A Novel Chronic *in vivo* Oral Cadmium Exposure-Washout Mouse Model for Studying Cadmium Toxicity and Complex Diabetogenic Effects

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Abstract

Type II diabetes mellitus (T2DM) is characterized by insulin resistance, β-cell dysfunction and hyperglycemia. In addition to well known risk factors such as lifestyle and genetic risk score, accumulation of environmental toxicants in organs relevant to glucose metabolism is increasingly recognized as additional risk factors for T2DM. Here, we describe the development of an *in vivo* oral cadmium exposure with washout and high fat diet (HFD) model in C57BL/6N mice with islet cadmium bioaccumulation comparable to that found in human islets. Significant gender effect in phenotype was observed. In males, cadmium exposure followed by washout and HFD results in islet cadmium accumulation that is associated with decreased plasma insulin during hyperglycemia without changes in either body weight or kidney function. In females, cadmium-mediated dysglycemia is associated with islet cadmium accumulation and elevated plasma insulin during GSIS immediately following cadmium exposure. However, following washout with HFD, females displayed an increase in systemic glucose clearance without changes in either plasma insulin or body weight. No effect of cadmium on insulin secretion was observed in islets *ex vivo*, highlighting differences between *in vivo* and *ex vivo* cadmium exposure models. In summary, our oral *in vivo* cadmium exposure-washout with HFD model resulted in islet cadmium bioaccumulation that is relevant in the context of environmental cadmium exposure in humans. Here, we showed that islet cadmium bioaccumulation is associated with a complex cadmium-mediated changes in glucose clearance and β-cell function. The model described here will serve as a useful tool to further examine the relationship between cadmium exposure, islet cadmium bioaccumulation, dysglycemia and their underlying mechanisms.

Introduction

Type II diabetes mellitus (T2DM) is characterized by insulin resistance and dysfunction of insulin-secreting β-cells within pancreatic islets of Langerhans (Petersmann, Muller-Wieland et al. 2019). The development of T2DM is dependent upon the interplay of many factors. Several genetic variations (Todd, Srinivasan et al. 2018, Banerjee, Vats et al. 2019), intrauterine environment (Fernandez-Twinn, Hjort et al. 2019) and lifestyle-related factors including obesity, diet, physical inactivity and smoking have been associated with susceptibility of T2DM (Kolb and Martin 2017). In addition to these risk factors, increasing evidence suggests that exposure to environmental toxicants such as arsenic (Martin, Gonzalez-Horta et al. 2015, Kuo, Moon et al. 2017, Beck, Chandi et al. 2019, Li, Douillet et al. 2020) and cadmium (Schwartz, Il'yasova et al. 2003) as contributors to the development of T2DM.

To date, studies in humans and animal models describing the relationship between Cd exposure on diabetes are inconsistent. Some epidemiologic studies showed an association between Cd exposure and impaired glucose homeostasis (Bell, Early et al. 1990, Schwartz, Il'yasova et al. 2003, Afridi, Kazi et al. 2008, Fitzgerald, Olsen et al. 2020) while others reported the lack of an association (Barregard, Bergstrom et al. 2013, Menke, Guallar et al. 2016). Cadmium is a transition metal with toxic properties that is found in low levels in fruits, grains and vegetables grown on Cd-containing soil (Jolly, Islam et al. 2013), drinking water (Dalmieda and Kruse 2019) and cigarette smoke (Scherer and Barkemeyer 1983). Exposure to environmental cadmium through ingestion of Cd-containing food components and inhalation of cigarette smoke results in gradual

accumulation of Cd in various organs including the pancreas (Wong, Allen et al. 2017). In mice, short term Cd exposure *in vivo* given as subcutaneous injections resulted in bioaccumulation of Cd in pancreatic islets and dysglycemia (Fitzgerald, Olsen et al. 2020). In dispersed mouse islet cell culture, bioaccumulation of intracellular Cd was associated with a blunted glucose-stimulated insulin secretion (El-Muayed, Raja et al. 2012). Further studies examining the effects and mechanisms of sustained, oral Cd in insulin-producing β-cells are warranted. Cadmium has a long tissue half-life of up to 30 years and thus, results in gradual Cd accumulation in human tissues over decades (Elinder, Lind et al. 1976, Jarup and Akesson 2009) with the highest levels in the kidney cortex reached at age 50 (Friberg 1984). Replicating similar rates of tissue Cd accumulation in a murine model is of high importance for examining the effect of Cd at tissue levels but at the same time, poses unique challenges relating to the long half-life of Cd and its low oral bioavailability of 2-4% (Schilderman, Moonen et al. 1997).

In the present study, we established a novel *in vivo* oral Cd exposure murine model with an initial oral Cd exposure phase followed by a washout phase with high fat feeding aimed at replicating chronic islet Cd accumulation in the setting of insulin resistance in humans over a reasonable time frame. Our Cd exposure-washout with high fat feeding mouse model results in near baseline systemic Cd levels while achieving islet Cd bioaccumulation that are within range of those found in native human islets from the general population (Wong, Allen et al. 2017). We used this model to investigate the effects of Cd bioaccumulation in pancreatic islets on β-cell function at islet Cd concentrations that are relevant to human environmental Cd exposure.

85

86 **Methods**

87 **Cadmium exposure in mice**

88 5-6 weeks old, C57BL/6N mice were exposed to either vehicle or 0.5 or 1mM $CdCl_2$ (56.2
89 or 112.4ppm, Rigaku, Cat #: 1008154) in deionized drinking water (18.2μΩ, Millipore)
90 acidified with HCl (30μM, Fisher Scientific) for varying duration as indicated. During Cd
91 exposure, mice were fed regular chow (Envigo, Cat #: 7912) *ad libitum* unless otherwise
92 stated. In some experiments, mice were transitioned from $CdCl_2$-containing drinking
93 water to vehicle drinking water during a subsequent washout phase. Vehicle water was
94 given with adjusted calories high fat rodent chow (Envigo, Cat #: TD88137, TD180240,
95 42% from fat) *ad libitum* during the washout phase. Drinking water was changed once a
96 week to ensure freshness throughout all experimental phases. At the conclusion of the
97 study, mice were euthanized and tissues were collected for further analysis. All animal
98 experiments were reviewed and approved by the Institutional Animal Care and Use
99 Committee of Northwestern University.

100

101 ***In vivo* glucose tolerance tests and glucose-stimulated insulin secretion**

102 Standard glucose tolerance test (GTT) was performed by injecting a glucose bolus (50%
103 dextrose, pH 4.2, Hospira, Cat #: 000622) into the intraperitoneal cavity at a dose of 1g/kg
104 body weight following an overnight fast of 16-18 hours (Ayala, Samuel et al. 2010). Blood
105 was drawn through a tail capillary before glucose injection (t=0 min) and at 2, 5, 15, 30,
106 60 and 120 minutes post-glucose load for blood glucose determination. *In vivo* glucose-
107 stimulated insulin secretion (GSIS) was performed using a higher glucose dose of 3g/kg

108 body weight (Ayala, Samuel et al. 2010). Blood sampling time points from tail capillary
109 for glucose measurements during GSIS were similar to that of GTT tests. Blood glucose
110 concentrations were measured using Accu-Chek Nano glucose meter (Roche
111 Diagnostics). For GSIS, 20μl of blood was also collected in heparinized microvette tubes
112 (Sarstedt, Germany, Cat #: CB300LH) before glucose injection (t=0min) and at 2, 5, 15
113 and 30 minutes post-glucose load for plasma insulin analysis. Plasma samples were
114 stored at -80°C until measurements of plasma insulin that was performed using ELISA
115 kits (Crystal Chem, Cat #: 90080). In some experiments, additional plasma samples were
116 obtained for determination of proinsulin levels.
117
118 **Hyperglycemic clamp**
119 Hyperglycemic clamps were performed by the Mouse Metabolic Phenotyping Center
120 (MMPC), Vanderbilt University, Tennessee. Briefly, 5 days prior to clamp studies,
121 catheters were implanted into the carotid artery and jugular veins for sampling and
122 infusion respectively. Hyperglycemic clamps were performed in unrestrained, conscious
123 mice following 5 hours fast (Ayala, Samuel et al. 2010). At t=0 min, blood glucose was
124 raised and continuous glucose infusion rate adjusted to maintain hyperglycemic plateau
125 between 250-300mg/dl (13.8-16.7mM) based on arterial blood glucose measurements at
126 10 min intervals. Mice received heparinized saline- washed mouse erythrocytes from
127 donors at 5μl/min to prevent a drop in hematocrit. Baseline arterial blood glucose, insulin
128 and c-peptide were measured at -15 and -5 minutes prior to clamp. Arterial blood
129 sampling for clamp insulin was measured at 5, 10, 15, 20, 40, 60, 80, 100 and 120 minutes
130 during clamp. Arterial c-peptide was measured at 15, 100 and 120 minutes during clamp.

