and Dpp4hep−/− mice fed a HFHC diet for four weeks (Supplemental Figure 2B), but DPP4 plasma concentration was unchanged (Supplemental...
Accordant with the clamp data, Dpp4<sup>hep-/-</sup> mice had significantly reduced glucose excursion AUC post-injection of pyruvate compared with littermate Dpp4<sup>GFP</sup> controls (Figure 2A). Next, we examined whether reduced hepatic glucose production in Dpp4<sup>hep-/-</sup> mice is mediated by DPP4’s action on GLP-1 receptor (GLP-1R) signaling. The decrease in plasma glucose following pyruvate injection was abrogated with the administration of exendin-9-39, a compound which blocks signalling through the GLP-1R (35), 15 minutes prior to injection of pyruvate (Figure 2B). Dpp4<sup>hep-/-</sup> mice showed no change compared to Dpp4<sup>GFP</sup> in an arginine tolerance test, indicating that islet responsivity to acute depolarization was normal (Supplemental Figure 2D). In addition, in response to arginine, mice lacking hepatocyte Dpp4 did not demonstrate any differences in blood glucose, active GLP-1, insulin, or glucagon (Supplemental Figures 2E-G). GLP-1 has been reported to circulate through the portal circulation and the lymphatics to enter systemic circulation at the thoracic duct (36, 37). To evaluate how DPP4 in hepatocytes may influence the bioactivity of incretin hormones at each of these sites (i.e., portal or systemic concentration), we administered oral glucose to Dpp4<sup>hep-/-</sup> and control mice and sampled portal vein blood via cannulation followed by systemic blood 15 minutes later by cardiac puncture. DPP4 activity but not protein levels within the portal vein (PV) was significantly reduced in the Dpp4<sup>hep-/-</sup> mice compared with Dpp4<sup>GFP</sup> mice (Figure 2C, D). In addition, active GLP-1 and active GIP levels were elevated ~4-fold in the local portal circulation in Dpp4<sup>hep-/-</sup> mice compared with Dpp4<sup>GFP</sup> mice (Figure 2E, F). On the other hand, no significant differences in active GLP-1 and GIP were noted in plasma isolated immediately in the same mice by cardiac puncture (CP) (Figure 2E, F). Despite the increase in circulating incretin, plasma insulin and glucagon levels were unchanged in local hepatic and systemic circulations (Figure 2G-H).

To investigate the role of DPP4 in hepatocytes vs. other sites which contribute to circulating DPP4, including Tie2+ endothelial cells and immune cells, we used Dpp4<sup>EC-/-</sup> mice. Previously, we have reported that deletion of Dpp4 from the Tie2+ endothelial cells (Dpp4<sup>EC-/-</sup>) of
high-fat-fed mice resulted in increased systemic concentrations of GLP-1 and improved glucose excursion, but did not affect HGP (38). As expected, in contrast to Dpp4^hep^-/^-, Dpp4^EC-/^- had no decrease in hepatic Dpp4 mRNA expression (Supplemental Figure 2H) and, consistent with previous reports (38), systemic fasting plasma DPP4 activity and DPP4 protein levels (Supplemental Figure 2I, J) were significantly decreased in Dpp4^EC-/^- mice compared to controls. Furthermore, a pyruvate tolerance test showed no change in HGP in HFHC-fed Dpp4^EC+/^+ and Dpp4^EC-/^- mice previously administered saline or exendin 9-39 (Figure 2I, J), consistent with our previous hyperglycemic-euglycemic clamp analyses (38).

To directly determine how DPP4 in these different tissue settings governs incretin bioactivity and HGP, we measured the portal concentrations of DPP4 and incretins, and also examined pyruvate tolerance in HFHC-fed Dpp4^EC+/^+ and Dpp4^EC-/^- mice. While portal DPP4 activity was unchanged (Figure 2K), DPP4 protein levels in the PV were significantly decreased in Dpp4^EC-/^- mice (Figure 2L). Furthermore, levels of active GLP-1 and active GIP were unchanged in the portal circulation in Dpp4^EC-/^- mice, but systemic concentrations of incretins were increased 2.5-fold (GLP-1) or trended 2-fold higher (GIP) in samples isolated by cardiac puncture (Figure 2M, N). Levels of insulin and glucagon at either sampling site (Figure 2O, P) were unaltered. Together, these results suggest that the reduction in HGP observed in HFHC-fed Dpp4^-/- mice is driven by the loss of Dpp4 in hepatocytes and not Tie2^+ endothelial cell populations. Furthermore, this improved suppression of HGP is associated with elevated incretin concentrations within the PV, not the systemic circulation.

**Dyslipidemia and liver steatosis are unaffected by the genetic elimination of Dpp4 in aged HFHC-fed mice**

Insulin resistance, de novo lipogenesis and dysregulated blood glucose are key hallmarks in the progression of NAFLD as part of the multiple-hit hypothesis (39). To determine whether the reduction in HGP observed following the deletion of Dpp4 from the whole animal or
liver only can prevent MALFD progression, we fed aged 6-month-old mice either a standard laboratory diet (SLD) or HFHC diet for 24 weeks. In a small subset of aged mice (n=2/group), we performed similar portal vein cannulations and cardiac punctures and observed trends consistent with those shown in Figure 2 (Supplemental Table 1). Analysis of Dpp4 mRNA in whole liver extracts revealed elimination in all expected genotypes (Figure 3A). DPP4 activity in plasma and liver was absent from Dpp4^−/− on SLD and HFHC and reduced by 60% and 75% in Dpp4^hep^−/− mice compared to controls (Figure 3B, C). Agreeing with our previous results (22), deletion of hepatocyte Dpp4 led to sustained reductions in plasma and hepatic DPP4 activities, with little change in circulating DPP4 protein levels (Figure 3D). Deletion of hepatocyte-specific DPP4 in these aged mice did not affect glucose tolerance as only whole-body deletion of DPP4 increased systemic active GLP-1 and led to reduced blood glucose excursion during oGTT (Supplemental Figure 3A,B), consistent with previous work (22, 23). Glycogen concentrations were unchanged in all settings (Supplemental Figure 3C). In both SLD-fed and HFHC-fed Dpp4^−/− mice, lack of Dpp4 did not affect HDL, LDL or total cholesterol levels in plasma (Figure 3E-G). However, fasting plasma triglycerides were significantly reduced in Dpp4^−/− mice (Figure 3H). Biochemical measurement and histochemical analysis with Oil-Red-O staining in livers revealed no differences in neutral lipid concentrations between genotypes (Figure 3I-M). Hepatic gene expression analysis also revealed that sterol regulatory element-binding transcription factor 1 (Srebf1) mRNA expression was significantly up-regulated in HFHC-fed Dpp4^−/− mice but unchanged in SLD-fed mice and HFHC-fed Dpp4^hep^−/− mice, compared to controls (Figure 3N). Microsomal triglyceride transfer protein (Mttp) mRNA expression was significantly decreased in SLD-fed Dpp4^−/− mice compared to controls but unchanged in all genotypes under HFHC-feeding (Figure 3O). In contrast, hepatic forkhead box protein O1 (FoxO1) expression was unchanged between all genotypes under both diets (Figure 3P). Taken together, these data suggest that hepatic lipid accumulation is largely unaffected by whole-body or hepatocyte-specific elimination of Dpp4 gene in aged, SLD- or HFHC-fed mice.
**Systemic, not hepatic, loss of Dpp4 in aged mice increases hepatic fibrosis**