131 Plasma insulin and plasma c-peptide were measured using radioimmunoassay (EMD
132 Sigma, Cat #: RI-13K) and bead immunoassay (Luminex, R&D Systems) respectively that
133 were used according to manufacturer's instructions. Following completion of clamp
134 studies, mice were returned to regular housing for at least 48 hours with food and water
135 available *ad libitum* prior to islet perifusion studies.
136

137 **Pancreatic islet isolation**

138 Islet isolation was performed as previously described (El-Muayed, Raja et al. 2012). In
139 brief, C57BL/6N male mice were anaesthetized with intraperitoneal injection of ketamine
140 (10mg/ml)/xylazine (1mg/ml) and the abdominal cavity opened to expose the common
141 bile duct. Pancreas was perfused with 3ml collagenase (0.5mg/ml, Sigma, Cat #: C7657)
142 dissolved in HBSS buffer (Corning, Cat #: 21-020-CV) with phenol red through the
143 common bile duct. Inflated pancreases were excised, washed in phenol red-free HBSS
144 buffer (Gibco, Cat #: 14175-103) and digested at 37°C for 15 minutes. Islets were purified
145 from digested pancreas tissue by centrifugation in Biocoll density gradient (EMD Millipore,
146 Cat #: L6155) at 4°C, 2500rpm for 20 minutes. Purified islets were hand-picked under a
147 microscope (Leica Microsystems) and transferred into clean microcentrifuge tubes. Islets
148 were washed 3 times in ice-cold PBS (Corning, Cat #: 21-031-CV) at 4°C, 500xg for 2
149 minutes and stored at -20°C for ICP-MS metal analysis or used for GSIS experiments as
150 described.
151

152 **Islet perifusion**

153 Islet perifusion was performed by MMPC Core Facility, Vanderbilt University, Tennessee.
154 In brief, following isolation, islets were handpicked under the microscope for functional
155 assessment. Islet function was assessed by perifusion using approximately 50 islets (~55
156 islet equivalents (IEQ)/per perifusion chamber) at a baseline of 5.6mM glucose, and
157 stimulated with 16.7mM glucose, 16.7mM glucose + 100μM 3-isobutyl-1-methyl-xanthine
158 (IBMX, Sigma, Cat #: I5879), 16.7mM glucose + 20mM Arginine/HCl (Sigma, Cat #:
159 A6969), 5.6mM glucose + 300μM Tolbutamide (Sigma, Cat #: T0891), and 5.6mM
160 glucose + 20mM KCl. Perifusate fractions were collected at the flow rate of 3ml/min and
161 insulin was measured using radioimmunoassay kit (Millipore, Cat #: RI-13K). Insulin
162 secretion were normalized to IEQ.
163

164 **Measurement of islet and blood metal content**

165 Measurements of islet Cd were performed as previously reported (Wong, Allen et al.
166 2017). In brief, 100 islets or 30μl of blood was collected in metal-free, trace metal grade,
167 HCl-washed, 1.7ml polypropylene tubes (Denville, Cat #: C-2170). Ceramic scissors and
168 plastic instruments were used for tail nicking and blood collection. Blood and islet
169 samples were dried and heated at 80°C for 30 minutes. Dried blood and islet samples
170 were dissolved in ultra trace elemental grade (TEG) 68% HNO_3 (Fisher Scientific, Cat #:
171 A467-500), heated to 80°C for 30 minutes followed by dilution with trace metal grade
172 water (EMD, Cat #: WX0003-6) and stored at -20°C. Samples were further diluted with
173 trace metal grade water acidified with TEG HNO_3 to a final concentration of 2.765N prior
174 to submission for Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analysis, as
175 previously reported (Wong, Allen et al. 2017). Islet metal concentrations were normalized

176 to total islet protein. Total islet protein in hydrolyzed samples was measured using a BCA
177 assay kit (Thermo Scientific, Cat #: 23235) following pH neutralization with NaOH.
178 Accuracy of BCA assay protein measurements using this procedure was validated using
179 BSA standards. Negative ICP-MS output values were imputed to zero. Certified metal-
180 free pipette tips (VWR, Cat #: 53508-918), conical tubes (VWR Cat #: 89049-170, 89049-
181 174) and 1.7ml polypropylene microcentrifuge tubes (Denville, Cat #: C-2170) were used
182 throughout the collection and analysis process. All steps were performed in an epoxy-
183 coated laminar flow hood with trace element grade air filter (Baker, UK).
184

185 **Zn^{67} turnover in MIN6 cells**

186 MIN6 cells (immortalized murine β-cell line) were exposed to either vehicle or 0.5μM
187 $CdCl_2$ for 48 hours as previously described (El Muayed, Raja et al. 2012). Growth media
188 was supplemented with 10μM $ZnCl_2$ to achieve a total Zn^{2+} concentration of 15μM in
189 culture (native media contains 5μM Zn^{2+}). This exposure regimen results in significant
190 accumulation of Cd (El Muayed, Raja et al. 2012). At 1, 2 or 4 hours prior to harvest,
191 culture media was changed and fresh media containing the rare, non-radioactive Zn^{67}
192 tracer was added as $Zn^{67}Cl_2$ at a concentration of 10μM (in addition to the 5μM natural
193 Zn^{2+} mix found in media). At harvest, samples were washed twice in PBS and incubated
194 in 0.05% trypsin (37°C, 4 min). Following trypsinization, samples were transferred into
195 fresh microcentrifuges tubes, centrifuged (500rcf, 3 min) and further washed in PBS.
196 Pellets were stored at -20°C until processed for metal profile including Zn isotopes by
197 ICP-MS. The rate of divalent Zn^{67} uptake was used as a proxy measure for Zn turnover.
198

199 **Statistical analysis**

200 Two-tailed t-tests were performed to compare differences between control and test
201 groups (Prism, v9.3.1). One way ANOVA with post hoc Tukey's test for multiple
202 comparison was performed to compare differences between more than two groups.
203 Where appropriate, two-way ANOVA with repeated measures was performed to compare
204 glucose and insulin secretion over time (Prism, v9.3.1). Linear regression analysis was
205 performed between islet Cd levels, islet Zn levels, glucose AUC and insulin AUC in both
206 male and female datasets (SAS). Values are expressed as mean ± standard error of the
207 mean (SEM). $p<0.05$ were considered statistically significant.

208

209 **Results**

210 **Development of the newly established *in vivo* Cd exposure-washout model**

211 In initial experiments, we exposed male C57BL/6 mice to either vehicle (V) or 1mM $CdCl_2$
212 in drinking water continuously for 12 months. There were no differences in either glucose
213 tolerance (Suppl. Fig 1A), glucose area under the curve (Suppl. Fig 1B), glucose-
214 stimulated insulin secretion or body weight (Suppl. Fig 1C) between Cd-exposed and V
215 controls in this initial model of Cd exposure without insulin resistance. Next, we performed
216 experiments aimed at examining the effects of Cd exposure with high fat diet-induced
217 insulin resistance (Ayala, Samuel et al. 2010). To ameliorate the well described
218 anorexigenic effects of Cd, we employed an initial Cd exposure phase, during which mice
219 were exposed to either 0.5 or 1mM $CdCl_2$ in their drinking water, followed by a washout
220 phase (without Cd exposure) with high fat diet. Animal cohorts and their relevant outcome
221 parameters that were performed in this study are summarized in Suppl. Table 1.

222

223 We exposed male mice to either V or 1mM $CdCl_2$ for 4 weeks followed by a washout
224 phase (without high fat diet (HFD)). Following 4 weeks of Cd exposure, we observed a
225 significant increase in both islet Cd accumulation (79.40 ± 3.40nmol/g protein vs 0.27 ±
226 0.05nmol/g protein, $p<0.001$, Fig 1A) and systemic blood Cd (0.9434 ± 0.0078μM vs
227 0.0033 ± 0.0003μM, $p<0.001$, Fig 1B) in Cd-exposed mice compared to V controls.
228 However, at 16 weeks into the washout phase (without HFD), we continue to observe an
229 increase in islet Cd accumulation (Fig 1A) but with systemic blood Cd levels (Fig 1B) near
230 baseline levels in Cd-exposed mice. While blood Cd levels decreased during the washout
231 phase to near baseline levels (Fig 1B), islet Cd in Cd-exposed mice remained significantly
232 elevated following the washout phase (Fig 1A). This demonstrates that the
233 bioaccumulated Cd in murine islets as a consequence of Cd exposure *in vivo* remains
234 and persists in islets long after the cessation of Cd exposure despite the return of systemic
235 Cd levels to baseline during the same time period. For comparison, Cd concentration
236 median and range found in native human islets are 26.1nmol/g protein and 4.4-
237 94.7nmol/g protein respectively (Wong, Allen et al. 2017). Therefore, islet Cd
238 concentrations found in our model are comparable to that found in human islets from the
239 general US population (Wong, Allen et al. 2017). To further confirm this observation, we
240 exposed a separate cohort of male mice with either V or 1mM $CdCl_2$ in their drinking water
241 for 19.5 weeks followed by a washout phase with HFD for either 3 or 15 weeks. At 3
242 weeks into the washout phase, both islet Cd ($p<0.001$, Fig 1C) and blood Cd levels (Fig
243 1D) were elevated in Cd-HFD mice compared to V-HFD controls. At 15 weeks into the
244 washout phase, islet Cd levels remain significantly elevated in Cd-HFD mice ($p<0.01$, Fig