Soluble, circulating DPP4 has been shown to be a marker of liver fibrosis (40). Thus, we examined if systemic and hepatic loss of Dpp4 in mice affected liver fibrosis. Liver damage markers, such as alanine aminotransferase (ALT), aspartate transaminase (AST) and alkaline phosphatase, were not significantly different between all groups and their respective controls (Figure 4A-C). Liver size normalized to tibia length was also unchanged (data not shown). To our surprise, mRNA levels of fibrosis markers, including Col1a1, Col3a1, Mmp2, Mmp11, Des, and Ddr2 were elevated in HFHC-fed Dpp4−/− mouse livers (Figure 4D-I), suggesting worsening fibrosis in the Dpp4−/− mice. However, this elevated gene expression was not observed Dpp4hep−/− mouse livers. No changes of expression were noted in fibrosis and hepatic stellate cell activation factors, Mmp9, Gfap and Vim, between any of the genotypes (Supplementary Figure 4A-C) while both Lrat and Pcdh7 were significantly reduced in SLD-fed Dpp4−/− mice compared with controls (Supplementary Figure 4D, E). Consistent with gene expression, visualization of collagen with picrosirius red staining revealed that HFHC-fed Dpp4−/− mice had elevated fibrotic area in the liver, whereas it was relatively unchanged in Dpp4GFP and Dpp4hep−/− mice (Figure 4J-M). Supporting increased fibrosis, blinded meta-analysis of histological data in viral hepatitis (METAVIR) histopathological scoring of liver samples revealed a shift of 33% in Dpp4−/− mice to level 4 relative to littermate controls, while no Dpp4hep−/− mice were scored in this range (Figure 4N). Overall, these data suggest that systemic, not hepatic, loss of Dpp4 increases liver fibrosis.

**Global, but not hepatic, loss of DPP4 increases expression of genes associated with adaptive immunity, inflammasome and senescence-associated genes and pathways**

To gain molecular insights into the inflammatory responses in the liver mediated by loss of Dpp4, we conducted NanoString mRNA analysis on liver tissue using an immunology panel of
over 500 immune-related genes. All Dpp4<sup>hep</sup>−/− mice were confirmed by qRT-PCR with primers specific for the recombined Dpp4 flox sites (Figure 3A), given the location of the flox deletion site towards the C-terminal end of the Dpp4 transcript and modest reduction in gene expression detected by NanoString probes (Supplemental Figure 5A). Supervised hierarchical clustering analysis of differentially expressed genes in Dpp4<sup>−/−</sup> mice revealed a distinct cluster of genes that were up-regulated in SLD- and HFHC diet-fed Dpp4<sup>−/−</sup> mice compared to Dpp4<sup>+/+</sup> and Dpp4<sup>GFP</sup> vs. Dpp4<sup>hep</sup>−/− (Supplemental Figure 5B, C). We identified differentially expressed genes that were distinct and overlapping among SLD-fed Dpp4<sup>−/−</sup>, HFHC-fed Dpp4<sup>−/−</sup> and HFHC-fed Dpp4<sup>hep</sup>−/− livers (Supplemental Figure 5D). Pathway analysis in each comparison was performed, showing that only cytokine signaling in SLD-fed Dpp4<sup>−/−</sup> mice was significantly up-regulated compared to Dpp4<sup>+/+</sup> mice (Figure 5A). Notably, 18 pathways, including adaptive and innate immune pathways, inflammasome, toll-like receptor signaling, oxidative stress and TGFβ signaling, were all significantly up-regulated in HFHC-fed Dpp4<sup>−/−</sup> compared to Dpp4<sup>+/+</sup> mice (Figure 5B). Consistent with no difference in liver fibrosis (Figure 4H), all pathways were indistinguishable between HFHC-fed Dpp4<sup>GFP</sup> and Dpp4<sup>hep</sup>−/− mouse livers (Figure 5C), suggesting that hepatocyte DPP4 was not influencing the immunological response. We validated these results by immunostaining for the top differentially expressed transcript, Marco, in mice fed the HFHC diet. Consistent with the NanoString analysis, Marco staining was significantly reduced in the HFHC diet fed Dpp4<sup>−/−</sup> mouse livers compared to controls (Supplementary Figure 6A-G). Additionally, Marco expression in Dpp4<sup>GFP</sup> mice exhibited a spread of high and low expressing livers which was recapitulated with immunostaining (Supplementary Figure 6C, D, F, G). When we probed gene expression within the inflammasome pathway, whose activation is a contributing factor in the initial progression of NAFLD (41), we found that all genes (App, Bcl2, Nfkb1, Nfkb2 and Rela) were significantly upregulated in HFHC-fed Dpp4<sup>−/−</sup> mice (Figure 5D). In contrast, only App and Rela were significantly upregulated in SLD-fed Dpp4<sup>−/−</sup> mice compared to controls, and Rela was
significantly downregulated in \textit{Dpp4}^{hep/-} mice (\textbf{Figure 5D}). Similarly, 19 of 28 NF-kB signaling pathway genes were significantly upregulated in HFHC-fed \textit{Dpp4}^{/-} mice, whereas many genes were unchanged, or downregulated, in SLD-fed \textit{Dpp4}^{/-} mice and HFHC-fed \textit{Dpp4}^{hep/-} mice, compared to respective controls (\textbf{Figure 5E}). To further complement this analysis, we analyzed mRNA expression of known chemokine substrates of DPP4 in whole liver extracts. Consistent with its role in NAFLD progression (42), \textit{Ip-10} (\textit{Cxcl10}) gene expression was significantly upregulated in HFHC-fed \textit{Dpp4}^{/-} mice compared to controls, but unchanged in SLD-fed mice and HFHC-fed \textit{Dpp4}^{hep/-} mice compared to controls (\textbf{Figure 5F}). Expression of the gene, regulated on activation, normal T cell expressed and secreted (\textit{RANTES}; \textit{Ccl5}), which is associated with severe liver fibrosis (43), was also significantly up-regulated in HFHC-fed \textit{Dpp4}^{/-} mice (\textbf{Figure 5G}), whereas no changes in \textit{Mcp-1} (\textit{Ccl2}) or \textit{Eotaxin} (\textit{Ccl11}) gene expression were noted (\textbf{Figure 5H, I}). In contrast, gene expression of \textit{Ip-10}, \textit{RANTES}, \textit{Mcp-1} and \textit{Eotaxin} were unchanged between HFHC-fed \textit{Dpp4}^{GFP} and \textit{Dpp4}^{hep/-} mice (\textbf{Figure 5F-I}).