245 1C) while systemic Cd levels returned to near baseline values ($p<0.05$, Fig 1D) when
246 compared to V-HFD controls. Islet Cd bioaccumulation in Cd-HFD mice at both 3 and 15
247 weeks into the washout phase were observed in the absence of significant differences in
248 islet Zn content (Fig 1E). Body weight gain were similar between V-HFD and Cd-HFD
249 groups throughout the exposure and washout phases with the exception of one time point
250 at 26 weeks of age ($p<0.05$, 33.5 ± 2.4 g vs 29.8 ± 2.6 g for V-HFD and Cd-HFD groups
251 respectively), demonstrating that this Cd exposure-washout with HFD regiment was
252 successful in mitigating the well-known anorexigenic effects of Cd in Cd-exposed mice
253 (Fig 1F).
254
255 **Cadmium results in transient impairment in glucose clearance in the setting of**
256 **insulin resistance**
257 To examine the effects of islet Cd accumulation in the setting of insulin resistance in our
258 exposure-washout model, we exposed male mice with either V (n=7) or 1mM $CdCl_2$ (n=6)
259 in their drinking water simultaneously with HFD. Mice were exposed to V or Cd for $20 \pm$
260 3 weeks followed by a washout phase. During the washout phase, Cd-exposed mice
261 were placed on vehicle drinking water and both mice groups continue to receive HFD. At
262 11-12 weeks into the washout phase with HFD, we performed GSIS in both the V and
263 Cd-exposed mice. Cd-HFD mice showed lower systemic glucose clearance ($p<0.05$, Fig.
264 2A, $p<0.01$ Fig. 2B) without changes in either plasma insulin (Fig. 2C) or fasting body
265 weight (Fig. 2D). However, at 24 weeks into the washout phase with HFD, Cd-HFD mice
266 did not show differences in systemic glucose disposal following a glucose tolerance test
267 (Fig. 2E). As with our initial pilot experiments, islet Cd (53.6 ± 9.4nmol/g protein, $p<0.001$,

268 Fig. 2F) in Cd-HFD mice remained elevated compared to V-HFD controls at 31 weeks
269 into the washout phase despite systemic Cd levels being close to baseline (-0.0316 ±
270 0.0004μM vs. 0.06 ± 0.03μM, $p<0.01$, Fig. 2G).
271
272 **Islet cadmium bioaccumulation reduces plasma insulin during hyperglycemic**
273 **clamp without significant changes in *ex vivo* islet insulin secretion**
274 Next, we investigated the effects of islet Cd bioaccumulation on insulin and glucose
275 homeostasis in the setting of insulin resistance. To do this, we exposed age-matched,
276 male mice to either vehicle (n=6) or 0.5mM $CdCl_2$ (n=7) in drinking water for 17 weeks.
277 At the end of 17 weeks Cd exposure, Cd-exposed mice were transitioned to regular
278 vehicle water (i.e. washout phase) and both V and Cd-exposed mice received high fat
279 diet (HFD, 42% fat diet) for 12 weeks prior to hyperglycemic clamp studies. In the setting
280 of hyperglycemia, maintained at 250-300mg/dL arterial glucose (Fig. 3A) by continuous
281 glucose infusion (Fig. 3B), Cd-exposed mice on HFD (Cd-HFD) showed lower plasma
282 insulin levels compared to V mice on HFD (V-HFD) at 120 min ($p<0.05$, Fig. 3C). The
283 decrease in plasma insulin observed in Cd-HFD mice during hyperglycemic clamp
284 occurred in the absence of changes in either plasma c-peptide (Fig. 3D) or body weight
285 (Fig. 3E). To assess Cd accumulation on *ex vivo* islet insulin secretion, we performed
286 islet perifusion and stimulation with glucose and several insulin secretagogues in islets
287 from V-HFD (n=5) and Cd-HFD (n=5) mice. Islet insulin release (Fig. 3F) and total islet
288 insulin content (Fig. 3G) were similar between Cd-HFD and V-HFD control islets in
289 response to 16.7mM glucose, 100uM BMX, 20mM Arginine, 300uM Tolbutamine and
290 20mM KCl. This suggests that the mechanisms of Cd-induced decrease in plasma insulin

291 observed during hyperglycemic clamp is independent of changes in the rate of insulin
292 secreted per islet.
293
294 **Gender dichotomy of cadmium-mediated glycemic effects *in vivo***
295 Next, we investigated the gender dichotomy of Cd-mediated glycemic effects. To do this,
296 we exposed age-matched, male and female mice to either V (n=9-11) or a higher 1mM
297 $CdCl_2$ (n=9-10) dose in drinking water for 18 weeks. Cd-exposed mice were transitioned
298 to vehicle drinking water following 18 weeks of Cd exposure during the washout phase.
299 Both groups of V and Cd-exposed mice received HFD (42% from fat) during the washout
300 phase. Following the Cd exposure phase and prior to the washout phase, V and Cd-
301 exposed, male mice did not display any differences in whole body glucose clearance (Fig.
302 4A) and GSIS (Fig. 4B). In contrast, Cd-exposed female mice displayed significantly
303 higher plasma insulin levels 30 minutes after a glucose load compared to their V controls
304 ($p<0.05$, Fig. 4B). This increase in plasma insulin was not associated with changes in
305 either whole-body glucose clearance (Fig. 4A) or fasting body weight (Fig. 4C) and may
306 signify an increase in insulin resistance. Following 20 $\pm$ 1 week into the washout phase
307 with HFD, we did not observe significant differences in GSIS between V-HFD and Cd-
308 HFD males (Figs. 4D, 4E and 4F). In contrast, at the same time point, we observe a
309 significantly higher whole-body glucose clearance in Cd-HFD females compared to their
310 V-HFD control counterparts ($p<0.05$, Fig. 4D and 4E). This increase in whole body
311 glucose clearance in Cd-HFD females was not associated with changes in either plasma
312 insulin (Fig. 4F) or fasting body weight (Fig. 4G). Islets from Cd-HFD female mice showed
313 significant islet Cd bioaccumulation (119.3 $\pm$ 14.0nmol/g protein) compared to their V-

314 HFD female controls (29.3 ± 17.3nmol/g protein) ($p<0.01$, Fig. 4H). Taken together, these
315 findings suggest Cd-mediated changes in glucose homeostasis where islet Cd
316 bioaccumulation is associated with Cd-mediated dysglycemia in females but not in males.
317 It is of note that serum creatinine levels in Cd-HFD males were similar to that of V-HFD
318 males (Table 1) suggesting that this regiment of Cd exposure (1mM $CdCl_2$ for 18 weeks
319 followed by washout and HFD for 26 weeks) did not affect kidney function, which is an
320 important consideration when interpreting dysglycemia data given that renal failure results
321 in prolonged half-life of insulin. To further evaluate the relationship between Islet Cd
322 bioaccumulation and Cd-mediated dysglycemia in each gender, we performed linear
323 regression analysis between islet Cd content and insulin AUC in both males and females.
324 When the male and female datasets were pooled and analyzed, we observed a significant
325 gender effect on insulin AUC ($p<0.05$, Table 2). To determine if the gender effect was
326 male or female-dependent, we repeated the linear regression analysis in males and
327 females separately. We observed that islet Cd content was negatively associated with
328 insulin AUC in males ($p<0.05$, Table 2) but not in females. There was no correlation
329 between islet Zn and insulin AUC in both males and females (Table 2). There was also
330 no correlation between glucose AUC and insulin AUC in both males and females (Suppl.
331 Table 2). There were no differences in plasma proinsulin or proinsulin:insulin ratio in
332 selected experiments (results not shown).
333
334 ***Ex vivo* cadmium accumulation in intact murine and human islets did not result in**
335 **measurable changes in insulin secretion**

336 To compare the rate of Cd accumulation between human and murine islets, we exposed
337 human islets (n=3/group) and mouse islets (n=6/group) to either V or 0.1μM $CdCl_2$ for 48
338 hours in culture and measured islet Cd content. Islets originating from the same donor
339 or mouse were divided equally between groups. Cd-exposed human islets (121.7 ±
340 25.8nmol/g protein) had significantly higher intracellular Cd levels compared to vehicle
341 control islets (21.1 ± 7.0nmol/g protein) following 48 hours of Cd exposure ($p<0.05$, Fig.
342 5A). Cd-exposed murine islets (38.3 ± 3.0nmol/g protein) also showed significantly higher
343 intracellular Cd levels compared to vehicle control islets (7.6 ± 1.2nmol/g protein)
344 following 48 hours of Cd exposure ($p<0.001$, Fig. 5A). Cd levels in human islets exposed
345 to Cd *ex vivo* was significantly higher compared to mouse islets exposed under the same
346 conditions ($p<0.0001$, Fig 5A). Human islets contained lower Zn levels compared to
347 mouse islets at baseline ($p<0.01$, Fig 5B), suggesting significant differences in divalent
348 metal handling between human and mouse islets. However, Cd exposure did not
349 significantly affect islet Zn content in both human and mouse islets (Fig 5B). There were
350 also no significant differences in total islet proinsulin to insulin ratio in response to Cd
351 exposure in mouse islets *ex vivo* (data not shown).
352
353 ***Ex vivo* cadmium accumulation did not affect islet Zn content or β-cell Zn turnover**
354 Given that Cd has been shown to interact with many Zn transport and binding proteins
355 (Himeno, Yanagiya et al. 2009, Friedman 2014), we compared islet Zn levels following *in*
356 *vivo* Cd accumulation and examined Zn turnover following islet Zn accumulation. To do
357 this, we exposed MIN 6 cells (immortalized mouse β-cell line) to either V or 0.5μM $CdCl_2$
358 in media for 48 hours. Following 48 hours of $CdCl_2$ incubation, media was changed and