Cellular senescence has been identified to be involved in the transition from liver steatosis to a more severe phenotype involving hepatocyte ballooning and elevated fibrosis (44). DPP4 has been identified on the surface of senescent cells, preferentially sensitizing them to cytotoxicity by natural killer cells (45). Therefore, we were prompted to evaluate gene expression of senescence-associated secretory phenotype (SASP) factors (46) in our models. Our liver NanoString analysis identified increased expression of \textit{Trp53} in both SLD-fed and HFHC-fed aged \textit{Dpp4}^{/-} compared to their respective controls (\textbf{Figure 5J, K}). We additionally measured genes associated with p53 signaling (47, 48). The mRNA level of \textit{Ankrd1} was increased in SLD- and HFHC-fed \textit{Dpp4}^{/-}, but unchanged in \textit{Dpp4}^{hep/-} compared to controls (\textbf{Figure 5M}). \textit{Cdkn1a} expression was unchanged across both diets, and all genotypes (\textbf{Figure 5N}), while \textit{Cdkn2a} was significantly decreased in HFHC-fed \textit{Dpp4}^{/-} mice only (\textbf{Figure 5O}). When we analyzed protein levels of chemokine and cytokine SASPs, 8-weeks after starting the diet, CXCL1 levels were significantly increased in SLD-fed \textit{Dpp4}^{/-} mice (\textbf{Supplemental Figure})
7A), while IL6 was significantly increased in HFHC-fed Dpp4^+/− mice (Supplemental Figure 7B).

Other plasma cytokines including Il1β, IFNγ, IL10, and IL2 were unchanged (Supplemental Figure 7C-H). However, in HFHC-fed, IL4 was significantly decreased in Dpp4^+/− mice compared to controls (Supplemental Figure 7I), while it was increased in Dpp4^hep−/− as was IL5 at both 8 weeks and endpoint (Supplemental Figure 7I, G). Few other significant changes were noted in plasma or within liver tissue at endpoint (Supplemental Figure 7J-Y).

Immune-related genes are up-regulated in F4/80+ cells of SLD-fed Dpp4^+/− mice but HFHC feeding reduces DPP4 expression in F4/80+ cells

Roles of both liver-resident macrophages and recruited monocyte-derived macrophages in NAFLD progression (49) and liver fibrosis (50) have been established. Additionally, DPP4 is known to be up-regulated when macrophages are polarized with pro-inflammatory stimuli while implicated in macrophage polarization and activation to mediate inflammation (51). We therefore probed if liver-resident macrophages were causal in driving the increased inflammation in the livers of mice with global Dpp4 deletion. We isolated F4/80+ cells from the liver and conducted NanoString mRNA analysis using the same immunology panel described above. Surprisingly, a large cluster of immune-related genes were up-regulated in F4/80+ cells of SLD-fed Dpp4^+/− mice compared to controls. However, these same genes were not differentially expressed in HFHC-fed Dpp4^+/− mice (Supplemental Figure 8A, C). While in HFHC-fed Dpp4^hep−/− mice, two distinct clusters were revealed to be significantly altered compared to controls (Supplemental Figure 8B, C). Pathway analysis revealed significant increases in NF-κB signaling, adaptive and innate immune system and cytokine signaling (Figure 6A) in SLD-fed Dpp4^+/− mice compared to controls. However, no pathways were significantly altered in HFHC-fed Dpp4^−/− (Figure 6B) and Dpp4^hep−/− mice (Figure 6C). In the isolated F4/80+ cells, unexpectedly, Dpp4 was downregulated in HFHC-fed Dpp4^+/+ mice compared to SLD-fed Dpp4^+/+ mice. Its expression was unchanged between HFHC-fed Dpp4^GFP and Dpp4^hep−/− mice, confirming
deletion was restricted to hepatocytes (Figure 6D). To further understand potential differences in F4/80+ cells’ composition within the liver, we assessed differences in the abundance of transcripts associated with characterized populations. We found Adgre1 was up-regulated in HFHC-fed Dpp4−/− mice (Figure 6E), and Ccr2 trended to increase in HFHC-fed Dpp4−/− mice compared with controls. These markers remained unchanged in SLD-fed Dpp4−/− and Dpp4hep−/− mice versus their respective controls (Figure 6F). However, no differences in F4/80 immunostaining were noted (Supplementary Figure 9A-E) between the HFHC-fed groups. Additionally, no changes were observed in macrophage polarization and population markers, Itgax (Figure 6G), Mrc1 (Figure 6H), Cd163 (Figure 6I), Arg1 (Figure 6J) and Clec4f (CLC4F) (Figure 6K) between genotypes and their respective controls. Consistent with these results, CLEC4F immunostaining revealed no differences between HFHC-fed groups (Supplementary Figure 9 A-D,F). Trp53 was up-regulated in SLD-fed Dpp4−/− and downregulated in HFHC-fed Dpp4hep−/−, while Cxcr4 was up-regulated in both (Figure 6L). SLD-fed Dpp4−/− mice had significant changes in many components of the NF-κB signaling pathway but few of these patterns were observed in HFHC-fed Dpp4−/− or Dpp4hep−/− mice (Figure 6M). These data reveal an unexpected complex relationship between DPP4 and liver resident F4/80+ cells immunological profiles associated with diet composition.

Liver-specific insults affect circulating DPP4 protein concentrations

Given its strong correlation with adipose tissue accumulation and obesity in both humans and mice, soluble, circulating DPP4 was initially characterized as an adipokine (13, 22). Recent studies with adipocyte-specific targeting of DPP4 have determined that while adipocytes shed a small amount of DPP4 (22, 52), hepatocytes account for the significant elevation in DPP4 observed in high-fat diet feeding and metabolic dysregulation (22, 24). Further, liver DPP4 expression is elevated in NAFLD (20, 21). Comprehensive studies in cultured hepatocytes have determined that a combination of leptin and palmitic acid stimulates a 6-fold
increase in Dpp4 mRNA expression (53). To test the necessity for adiposity and peripheral insulin resistance in vivo for elevated enzymatically active circulating DPP4, we assessed plasma DPP4 activity in HFHC-fed phosphatidylethanolamine N-methyltransferase (Pemt−/−) mice, which are a lean model of hepatomegaly and hepatic steatosis due to disruption in de novo synthesis of choline (54) (Supplemental Figure 10A). Pemt−/− mice do not develop obesity with high-fat feeding, retain insulin sensitivity and have lower leptin concentrations compared with littermate controls (54, 55). Notably, systemic DPP4 activity was increased 4-fold relative to controls (Supplemental Figure 10B), suggesting dysregulation of hepatic lipid pathways is related to increased DPP4 activity, independent of the development of obesity. Unexpectedly, Dpp4 mRNA level in the liver was unchanged (Supplemental Figure 10C). However, mRNA expression of candidate sheddases was significantly increased, including Mmp9, Mmp2, Adamst but not Adam17 (Supplemental Figure 10D-G).