359 10μM Zn^{67} tracer was added to fresh culture media (equivalent to 66% of total Zn in media)
360 and the rate of cellular Zn^{67} uptake was measured at 1, 2 and 4 hours following the start
361 of Zn^{67} tracer exposure. The rate of cellular Zn^{67} uptake was used as a proxy
362 measurement for cellular Zn turnover. We observed that the rate of Zn^{67} accumulation
363 were similar between V and Cd-exposed MIN6 cells, suggesting that cellular Cd
364 accumulation *in vitro* did not affect cellular Zn turnover (Suppl Fig. 2). Overall, islet Zn
365 content was not significantly different between Cd-HFD mice and V-HFD controls from
366 any of our animal cohorts (Suppl Table 1).
367

368 **Discussion**

369 Here, we report the development of a chronic, oral Cd exposure mouse model that results
370 in islet Cd bioaccumulation that is comparable to that found in human islets from the
371 general US population (Wong, Allen et al. 2017). In males, Cd exposure followed by
372 washout with HFD resulted in islet Cd bioaccumulation that is associated with decrease
373 plasma insulin during hyperglycemia in the absence of changes in *ex vivo* islet insulin
374 secretion, body weight and kidney function. Concomitant exposure of Cd and HFD results
375 in transient impairment in systemic glucose clearance without changes in either plasma
376 insulin or body weight. In females, Cd exposure followed by washout with HFD was
377 associated with islet Cd bioaccumulation and elevated plasma insulin during GSIS without
378 changes in whole body glucose clearance immediately following Cd exposure (prior to
379 washout phase). However, following the washout phase with HFD, Cd-HFD females
380 displayed lower systemic glucose clearance during GSIS without changes in either
381 plasma insulin or body weight.

382

383 There are several benefits to our oral exposure-washout *in vivo* mouse model. Firstly, it
384 ameliorated the anorexigenic effects of Cd, thereby resulting in a similar mean body
385 weight gain between V and Cd-exposed animals. Secondly, this oral exposure-washout
386 regiment achieves target tissue (pancreatic islets) Cd bioaccumulation at concentrations
387 comparable to those found in human islets while reducing systemic blood Cd
388 concentrations at the end of the washout phase and minimizing generalized systemic
389 toxic effects. Islet Cd range in Cd-exposed mice is consistent with islet Cd range that we
390 have previously reported in human islets (4.4 - 94.7nmol/g protein) (Wong, Allen et al.
391 2017), hence making this *in vivo* experimental Cd dose relevant in the context of human
392 environmental Cd exposure. Furthermore, the continued oral Cd exposure through
393 drinking water was employed to resemble environmental exposure routes seen in the
394 general population. The oral Cd dose of 1mM was chosen to compensate for low oral Cd
395 bioavailability and life-long tissue accumulation as reported in humans (Jarup, Rogenfelt
396 et al. 1983, Schilderman, Moonen et al. 1997).

397

398 Several epidemiological studies have described contradictory findings between Cd
399 exposure and dysglycemia. Some have reported a statistically significant, positive
400 association between Cd exposure and dysglycemia (Schwartz, Il'yasova et al. 2003,
401 Wallia, Allen et al. 2014, Nie, Wang et al. 2016) while others have reported the lack of a
402 statistically significant association (Barregard, Bergstrom et al. 2013, Moon 2013, Menke,
403 Guallar et al. 2016). Discrepancies in observations could be attributed to differences in
404 study design, study power, sample analyzed (blood or urine), genetics and population

405 exposure levels. Inconsistencies between Cd exposure and dysglycemia have also been
406 reported in Cd-induced diabetic animal models. Some studies have shown that *in vivo*
407 Cd exposure resulted in hyperglycemia and β-cell dysfunction (Trevino, Waalkes et al.
408 2015, Fitzgerald, Olsen et al. 2020) while others have demonstrated normal glucose
409 homeostasis with modest decrease in insulin secretion following Cd exposure *in vivo* (Li,
410 Li et al. 2019). Animal strain, dose, duration and route of administration of Cd should be
411 considered when interpreting data reported in Cd-induced animal models of dysglycemia.
412 Additionally, short duration Cd exposure may result in acute systemic toxic effects
413 especially with elevated blood Cd levels in the absence of a washout period.
414
415 Pancreatic β-cells accumulate Cd in a dose- and time-dependent manner *in vitro* (El-
416 Muayed, Raja et al. 2012). Measurable levels of Cd have also been detected in human
417 islets (Wong, Allen et al. 2017). However, the molecular mechanisms of Cd^{2+} transport
418 into β-cells are largely unknown. DMT1 (Lortz, Schroter et al. 2014), ZnT (Kambe, Narita
419 et al. 2002, Chimienti, Favier et al. 2005, Cai, Kirschke et al. 2018) and ZIP (Lawson,
420 Maret et al. 2017) are metal transport proteins that are abundantly expressed in
421 pancreatic β-cells. A known iron transporter, DMT1 has been reported to also effectively
422 transport several other divalent metals such as Fe^{2+}, Mn^{2+}, Ni^{2+} and Co^{2+} (Arredondo,
423 Munoz et al. 2003, Garrick, Dolan et al. 2003). ZIP and ZnT are known to facilitate Zn^{2+}
424 entry into and exit from the cytosol respectively (Lemaire, Chimienti et al. 2012, Lawson,
425 Maret et al. 2017). In addition to transporting the divalent cation Zn^{2+}, ZIP proteins can
426 bind to and transport Cu^{2+}, Ni^{2+} (Antala and Dempski 2012) and Mn^{2+} (Winslow, Limesand
427 et al. 2020). In addition to its role in Zn^{2+} transport, ZnT10 is also involved in the regulation

428 of manganese homeostasis, which is now viewed as its main function (Winslow,
429 Limesand et al. 2020). Taken together, these reports suggest that DMT1, ZIP and ZnT
430 proteins are not divalent cation-specific. As Cd^{2+} is also a divalent cation, it is plausible
431 that Cd^{2+} could bind to and be transported into and out of the cytosol through any of these
432 divalent metal transport proteins. This has been shown for ZIP8, ZIP14 and ZnT1 (Dalton,
433 He et al. 2005, Ohana, Sekler et al. 2006, Girijashanker, He et al. 2008).
434
435 We have previously shown that the accumulation of Cd in β-cells at environmentally
436 relevant levels leads to impaired β-cell function *ex vivo* (El-Muayed, Raja et al. 2012).
437 These experiments were carried out in dispersed murine islets as well as in an
438 immortalized murine β-cell line, MIN6. In contrast, our current experiments did not show
439 any differences in *ex vivo* function of intact islets following either *in vivo* or *ex vivo* Cd
440 accumulation. It is likely that this difference is due to a protective effect of an intact islet
441 structure. We also observed a higher rate of *ex vivo* Cd accumulation in human islets
442 compared to mouse islets, possibly due to differences in the composition and function of
443 metal transporters (especially divalent metal transporters) between human and mouse
444 islets. This observation highlights the value of assessing tissue level Cd concentrations
445 when evaluating its toxic effects. Accumulation of Cd *in vivo* have been associated with
446 hyperglycemia and decrease glucose-stimulated insulin secretion in a model of shorter
447 term exposure (Fitzgerald, Olsen et al. 2020). The addition of a washout phase in our
448 current model provides the added advantage of mitigating acute toxic effects of Cd,
449 thereby facilitating a better correlation between target tissue Cd levels and its resulting
450 toxic effects.

451

452 The underlying molecular mechanisms of intracellular Cd bioaccumulation on β-cell
453 cytotoxicity and function are not well studied. Chang et al. (2013) showed that intracellular
454 Cd accumulation *in vitro* resulted in β-cell apoptosis that is mediated by the JNK-regulated,
455 mitochondria-dependent pathway (Chang, Hsu et al. 2013). Cd-mediated pancreatic β-
456 cell death was also shown to be mediated by Ca^{2+}-dependent, JNK activation and CHOP-
457 related signaling pathways (Huang, Kuo et al. 2019). It has also been hypothesized that
458 Cd may interfere with the trafficking of Zn in β-cells given the high Zn turnover present in
459 β-cells and the overlap between Cd and Zn transport pathways. However, the lack of
460 change in islet Zn concentration in response to Cd accumulation provides evidence
461 against this hypothesis, at least at the whole cell level. However, this does not rule out
462 changes in Zn distribution within cells at the protein or organelle level in response to Cd
463 accumulation. Taken together, our current findings and published literature indicate that
464 accumulation of Cd in pancreatic islets is cytotoxic to β-cells, thus resulting in Cd-
465 mediated β-cell apoptosis that consequently affects total insulin secretory capacity *in vivo*.
466 Given the diverse intracellular targets of Cd, each with a different concentration-effect
467 relationship, it is not surprising that Cd effects that are observed at a whole cell and
468 organism level are strongly dependent on exposure duration and tissue concentration.
469

470 Cd-mediated molecular mechanisms *in vivo* and *ex vivo* are likely distinct from one
471 another which, in turn, could affect Cd toxicology differently under both conditions. This
472 may be due to the uptake, organification and toxicokinetic properties of Cd. *In vivo*, Cd
473 is primarily bound to albumin or metallothionein (Jin, Nordberg et al. 1986, Nordberg

474 2004). *Ex vivo*, the primary form of Cd in culture is Cd^{2+}. Recently, we reported
475 differences in Cd-mediated islet transcriptome changes between *in vivo*- and *ex vivo*-
476 exposed islets where we did not find any overlap in Cd-induced differentially expressed
477 genes in both groups (Wong, Wang et al. 2021).
478
479 Here, we report the development of a chronic, oral Cd exposure mouse model that results
480 in islet Cd bioaccumulation that is relevant in the context of environmental Cd exposure
481 in humans. Our *in vivo* Cd exposure-washout mouse model showed islet Cd
482 bioaccumulation that is associated with subtle and complex Cd-mediated effects on
483 glucose clearance and plasma insulin levels. Further research examining the relationship
484 between Cd exposure, islet Cd bioaccumulation, dysglycemia and their underlying
485 mechanisms are needed to understand the effects of Cd-mediated β-cell dysfunction.
486

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

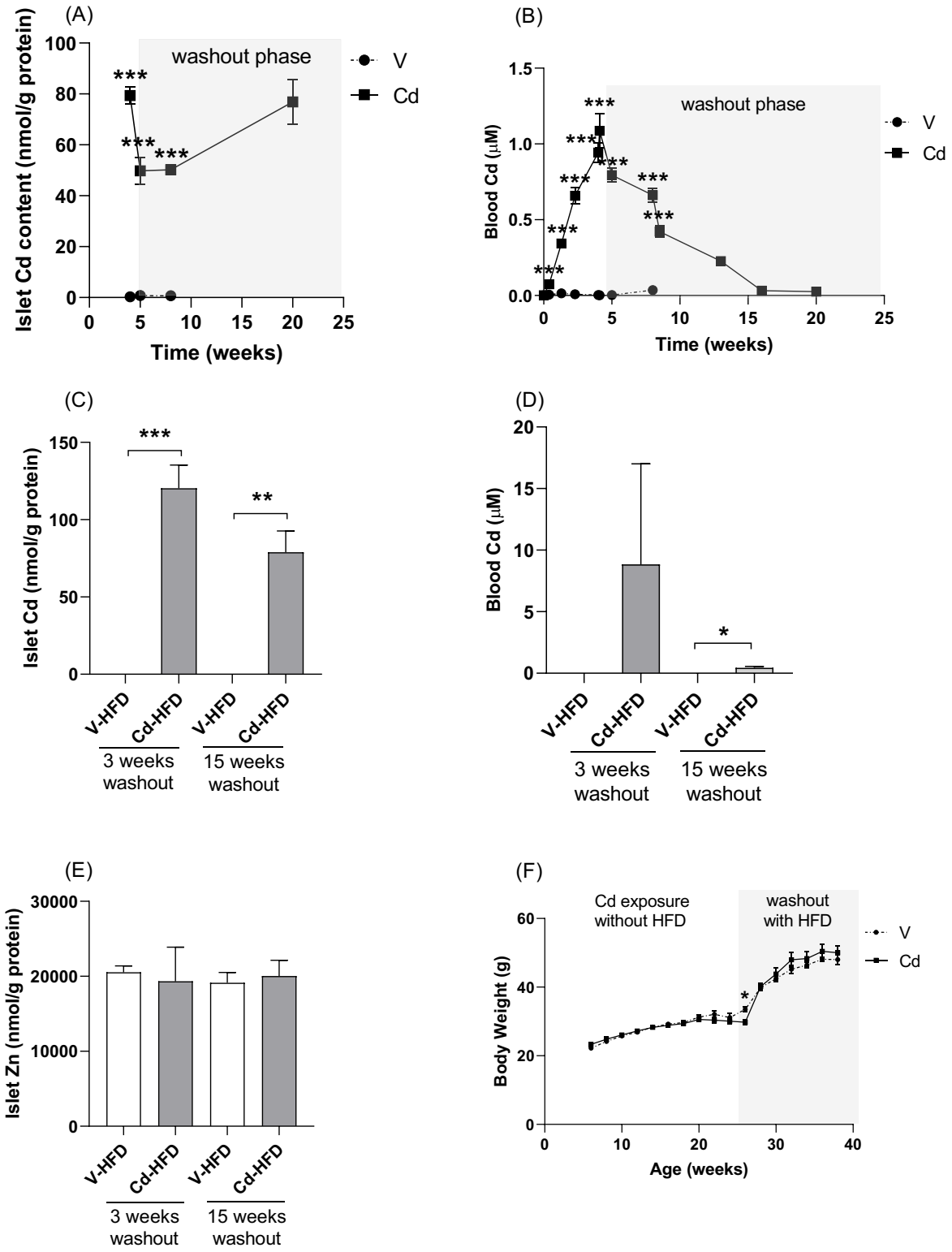
Authors' contributions

Winifred Wong: Conceptualization, Investigation, Validation, Formal analysis, Writing – original draft, review and editing. Janice Wang: Investigation, Validation, Writing-review and editing. Matthew Meyers: Investigation. Nathan Wang: Investigation. Rebecca Sponenburg: Investigation. Norrina Allen: Formal analysis. Joshua Edwards: Writing – review and editing. Malek El-Muayed: Conceptualization, Investigation, Formal

analysis, Funding acquisition, Writing-review and editing. All authors have read and approved the final manuscript.

512 **List of Figures**

513 Fig. 1: (A) Islet Cd content (normalized to total islet protein) and (B) blood Cd levels in
514 male mice exposed to either V (n=4-5) or 1mM $CdCl_2$ (n=4-8) for 4 weeks followed by 16
515 weeks of washout. (C) Islet Cd content, (D) blood Cd levels, (E) islet Zn content and (F)
516 body weight curve in male mice exposed to either V or 1mM $CdCl_2$ for 19.5 weeks
517 followed by 3 and 15 weeks washout with high fat diet (HFD). V-HFD, V-exposed followed
518 by washout and HFD; Cd-HFD, Cd-exposed followed by washout and HFD. Data are
519 expressed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.


(A)
Islet Cd content (nmol/g protein)
washout phase
V
Cd
Time (weeks)
(B)
Blood Cd (μM)
washout phase
V
Cd
Time (weeks)
(C)
Islet Cd (nmol/g protein)
V-HFD
Cd-HFD
V-HFD
Cd-HFD
3 weeks washout
15 weeks washout
(D)
Blood Cd (μM)
V-HFD
Cd-HFD
V-HFD
Cd-HFD
3 weeks washout
15 weeks washout
(E)
Islet Zn (nmol/g protein)
V-HFD
Cd-HFD
V-HFD
Cd-HFD
3 weeks washout
15 weeks washout
(F)
Cd exposure without HFD
washout with HFD
V
Cd
Body Weight (g)
Age (weeks)