In addition to steatosis, other liver-specific insults associated with elevated circulating DPP4 include infection with HCV (56). It has been well-established that chronic HCV infection is associated with metabolic disease (57). We have recently shown that in HCV patients treated with ombitasvir-a paritaprevir (with or without ribavirin) plus dasabuvir (PrOD), fasting glucose, insulin, and HOMA-IR are unchanged during treatment and follow-up post-treatment (58). We now sought to investigate whether elevated DPP4 levels are reversed following successful therapy for viral clearance in patients with metabolic dysfunction (HOMA-IR >2) that persists through the treatment and follow-up period. Plasma AST (Figure 7A) and ALT (Figure 7B) significantly decreased in all treatment groups and were maintained during follow-up. Not surprisingly, given the lack of change in metabolic disease parameters, high variability and no significant changes were observed in C-reactive protein concentrations (Figure 7C). Consistent with other studies utilizing interferon-alpha treatment (59, 60), DPP4 concentration in plasma significantly decreased within all PrOD regimens from baseline to 12 weeks post-treatment (Figure 7D). In comparison, DPP4 activity significantly decreased with ribavirin treatment from
baseline to follow-up in ribavirin-treated patients (Figure 7E). A similar trend to DPP4 protein was observed with known DPP4 substrates, IP-10 (Figure 7F), and MIP1α (Figure 7G). However, Eotaxin, another substrate, was unaffected (Figure 7H). Soluble intracellular adhesion molecule-1 was significantly decreased with treatment (Figure 7I). Taken together, DPP4 concentration and activity decrease with HCV treatment and viral clearance. However, this occurs independently of changes in metabolic parameters.

Discussion

Potentiation of GLP-1 action through receptor agonists has demonstrated efficacy in treating metabolic disease (61, 62); however, our knowledge of the effects of DPP4 elimination to potentiate endogenous GLP-1 action within the context of chronic liver disease progression is limited. The present data demonstrate that eliminating hepatocyte DPP4 in HFHC-fed mice decreases DPP4 activity and increases intact incretins in the portal circulation and reduces hepatic glucose production. These data are also consistent with patients in which DPP4i’s decrease HGP and are associated with reductions in glucagon (26, 34). In contrast, in HFHC-fed Dpp4EC-/-, we report increased levels of active GLP-1 in the systemic circulation and no effect on HGP as assessed by hyperinsulinemic- euglycemic clamp (38). Studies in mice have also demonstrated that intact GLP-1R signaling within the portal circulation is integral to glucose sensing (63). This is interesting given that post-prandial GLP-1 levels measured in the lymph are 5-6 times higher relative to sampling performed in portal plasma (36). Consistent with our data identifying different regulation of incretin bioactivity and modulation of glucose metabolism with DPP4 in hepatocyte vs. Tie2⁺ cells, recent reports have determined that elevated levels of active portal GLP-1 are disconnected from the classic definition of the incretin effect as increased portal circulation of GLP-1 does not potentiate nutrient-stimulated insulin secretion (64, 65). In studies performed in rats, samples taken 20 minutes following a high-fat diet meal detected elevated GLP-1 in the lymph collected from the mesenteric lymph duct rather than the
PV, while no difference was observed after a low-fat meal (66). Circulating DPP4 activity is lower in lymph than in plasma (36, 66). However, lymphatic endothelial cells have been reported to express DPP4, and modulation of levels with siRNA affects migration and function (67). Therefore, our current study is consistent with a model where, during HFHC diet-induced metabolic dysregulation, the deletion of Dpp4 within Tie2Cre+ cells increases the abundance of GLP-1 delivered to the systemic circulation, enabling the incretin effect and improves oral glucose tolerance but does not impact HGP. This is in contrast to Dpp4 in hepatocytes which when deleted in HFHC-fed mice increases GLP-1 bioactivity within the portal circulation and improves insulin-mediated suppression of hepatic glucose production. Our data align with results from hypoxia-inducible factor 1-alpha (Hif1α)hep-/- mice, demonstrating that elevation in DPP4 through activation of hepatocyte HIF1α reduces active activation GLP-1 in the portal circulation (53). Additionally, Baumeier et al. (25) demonstrated that hepatic Dpp4 overexpression resulted in decreased active glucose-stimulated GLP-1 in the vena cava after liver passage. Recent studies have documented that GLP-1R engagement in the portal circulation is reduced under high-fat diet feeding conditions (68), suggesting together with our data that multiple mechanisms converge to control GLP-1 action in the hepatic portal circulation, which may contribute to glucose dysregulation in mice upon high fat-feeding.

Elevated concentrations of plasma DPP4 are associated with liver disease severity and fibrosis (69). Consistent with this, hepatocyte-specific overexpression of DPP4 in mice results in increased hepatic steatosis, elevated liver enzymes and markers of inflammation (25). In the current study, we report DPP4 is elevated 4 fold in Pemt-/- mice which have both hepatomegaly and NASH (54), demonstrating that in addition to obesity, liver-specific insults can induce the release of DPP4. Surprisingly however, complete genetic elimination of Dpp4 or hepatocyte specific elimination in aged mice resulted in no changes in liver enzymes and the degree of steatosis. Consistent with our results, using liver-specific knockdown of DPP4 via therapeutic siRNA, in obese and diabetic db/db mice, no effect on liver enzymes or glucose tolerance was
observed (23). Both Ghorpade et al. and Varin et al. reported modest impact on the liver with longer-term targeting strategies (22, 24), suggesting that acute treatments targeting DPP4 more readily impact mouse lipid metabolism than long-term deletion of DPP4 which may be prone to metabolic adaptation.

In mice, DPP4i’s decrease liver fibrosis (70, 71); however, this was not recapitulated by genetically eliminating Dpp4 as PSR staining revealed a trend toward increased fibrosis, and expression of Col1a1 and Col3a1 were increased in Dpp4<sup>−/−</sup> mice. Chronic liver inflammation and immune reactions often precede fibrosis (72), therefore, we assessed mRNA expression of immune-related genes. Under HFHC-fed conditions, livers of Dpp4<sup>−/−</sup> mice but not Dpp4<sup><sup>hep</sup>−/−</sup> mice exhibited upregulation of transcripts associated with the inflammasome and markers of NF-kB signaling; pathways characterized to be activated in NAFLD (73, 74). This was surprising given that DPP4i’s have been reported to suppress NF-kB activation (75, 76). These results support important differences obtained by enzymatic inhibition vs. complete deletion of the DPP4 protein.

Recently, cellular senescence has been proposed as a key factor in NAFLD progression (77). Further, DPP4 has been identified as a surface protein that is enriched in senescent cells (45) and modulation of DPP4 in pre-senescent WI-38 cells (45) or in vascular endothelial cells (78) reduces markers of senescence. Additionally, senescence induced by glucocorticoid treatment, known to increase DPP4 transcriptionally (79) can be modulated by inhibition of DPP4 activity (80). In our study, life-long deletion of DPP4 in aged mice led to upregulation of Trp53, a component of the senescent machinery (81). Additionally, the expression of Ankrd1, Il1a, Ccl2 and Ccl3 were increased in HFHC-fed Dpp4<sup>−/−</sup> mice. A molecular link between p53 and DPP4 has been established in which p53 antagonizes ferroptosis by blocking DPP4 activity (82). However, the mechanistic link between DPP4, p53, cellular senescence and liver fibrosis could not be deduced from this study and warrants further investigation.
In addition to NAFLD, elevated DPP4 is also observed in other chronic liver diseases, such as HCV infection (15). Consistent with data from patients who received interferon treatment (56), plasma DPP4 concentrations, liver enzymes and cytokine levels were reduced after resolution of infection with Direct-Acting Antiviral therapy with or without ribavirin. DPP4 activity varied between treatment groups at baseline, but overall remained decreased from baseline at follow-up in all. Additionally, plasma AST, AST, IP-10, MIP1-a and sICAM all decreased with treatment. However, these changes were disassociated from changes in glucose regulation (58).

We acknowledge several shortcomings to our study including that the effects of exendin 9-39 cannot exclude the potential for signaling of glucagon through the GLP-1R (83-86), in addition to active GLP-1 (87). Additionally, while mice remained unrestrained during the hyperinsulinemic-euglycemic clamps, they were sampled by the tail vein which may contribute differential physiological responses to stress across genotypes. In addition, many of our inferences are dependent on transcript levels rather than direct protein quantitation due to the availability and reliability of relevant antibodies.

In summary, we have identified hepatocyte-derived Dpp4 as a key factor in regulating the bioactivity of GLP-1 in the portal vein and extend these findings to demonstrate that its elimination results in a reduction in hepatic glucose production. However, despite the elevation in GLP-1 and improvements in hepatic insulin sensitivity, we demonstrate a disconnect as markers of lipid metabolism, fibrosis and inflammation were unchanged or worsened in mice.

Methods

Animals: All studies were performed according to protocols approved by the University of Ottawa Animal Care Committee and in accordance with guidelines of the Canadian Council on Animal Care. Male mice were housed under a 12-h light/dark cycle and maintained on standard...
laboratory diet (SLD; Harlan Teklad, Mississauga, ON) or high fat, high cholesterol (HFHC) diet (TD.88137, Envigo-Teklad Custom Diets). Whole body $Dpp4^{-/-}$ mice, on a C57BL6 background, have been described (38, 88). To generate $Dpp4^{hep/-}$ mice, $Dpp4^{floxflo}$ adult mice were i.v. injected with $1.5 \times 10^{11}$ GC per mouse of AAV8.TBG.pi.egfp.wpre.bgh (AAV-GFP; control virus, #105535-AAV8) or AAV8.TBG.PI.CRE.rBG (AAV-Cre; #107787-AAV8) prior to the onset of HFHC diet feeding. Both AAV constructs were obtained from the University of Pennsylvania Vector Core Lab as a generous gift from James M. Wilson (Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA). B6.Cg-Tg(Tek-cre) 1Ywa/J mice were obtained from The Jackson Laboratory (Strain #: 008863, RRID: IMSR_JAX:008863) and bred with $Dpp4^{floxflo}$ to generate $Dpp4^{EC/-}$ mice. Experiments in $Pemt^{+/+}$ and $Pemt^{-/-}$ were approved by the University of Alberta’s Institutional Animal Care Committee. Mice were fed a high fat, high carbohydrate diet (F3282; Bio-Serv, Flemington, NJ) for 3 weeks and fasted for 12 hrs prior to sacrifice and tissue collection. Hyperinsulinemic-euglycemic clamps were performed in 12 week old $Dpp4^{+/+}$, $Dpp4^{-/-}$, $Dpp4^{floxflo}$, and $Dpp4^{hep/-}$ mice subjected to 12-16 weeks of HFHC diet. Experiments were performed in 18–20-week-old mice after 5 weeks of HFHC diet to validate our hepatic glucose production results. Lastly, to assess NAFLD disease progression, experiments were performed in 16–28-week-old mice fed a HFHC diet for 24 weeks sacrifice and tissue collection at 40-52 weeks. Eight mice died prematurely (1 $Dpp4^{hep/-}$, 2 $Dpp4^{GFP}$, 1 $Dpp4^{-/-}$ and 4 WT mice), and one $Dpp4^{GFP}$ mouse was omitted from analysis due to the presence of a very enlarged spleen. Aged, HFHC-fed $Dpp4^{hep/-}$ and $Dpp4^{GFP}$ mice were assessed for portal hormone concentrations (n=2), averages presented in Supplemental table 1. Mice were genotyped from genomic DNA (gDNA) isolated from tail samples and DNA recombination was confirmed in gDNA isolated from the liver. After DNA extraction and amplification, the PCR product was loaded on 1% agarose gels, separated for 30 min at 150V and the bands visualized under a blue light transilluminator.
Hyperinsulinemic-euglycemic clamp: Hyperinsulinemic-euglycemic clamps were performed as previously described (89). Briefly, a catheter was surgically placed into the right jugular vein, and mice were allowed to recover for 5 days. All mice regained their presurgical weights following surgery. The catheter was made accessible through an adaptor port implanted in the dorsal subscapular region. On the day of the clamp, mice were fasted for 5h and then infused with D-[3-3H]-glucose for 1h to evaluate basal glucose disposal. Human insulin (10 mU/kg/minute) infusate containing D-[3-3H]-glucose was then administered, and blood glucose levels were titrated with 50% dextrose to achieve and maintain euglycemia. Mice were not physically restrained and free to move around their cage, with blood samples taken via the tail vein. Basal and clamped rates of glucose disposal and hepatic glucose production (HGP) were calculated as described (89). All tissues were rapidly dissected, snap-frozen in liquid nitrogen, and stored at −80°C for later analyses.

Portal vein and cardiac puncture cannulation: Mice were given an oral bolus of glucose (2g/kg) and subsequently anesthetized with 4% isoflurane. Once fully unconscious, the abdomen was disinfected, and the mouse was placed on a heated surgical platform. A sagittal incision was made through the skin and fascia of the lower abdomen. A lateral transverse incision was made through the muscular layer to expose the abdominal contents. The portal vein was gently exposed by moving the intestines laterally toward the left body wall. Once exposed, the portal vein was cannulated with a 20-gauge butterfly needle 15 minutes after the glucose bolus and the needle was withdrawn to collect blood. Approximately one minute later, the beating heart was exposed by cutting through the diaphragm and thorax. An 18-gauge needle was used to collect blood from the right ventricle. Blood was aliquoted into two EDTA-coated capillary microvette tubes, one with 10% TED (vol/vol) (5000 KIU/ml Trasylol, 1.2 mg/ml EDTA and 0.1 nmol/L Diprotin A) and without, plasma was isolated after centrifugation (12,000rpm, 10min, 4°C) and stored at −80°C for later analyses.
**Pyruvate, arginine and glucose tolerance tests:** After a 16-hour fast, mice were intraperitoneally injected with either saline or exendin 9-39 (24nmol/kg body weight (90), Bachem), 15 minutes prior to injection with 2g/kg body weight pyruvate in sterile 0.9% saline. After a 4-hour fast, mice were intraperitoneally injected with 2g/kg body weight arginine in sterile 0.9% saline. For glucose tolerance tests, mice were fasted for 5h and given glucose in PBS (2g/kg body weight) in sterile 0.9% saline. Blood for glucose measurements (Glucometer, MediCure Canada, Ajax, Canada) was obtained from the tail vein before pyruvate injection and at 15, 30, 45, 60 and 90 minutes post-pyruvate injection, or before arginine injection and at 15 and 30 minutes post-arginine injection.