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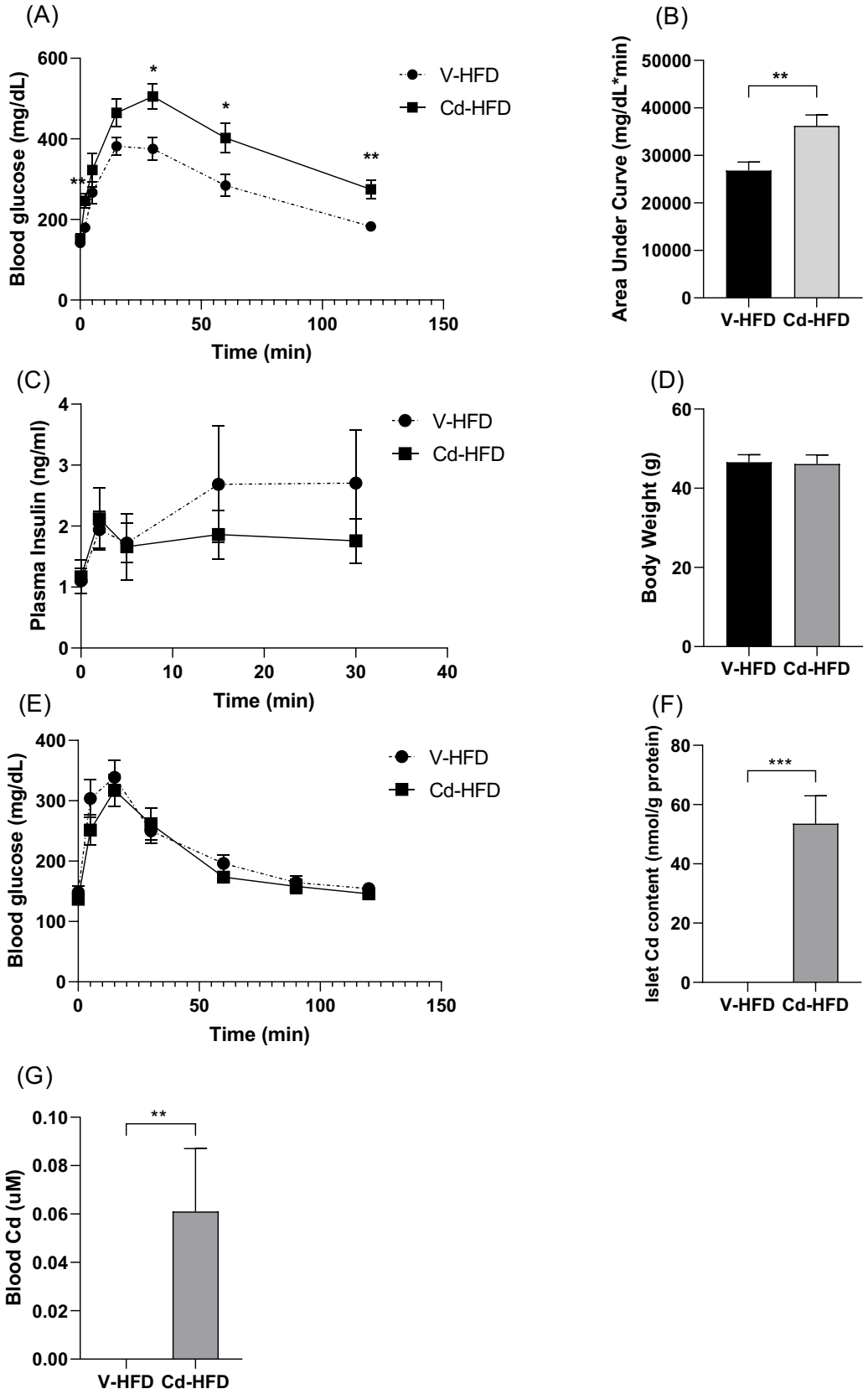
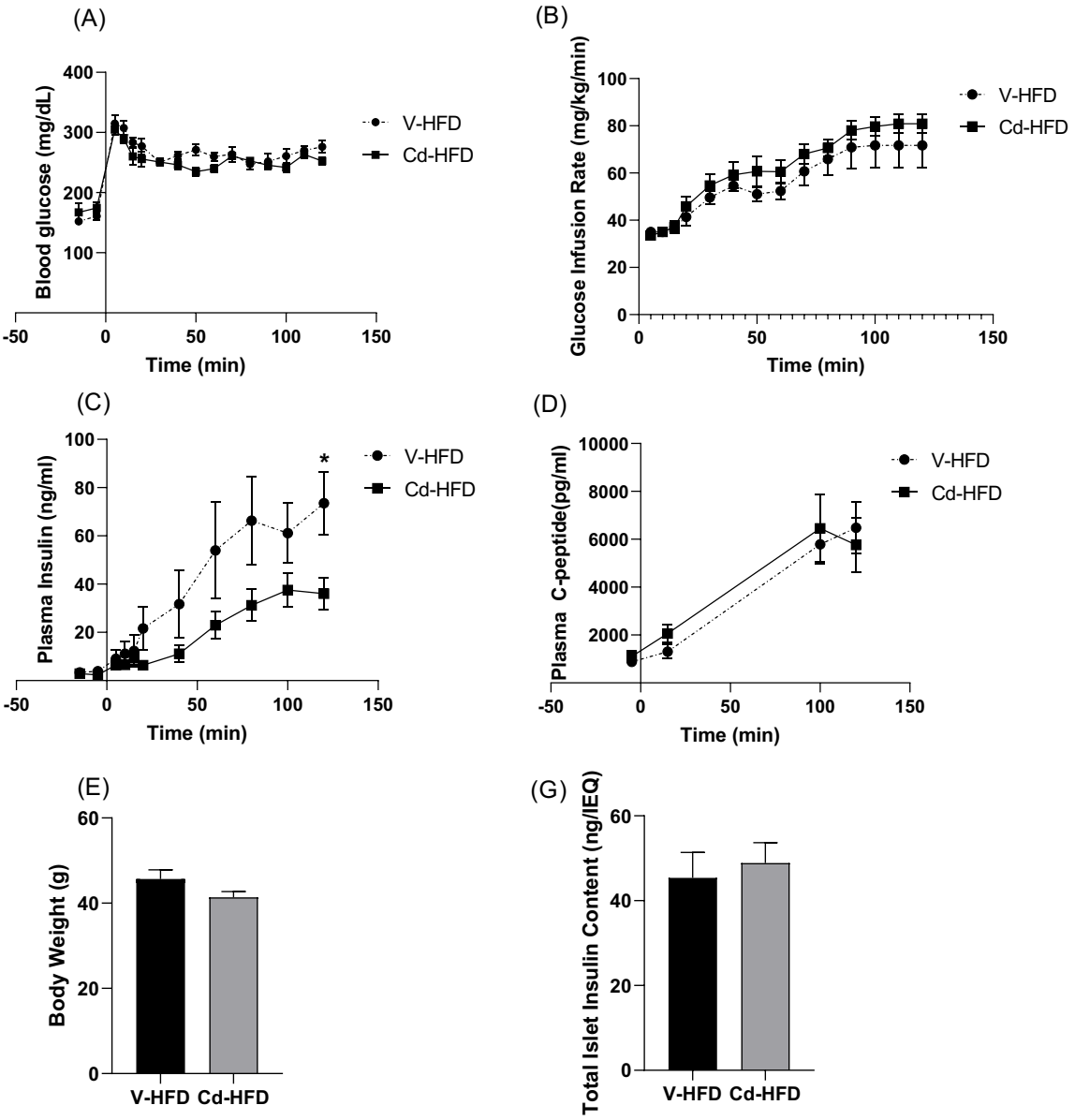
521

522 Fig 2: (A) Blood glucose, (B) area under the curve, (C) plasma insulin and (D) fasting
523 body weight during GSIS at 11-12 weeks into washout phase with HFD in male mice
524 exposed to either V (n=7) or 1mM $CdCl_2$ (n=6) on HFD. (E) Blood glucose during glucose
525 tolerance test 24 weeks into washout phase with HFD in V (n=7) or Cd-exposed (n=6)
526 male mice on HFD. (F) Islet Cd content and (G) blood Cd in male mice exposed to either
527 V (n=6) or 1mM $CdCl_2$ (n=6) for 19 weeks with HFD followed by 31 weeks of washout
528 with HFD. V-HFD, V-exposed followed by washout and HFD; Cd-HFD, Cd-exposed
529 followed by washout and HFD. Data are expressed as mean ± SEM. *$p<0.05$, **$p<0.01$,
530 ***$p<0.001$.


(A)
Blood glucose (mg/dL)
Time (min)
V-HFD
Cd-HFD
(B)
Area Under Curve (mg/dL*min)
V-HFD Cd-HFD
(C)
Plasma Insulin (ng/ml)
Time (min)
V-HFD
Cd-HFD
(D)
Body Weight (g)
V-HFD Cd-HFD
(E)
Blood glucose (mg/dL)
Time (min)
V-HFD
Cd-HFD
(F)
Islet Cd content (nmol/g protein)
V-HFD Cd-HFD
(G)
Blood Cd (uM)
V-HFD Cd-HFD

531

532 Fig. 3: (A) Arterial blood glucose, (B) glucose infusion rate, (C) plasma insulin, (D) plasma
533 c-peptide and (E) body weight during hyperglycemic clamp in mice exposed to either V
534 (n=6) or 0.5mM $CdCl_2$ (n=7) for 17 weeks followed by 12 weeks of washout with HFD on
535 V water. (F) Islet insulin secretion in response to high glucose and several insulin
536 secretagogues from V and Cd-exposed mice following 12 weeks of washout with HFD
537 (n=5/group). (G) Total islet insulin content following islet perifusion in V and Cd-exposed
538 mice islets following 12 weeks of washout with HFD (n=5/group). V-HFD, V-exposed
539 followed by washout and HFD; Cd-HFD, Cd-exposed followed by washout and HFD.
540 Data are expressed as mean ± SEM. *$p<0.05$.