**Blood and tissue collection:** All blood samples were collected in EDTA-coated capillary microvette tubes and plasma was isolated after centrifugation (12,000rpm, 10min, 4°C). During metabolic tolerance tests, blood was taken via tail vein. For terminal studies, mice were sacrificed by CO₂ inhalation and blood was obtained by cardiac puncture. For measurement of plasma active GLP-1 (Mesoscale) and active GIP (Crystal Chem), blood was mixed with 10% TED (vol/vol) and plasma stored at -80°C until further analysis. Plasma insulin (Alpco Diagnostics) and glucagon (Crystal Chem) levels were determined as per manufacturer's instructions. Analysis for plasma ALT, AST, ALP, TG, cholesterol, LDL and HDL were performed by the Pathology core at Toronto Centre for Phenogenomics. The Beckman Coulter AU480 clinical chemistry analyzer was used in combination with appropriate reagents (ALT, AST, TG, cholesterol, LDL, and HDL), calibrators (Beckman Coulter Lyophilized Chemistry Calibrator Levels 1 and 2), and quality control materials (Bio-Rad Liquid Assayed Multiqual 1 and 3). DPP4 activity was assessed using fluorometric assay (substrate: 10mM H-Gly-Pro-AMC HBr, Bachem cat. #I-1225, standard: AMC, Bachem cat. #Q-1025). DPP4 protein level was
measured using DPPIV/CD26 DuoSet ELISA kit (DY954; R&D System) following the
manufacturer's instructions.

**Picrosirius red and oil red O staining**: Liver tissue was fixed in 4% PFA and routinely
processed for paraffin embedding and cross-sectioned to obtain five μm-thick sections. The
slides were incubated with a 0.1% picrosirius red (PSR) solution and mounted with DPX
Mounting. Collagen accumulation was determined by the number of red-stained pixels using
ImageJ. Accumulation of fat droplets in the liver was visualized using Lipid (Oil Red O) Staining
Kit as per manufacturer protocol (BioVision, USA). A pathologist blinded to genotype assessed
the picrosirius red sections and provided a METAVIR score.

**Immunofluorescence staining**: Liver tissue was fixed in 4% PFA and routinely processed for
paraffin embedding and cross-sectioned to obtain five μm-thick sections. Sections were
dewaxed with toluene then rehydrated with graded washes of ethanol ending with water.
Antigen retrieval was performed using sodium citrate buffer (0.1M, pH 6, with 0.05% Tween-20)
in a microwave, then washed with DI water and PBS on a shaker. Sections were blocked with
10% donkey serum (D9663) for 30 minutes at room temperature. Antibodies against Marco
(mAB clone: EPR22944-66, cat#: ab259264, 1/200), F4/80 (mAB clone: CI:A3-1, cat#: MA1-
91124 1/250) and Clec4f (AF2784, 1/200) were incubated overnight at 4°C. Slides were washed
with PBS-T, then incubated with secondary antibodies Donkey anti-Rabbit IgG (H+L) Highly
cross-absorbed Alexa Fluor Plus 647 (A32795, 1/500), Donkey anti-Rat IgG (H+L) Highly cross-
absorbed Alexa Fluor Plus 647 (A48272, 1/500) and Donkey anti-Goat IgG (H+L) cross-
absorbed Alexa Fluor 555 (A-21432), 1/500) for 45 minutes at room temperature. Slides were
washed and nuclei were stained using 4’,6-diamidino-2-phenylindole (DAPI; Thermo Scientific
62248, 1/2000) for 5 minutes at room temperature, washed and mounted (Abcam, ab104135).
Images were obtained on a Zeiss AxioImager Z1 epifluorescent microscope with 20X or 63X oil-immersion objective and analyzed using Zeiss ZEN Blue microscopy software.

**Liver triglyceride and cholesterol content:** Total liver lipids were extracted using a modified Folch method (91). For SLD- and HFHC-fed mice, a 100 mg or 50 mg piece of liver tissue was homogenized in 4 ml chloroform/methanol (2:1, v/v) and processed as described previously (92). Lipids were quantified using Infinity Cholesterol or Triglyceride reagent at 540 nm.

**Hepatic F4/80+ cell isolation:** Fresh mouse livers were enzymatically digested using the components of a liver dissociation kit (kit #130-105-807, Miltenyi Biotec, USA) and the gentleMACS™ Dissociators were used for the mechanical dissociation steps as previously described (93). Hepatic F4/80+ cells were isolated from dissociated liver samples with Anti-F4/80 MicroBeads (Anti-F4/80 MicroBeads UltraPure, mouse, Miltenyi Biotec) as per manufacturer’s protocol and flash-frozen in liquid nitrogen before being stored at -80°C for mRNA extraction.

**RNA isolation, cDNA and qPCR:** Frozen liver and isolated hepatic F4/80+ cells were homogenized with TriReagent (Sigma Aldrich, St. Louis, MO) using a TissueLyser II system (Qiagen), and total RNA was extracted using manufacturer’s protocol. Reverse transcription was performed with the Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit. cDNA was subsequently used to assess mRNA expression by real-time quantitative PCR (QuantStudio 5 System, Thermo Fisher Scientific) with TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, 4444557) and TaqMan Gene Expression Assays (Thermo Fisher Scientific). The specific gene expression assays used are listed (Supplemental Table 1). Quantification of transcript levels was performed by the standard curve method, and expression levels for each gene were normalized to Actb (β-actin).

nCounter® Mouse Immunology Panel (catalog# XT-CSO-MIM1-12), were used where 100ng RNA was incubated with reporter and capture probes, consisting of 547 immunology-related mouse genes and 14 internal reference controls, for 16h at 65°C. Following hybridization, unbound probes were removed. According to the manufacturer’s instructions, assays were performed and quantified on the nCounter system, sample preparation station, and digital analyzer (NanoString Technologies).

Raw gene expression data were analyzed using NanoString’s software, nSolver v4.0.70, with the Advanced Analysis Module v2.0.115 with background subtraction. Genes with counts below a threshold of 20 were excluded from subsequent analysis. Data normalization was performed on background-subtracted samples using internal positive controls and selected housekeeping genes. Housekeeping genes were selected based on those that were consistent in all analyses across genotypes and diets: Sdha, Eef1g, Gapdh, Hprt, Polr2a, Rpl19, Oaz1, Tbp and Tubb5 for liver tissue, and Rpl19, Ppia, Oaz1, Eef1g, Sdha, Pol2a, Gusb, Tubb5, Gapdh and Hprt for F4/80+ cells. Differential gene expression analyses were performed using nSolver, which employs several multivariate linear regression models to identify significant genes (mixture negative binomial, simplified negative binomial, or log-linear model). Raw mRNA counts were log2 transformed and significance was determined using a T-test. Statistically significant, differentially expressed genes were identified as those with a p-value <0.05. Ratios of log2 normalized transcript count data were generated for SLD Dpp4^−/− mice vs baseline SLD WT mice, HFHC-fed Dpp4^−/− mice vs baseline HFHC-fed WT mice and HFHC-fed Dpp4^hep/− mice vs baseline HFHC-fed Dpp4^GFP mice. The NanoString data have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE218767.
Pathway scores generated from nSolver Advanced Analysis were standardized by Z-scaling. ClustVis (94) was used to perform supervised hierarchical clustering analysis and principal components analysis of log₂ transformed transcript count data and Z-scaled pathway scores.