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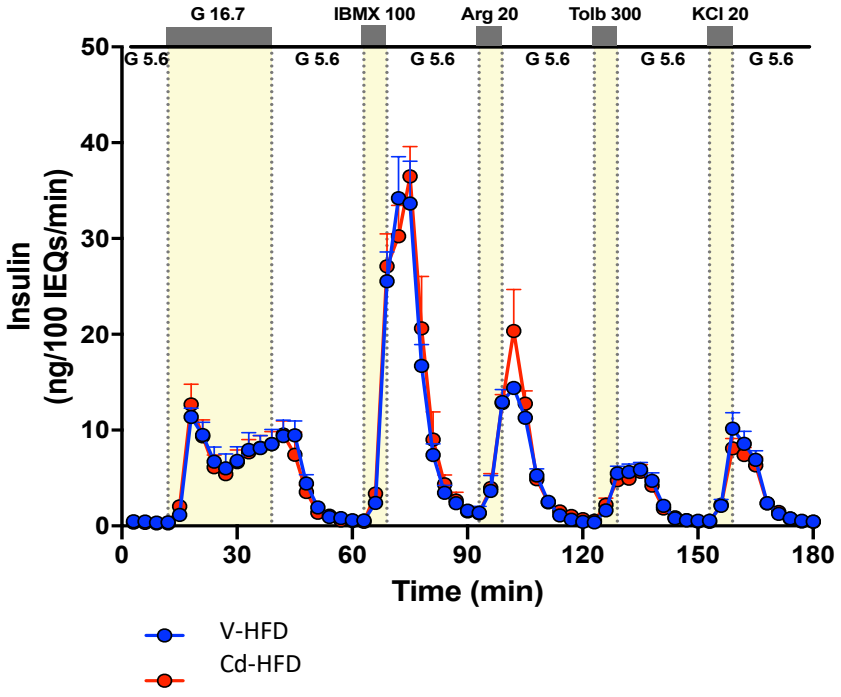
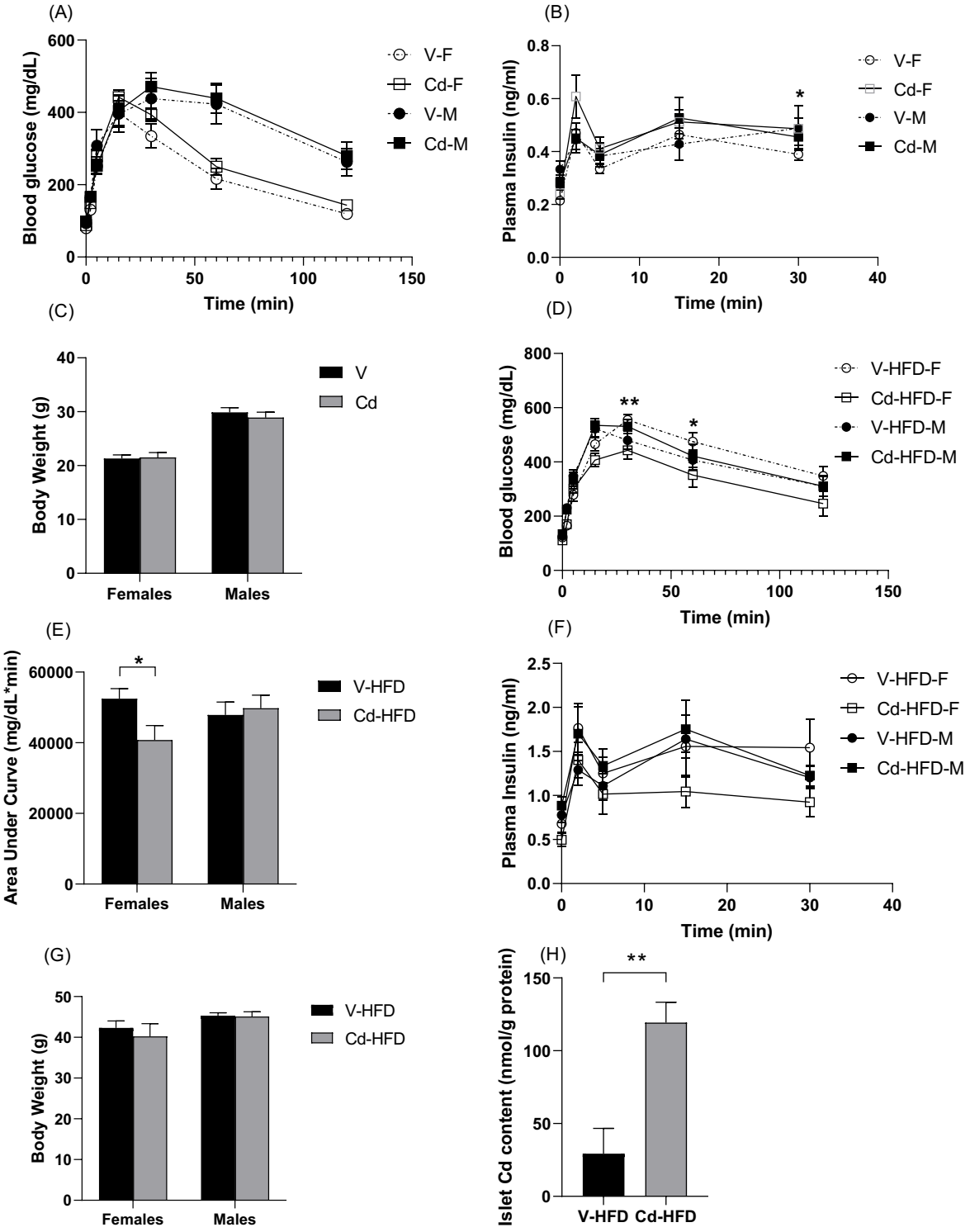


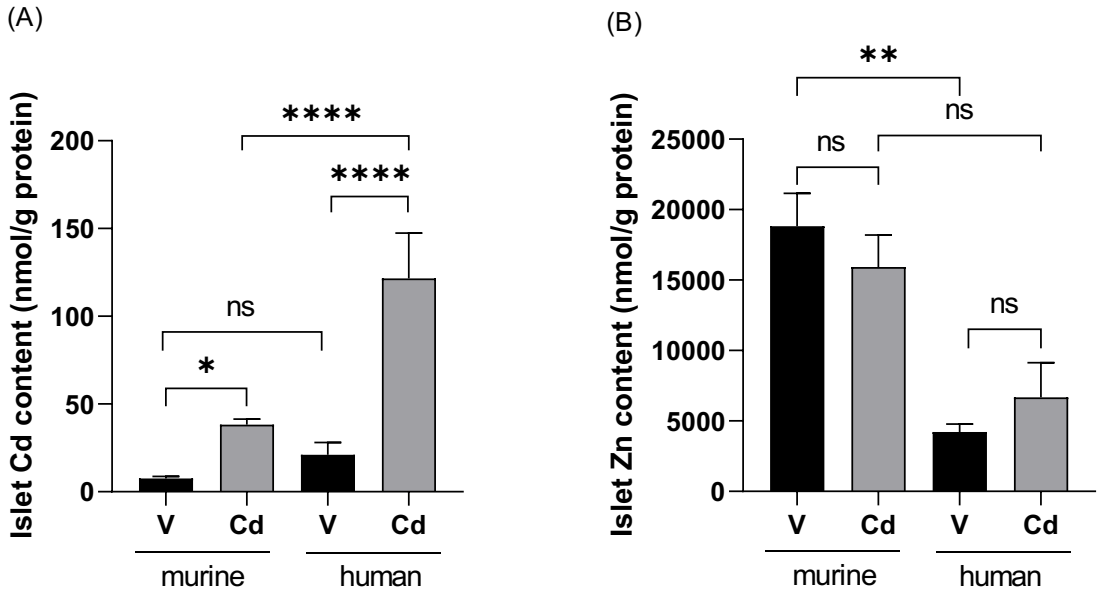
565 Fig. 4: (A) Blood glucose, (B) plasma insulin and (C) fasting body weight during *in vivo*
566 GSIS in age-matched male and female mice exposed to either V (n=10 (males), n=11
567 (females)) or 1mM $CdCl_2$ (n=10/gender) for 18 weeks. (D) Glucose curve and
568 corresponding (E) area under the curve, (F) plasma insulin and (G) fasting body weight
569 during GSIS in age-matched V-HFD and Cd-HFD male (n=9-10/group) and female (n=9-
570 10/group) mice 19-21 weeks into the washout phase with HFD. (H) Islet Cd content
571 (normalized for total islet protein) at sacrifice in V-HFD (n=9) and Cd-HFD (n=8) female
572 mice. V-F, Vehicle females; Cd-F, Cd-exposed females; V-M, Vehicle males; Cd-M, Cd-
573 exposed males; V-HFD, V-exposed followed by washout and HFD; Cd-HFD, Cd-exposed
574 followed by washout and HFD. Data are expressed as mean ± SEM. *$p<0.05$, **$p<0.01$.


(A)
Blood glucose (mg/dL)
Time (min)
V-F
Cd-F
V-M
Cd-M
(B)
Plasma Insulin (ng/ml)
Time (min)
V-F
Cd-F
V-M
Cd-M
(C)
Body Weight (g)
Females
Males
V
Cd
(D)
Blood glucose (mg/dL)
Time (min)
V-HFD-F
Cd-HFD-F
V-HFD-M
Cd-HFD-M
(E)
Area Under Curve (mg/dL*min)
Females
Males
V-HFD
Cd-HFD
(F)
Plasma Insulin (ng/ml)
Time (min)
V-HFD-F
Cd-HFD-F
V-HFD-M
Cd-HFD-M
(G)
Body Weight (g)
Females
Males
V-HFD
Cd-HFD
(H)
Islet Cd content (nmol/g protein)
V-HFD
Cd-HFD

575

576

577 Fig. 5: (A) Islet Cd and (B) islet Zn (normalized to total islet protein) in murine (n=6/group)
578 and human islets (n=3/group) exposed to either V or 0.1μM $CdCl_2$ for 48 hours. Data are
579 expressed as mean ± SEM. Adjusted p-values from one way ANOVA with post hoc
580 Tukey's test for multiple comparison. * p<0.05, **p<0.01, ****p<0.0001, ns: not significant.



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583 **List of tables**

584 Table 1: Serum Clinical Chemistry from male mice that were treated with either V (n=9)
585 or 1mM $CdCl_2$ (n=10) for 18 weeks followed by 26 weeks HFD thereafter. V-HFD, V-
586 exposed followed by washout with HFD mice; Cd-HFD, Cd-exposed followed by washout
587 with HFD mice. Data are expressed as mean ± SEM. *p<0.05.