**Human studies:** Participants were recruited between July 2015 and April 2016 from The Ottawa Hospital Viral Hepatitis Program (Ottawa, Canada). All participants were 18 years or older, planned to initiate HCV antiviral treatment, and provided signed informed consent to participate in a single-centre, open-label study [ClinicalTrials.gov (Identifier: NCT02734173)] which was approved by The Ottawa Health Science Network Research Ethics Board (REB #2015-0305). Three groups of patients were examined for this study: non-cirrhotic genotype 1a-infected participants receiving standard therapy plus ribavirin, non-cirrhotic genotype 1b-infected participants receiving standard therapy and compensated cirrhotic genotype 1a or 1b-infected participants dosed with standard therapy plus ribavirin and all had a HOMA-IR ≥ 2 (58). Patients were treated for 12 weeks with ribavirin and direct-acting antivirals, after which they achieved sustained virologic response (SVR), and they were followed up for an additional 36 weeks.

Inclusion and exclusion criteria, as well as methods for treatment are described (58). Plasma measurements were conducted by Laboratory Services at The Ottawa Hospital, as standard clinical procedure. Additional blood samples were treated with 1% Triton-X100, 0.3% tributyl phosphate and incubated at 37°C for 1h to destroy any virus. The concentrations of circulating factors in treated plasma were quantified using multiplexing immunobead assays analyzed using Mesoscale as described above. Plasma DPP4 concentration was quantified using the R-plex Human DPP4IV Antibody set (Mesoscale Diagnostics, cat. #F21YC) (95). To remove the variance in trait values attributed to sex and age differences for results described in Figure 7, a linear regression model compared the retrieved residuals (adjusted trait values) using the
unpaired t-test function available in R (version 4.0.5). Data are expressed as mean±SD and

\[P<0.05\] was considered statistically significant.

**Study Approval:** Animals were cared for in accordance with the Canadian Guide for the Care and Use of Laboratory animals (CCAC). All experimental procedures were approved under AUP#2909 and AUP#2029 by the University of Ottawa Animal Care and Veterinary Service. All participants were 18 years or older, planned to initiate HCV antiviral treatment, and provided signed informed consent to participate in a single-centre, open-label study [ClinicalTrials.gov (Identifier: NCT02734173)] which was approved by The Ottawa Health Science Network Research Ethics Board (REB #2015-0305).

**Statistics:** All data were plotted and statistical analysis were performed using GraphPad Prism (version 8.4.3). Data are expressed as mean ± standard error of the mean (SEM). Statistical differences between groups were evaluated by \(P<0.05\) was considered statistically significant.

**Author Contributions**

Conceptualization: MAD, MDF, EEM Methodology: KHK, MDF, EEM; Formal Analysis: NAT, Investigation: NAT, BV, MAN, NJ, EF, NMM, CAAL, NT, AAH, JRCN, CO, JNV, ILS, RS, SMP, ACC, AMC, RLJ, MAD, CLC, KHK, MDF EEM; Resources: MDF, EEM; Writing – Original Draft: NAT, BV, EEM; Writing – Review and Editing: all authors; Supervision: KHK, MDF, EEM, RJ; Project Administration: EEM; Funding Acquisition: EEM. Order of co-first authors was decided between the two authors and the corresponding author.

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Conflict of Interest: The Mulvihill lab receives funding from the Merck IISP program for preclinical studies unrelated to this work. The other authors have declared that no conflict of interest exists.
References


Figure 1: Hepatic glucose production is decreased in HFHC-fed $Dpp4^{+/+}$ and $Dpp4^{hep/-}$ mice. (A) Time-course of glucose infusion rates and plasma glucose, and endpoint GIR during hyperinsulinemic-euglycemic clamp of $Dpp4^{+/+}$ (n=6) and $Dpp4^{hep/-}$ (n=5) mice. (B) Glucose disposal rate (GDR), (C) basal and clamp hepatic glucose production (HGP) and (D) percent of HGP suppression under clamp conditions. Hepatic mRNA abundance (relative to Actb) for genes associated with (E) hepatic gluconeogenesis ($Gck$, $G6p$, $Pck$, $Gsk3b$ and $Pygl$) (F) insulin signaling ($Gcgr$, $Igf$ and $Igf1r$) in $Dpp4^{+/+}$ (n=11) and $Dpp4^{hep/-}$ (n=7) mice. (G) Time-course of plasma glucose and glucose infusion rates, and endpoint GIR during hyperinsulinemic-euglycemic clamp of $Dpp4^{GFP}$ (n=4) and $Dpp4^{hep/-}$ (n=5) mice. (H) GDR (I) basal and clamp HGP and (J) percent of HGP suppression under clamp conditions. Hepatic mRNA abundance (relative to Actb) for genes associated with (K) hepatic gluconeogenesis ($Gck$, $G6p$, $Pck$, $Gsk3b$ and $Pygl$) and (L) insulin signaling ($Gcgr$, $Igf$ and $Igf1r$) in $Dpp4^{GFP}$ (n=4) and $Dpp4^{hep/-}$ (n=11) mice. Data are presented as the mean ± SEM. Time-course data is analyzed by two-way ANOVA with Tukey’s multiple comparisons post hoc test, remaining data analyzed by unpaired students t-test with Welch’s correction, ns $p<0.05$, *$p=0.01$-$0.05$, **$p=0.001$-$0.01$. 

$Dpp4^{+/+}$ $Dpp4^{hep/-}$

$HFHC$ $HFHC$

$GIR$ $GIR$

$GDR$ $GDR$

$HGP$ $HGP$

$Glycemia$ $Glycemia$

$Gck$ $G6p$ $Pck$ $Gsk3b$ $Pygl$

$Gcgr$ $Igf1$ $Igf1r$

$Gck$ $G6p$ $Pck$ $Gsk3b$ $Pygl$

$Gcgr$ $Igf1$ $Igf1r$

$Glycemia$ $GIR$

$GIR$ $GIR$

$GDR$ $GDR$

$HGP$ $HGP$

$Glycemia$ $GIR$
Figure 2: Hepatic glucose production is decreased in Dpp4<sup>hep-/−</sup> but not Dpp4<sup>EC+/+</sup> via GLP-1 and GIP dependent pathways. Blood glucose prior to and during a pyruvate tolerance test (PTT) following (A) saline or (B) Exendin-9-39 i.p. injection and resulting area under the curve (AUC) in Dpp4<sup>GFP</sup> (n=4) and Dpp4<sup>hep-/−</sup> (n=4) mice. (C) DPP4 activity, (D) concentration, (E) active GLP-1, (F) active GIP, (G) insulin and (H) glucagon from the portal vein (PV) and cardiac puncture (CP) 15 minutes after glucose bolus in Dpp4<sup>GFP</sup> (n=4-12) and Dpp4<sup>hep-/−</sup> (n=4-11) mice. Blood glucose prior to and during a pyruvate tolerance test (PTT) following (I) saline or (J) Exendin-9-39 i.p. injection and resulting area under the curve (AUC) in Dpp4<sup>EC+/+</sup> (n=7) and Dpp4<sup>EC-/−</sup> (n=6) mice. (C) DPP4 activity, (D) concentration, (E) active GLP-1, (F) active GIP, (G) insulin and (H) glucagon from the portal vein (PV) and cardiac puncture (CP) 15 minutes after glucose bolus in Dpp4<sup>EC+/+</sup> (n=7-13) and Dpp4<sup>EC-/−</sup> (n=6-11) mice. Data are presented as the means ± SEM. Time-course data is analyzed by two-way ANOVA with Tukey’s multiple comparisons post hoc test, remaining data analyzed by unpaired students t-test with Welch’s correction, ns p<0.05, *p=0.01-0.05, **p=0.001-0.01.
Figure 3: Plasma and liver lipid profiles remain largely unchanged between genotypes. (A) Hepatic Dpp4 mRNA abundance (relative to Actb) (B) Plasma DPP4 activity. (C) Liver DPP4 activity. (D) Plasma DPP4 protein. (E) Plasma high-density lipoprotein (HDL), (F) low-density lipoprotein (LDL), (G) plasma cholesterol (chol), (H) plasma triglycerides (TG), (I) liver TG mass and (J) total cholesterol mass. Representative images of liver stained with Oil Red O in (K) SLD-fed Dpp4+/+ (n=5-7) and Dpp4−/− (n=4-6) mice, (L) HFHC-fed Dpp4+/+ (n=9-11) and Dpp4−/− (n=6) mice and (M) HFHC-fed Dpp4GFP (n=4) and Dpp4hep−/− (n=10-11) mice (scale bar = 200µm). Hepatic mRNA abundance (relative to Actb) of (N) Srebf1, (O) Mttp and (P) FoxO1. Data are presented as the means ± SEM, analyzed by unpaired students t-test with Welch’s correction, ns p<0.05, *p=0.01-0.05, **p=0.001-0.01, ***p=0.0001-0.001 and ****p<0.0001.
Figure 4: Fibrosis-related genes are up-regulated in HFHC-fed Dpp4−/− but not Dpp4hep−/− mice. (A) Alanine aminotransferase (ALT), (B) aspartate transaminase (AST) and (C) alkaline phosphatase (ALP). Liver mRNA abundance (relative to Actb) of (D) Col1a1, (E) Col3a1, (F) Mmp2, (G) Mmp11, (H) Des and (I) Ddr2. Representative images of liver stained with Picrosirius Red and quantitation of red-stained pixels in (J) SLD-fed Dpp4+/+ (n=6-7) and Dpp4−/− (n=5-6) mice, (K) HFHC-fed Dpp4+/+ (n=9-11) and Dpp4−/− (n=6) mice and (L) HFHC-fed Dpp4GFP (n=4) and Dpp4hep−/− (n=11-12) mice (scale bar = 250um) and (M) quantitation of red-stained pixels. (N) Liver METAVIR score prevalence. Data are presented as the means ± SEM, analyzed by unpaired students t-test with Welch’s correction, ns p<0.05, *p=0.01-0.05, **p=0.001-0.01, ***p=0.0001-0.001.
Figure 5: In whole liver tissue, immune-related genes and pathways are up-regulated in HFHC-fed Dpp4Δ mice, but unchanged in Dpp4ΔΔ mice. Pathway scores of immunological pathways in liver tissue of (A) SLD-fed Dpp4Δ+/+ (n=7) and Dpp4Δ−/− (n=5) mice, (B) HFHC-fed Dpp4Δ+/+ (n=11) and Dpp4Δ−/− (n=6) mice and (C) HFHC-fed Dpp4ΔFF (n=4) and Dpp4ΔΔΔ (n=11) mice. Log2 normalized mRNA counts of genes associated with (D) inflammasome pathways and (E) NF-kB signaling pathways. Liver mRNA abundance (relative to fed) was determined by qRT-PCR and analyzed using the unpaired students t-test with Welch’s correction, ns p<0.05, *p=0.01-0.05, **p=0.001-0.01, ***p=0.0001-0.001 and ****p<0.0001. nd = not detected.
Figure 6: In hepatic F4/80+ cells, immune related genes and pathways are up-regulated in SLD-fed Dpp4+/ mice, but unchanged in HFHC-fed Dpp4+/ and Dpp4hep-/- mice. Pathway scores of immunological pathways in F4/80+ cells isolated from liver tissue of (A) SLD-fed Dpp4+/ (n=6) and Dpp4-/- (n=6) mice, (B) HFHC-fed Dpp4+/+ (n=5) and Dpp4-/- (n=4) mice, and (C) HFHC-fed Dpp4+/ (n=3) and Dpp4hep-/- (n=7) mice. (D) Log2 normalized mRNA count of Dpp4 in F4/80+ cells isolated from liver. Liver mRNA abundance (relative to Actb) of (E) Adgre1 and (F) Ccr2. Liver mRNA abundance (relative to Actb) of (G) Ilgax, (H) Mrc1, (I) Cd163, (J) Arg1 and (K) Clec4f. (L) Log2 normalized mRNA counts of senescence associated signaling proteins (SASPs) in F4/80+ cells. Log2 normalized mRNA counts of genes associated with (M) NF-kB signaling pathway in F4/80+ cells in SLD-fed Dpp4+/ (n=7) and Dpp4-/- (n=5) mice, HFHC-fed Dpp4+/+ (n=11) and Dpp4-/- (n=5-6) mice and HFHC-fed Dpp4+/+ (n=11) and Dpp4hep-/- (n=11) mice. Box and whisker plots: box extends from the 25th to 75th percentiles, the whiskers go down to the smallest value and up to the largest. Data are presented as the means ± SEM, analyzed by unpaired students t-test with Welch’s correction, ns p<0.05, *p=0.01-0.05, **p=0.001-0.01, ***p=0.0001-0.001 and ****p<0.0001. nd = not detected.
Figure 7: Independent of metabolic parameters, DPP4 concentration and activity decrease with HCV clearance and viral treatment. Changes in plasma (A) AST, (B) ALT, (C) CRP levels, (D) DPP4 concentration, (E) DPP4 activity and changes in plasma cytokines and chemokines that are known substrates of DPP4, including (F) IP-10, (G) MIP1-ɑ, (H) Eotaxin and (I) sICAM in patients (n=5-6) with HOMA-IR >2 treated for HCV infection. Time points: BL = baseline, week 12 = end of treatment; follow up = 24 to 48 weeks after treatment.

Groups: RBV+ = hepatitis C (HCV) treatment containing ribavirin in participants without cirrhosis, RBV- = ribavirin free HCV treatment in participants without cirrhosis, RBV+FIB+ = HCV treatment containing ribavirin in participants with cirrhosis. To remove the variance in trait values attributed to sex and age differences, a linear regression model compared the retrieved residuals (adjusted trait values) using unpaired t-test. Data are presented as the mean ± SD and P<0.05 was considered statistically significant.