Analyte	V-HFD	Cd-HFD
ALP (U/L)	105.4 ± 11.5	105.1 ± 11.6
AST (U/L)	503.8 ± 90.4	522.2 ± 86.4
ALT (U/L)	611.7 ± 115.8	678.3 ± 158.1
Creatine kinase (U/L)	793.2 ± 504.9	349.7 ± 84.8
GGT (U/L)	0 ± 0	0 ± 0
Albumin (g/dL)	2.5 ± 0.1	2.6 ± 0.1
Total Bilirubin (mg/dL)	0.14 ± 0.02	0.15 ± 0.02
Total Protein (g/dL)	5.2 ± 0.2	5.4 ± 0.2
Globulin (g/dL)	2.7 ± 0.1	2.8 ± 0.1
Bilirubin – Conjugated (mg/dL)	0.06 ± 0.02	0.05 ± 0.02
BUN (mg/dL)	16.2 ± 2.0	16.2 ± 1.1
Creatinine (mg/dL)	0.10 ± 0.03	0.16 ± 0.05
Cholesterol (mg/dL)	314.8 ± 22.7	305.0 ± 20.0
Glucose (mg/dL)	376.3 ± 15.5	412.3 ± 27.3
Calcium (mg/dL)	8.5 ± 0.2	8.8 ± 0.3
Phosphorus (mg/dL)	7.4 ± 0.6	8.5 ± 0.7
Bicarbonate TCO_2 (mM)	17.3 ± 0.7	17.1 ± 1.6
Chloride (mM)	113.1 ± 1.3	108.3 ± 1.3*
Potassium (mM)	6.1 ± 0.5	6.2 ± 0.7
ALB/GLOB ratio	0.9 ± 0.03	0.9 ± 0.03
Sodium (mM)	150.6 ± 0.9	148.4 ± 1.4
BUN/Creatinine ratio	92.2 ± 27.8	58.3 ± 18.0
Bilirubin – Unconjugated (mg/dL)	0.09 ± 0.03	0.12 ± 0.03
Na/K ratio	26.0 ± 2.4	26.0 ± 2.6
Triglycerides (mg/dL)	67.0 ± 3.7	57.6 ± 4.9
C-reactive peptide (ng/ml)	15234.1 ± 815.1	14033.4 ± 574.7

588

589 Table 2: Linear regression analysis between islet Cd content, islet Zn content and insulin AUC in males alone (n=33),

590 females alone (n=29) and combined male and female mice (n=62). *p<0.05, ***p<0.001

		Model 1				Model 2			
Gender	**Predictor**	**Parameter Estimate**	**LCL Mean**	**UCL Mean**	**Pr > |t|**	**Parameter Estimate**	**LCL Mean**	**UCL Mean**	**Pr > |t|**
Males and females	**Gender**	5.65	-8.48	19.79	0.435	-17.36	-33.43	-1.29	0.039*
	Islet Cd	-0.15	-0.28	-0.02	0.028*	-0.09	-0.2	0.03	0.138
	Islet Zn	0.0005	$-12X10^{-5}$	0.001	0.115	0.0004	$-21X10^{-5}$	0.0009	0.217
	Age	-	-	-	-	1.36	0.75	1.98	<0.001***
Males	**Islet Cd**	-0.27	-0.48	-0.06	0.018*	-0.19	-0.37	-0.02	0.042*
	Islet Zn	0.0009	$-5 X 10^{-6}$	0.002	0.061	0.0007	$18X10^{-7}$	0.001	0.059
	Age	-	-	-	-	1.38	0.70	2.07	<0.001***
Females	**Islet Cd**	-0.04	-0.16	0.09	0.588	-0.02	-0.16	0.13	0.844
	Islet Zn	$-3X10^{-4}$	-0.001	0.0006	0.538	$-44X10^{-5}$	-0.002	0.0006	0.435
	Age	-	-	-	-	0.68	-1.85	3.2	0.604

591 LCL: lower control limits, UCL: upper control limits, Pr > |t|: t-distribution two-tailed p-value.

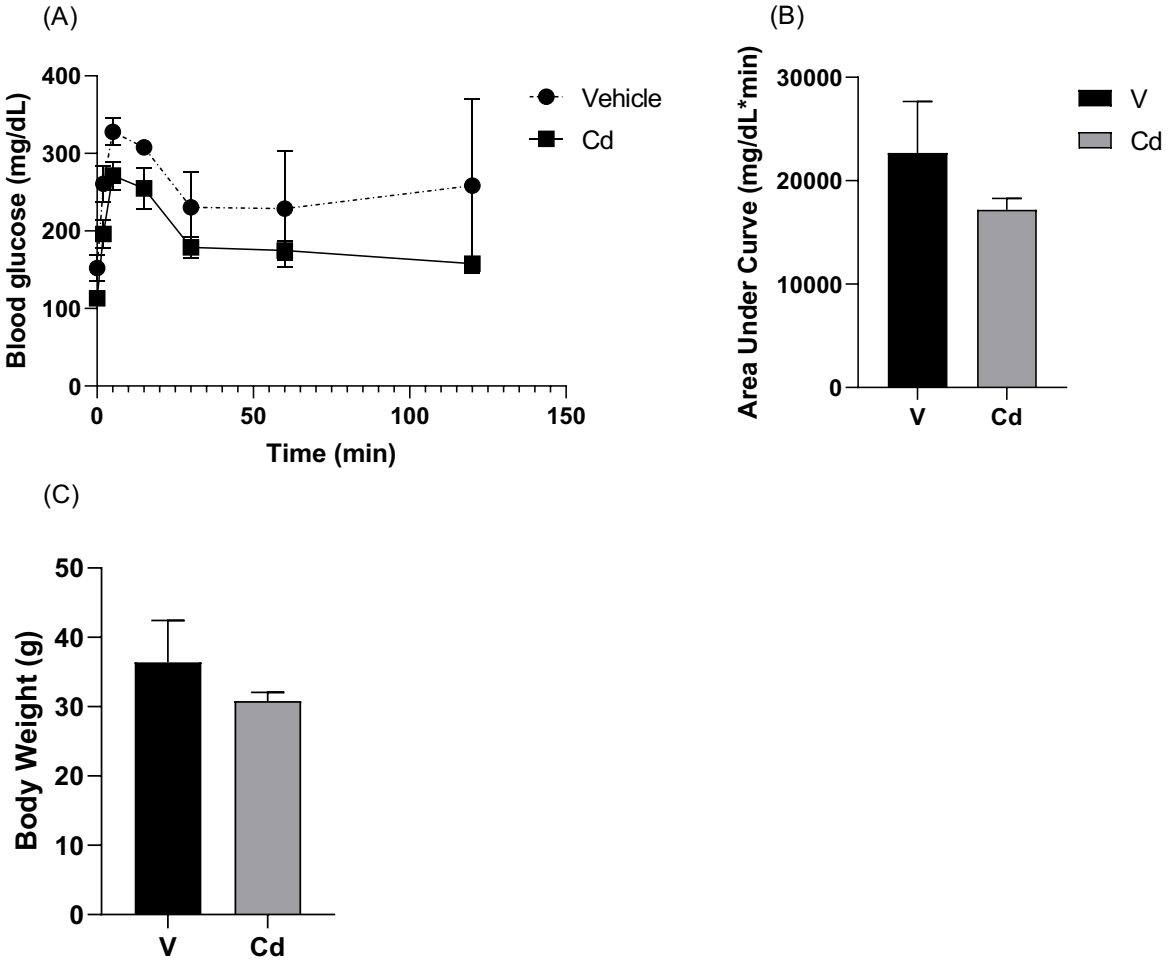
592

593 **Supplemental Figures**

594 Suppl Fig. 1: (A) Glucose tolerance test and corresponding (B) area under the curve (AUC)

595 and (C) body weight in mice exposed to either V (n=3) or 1mM $CdCl_2$ (n=5) in drinking

596 water for 12 months. Data are expressed as mean ± SEM.



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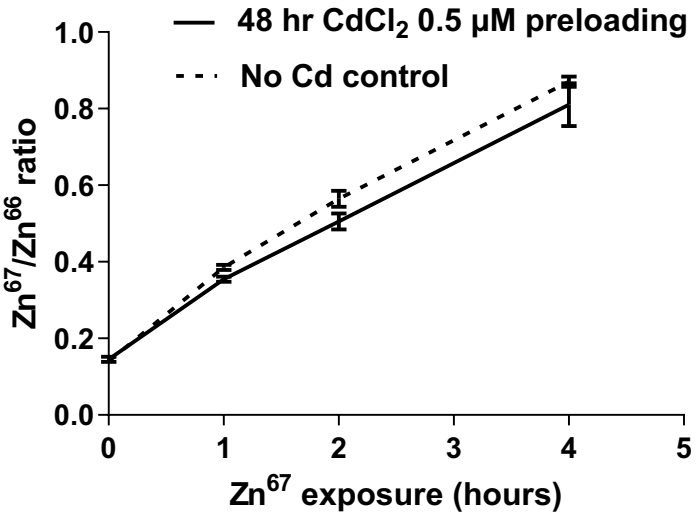
599 Suppl Fig. 2**:** Rate of non-radioactive, rare Zn isotope, Zn^{67} uptake as a proxy for Zn

600 turnover in MIN6 cells following exposure to either V or 0.5μM $CdCl_2$ for 48 hours (n=2).

601 Data are expressed as mean ± SEM.

602



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604

605 **Supplemental Table**

606 Suppl Table 1: List of cohorts with their relevant experimental and islet outcome parameters. Data are expressed as mean

607 ± SEM. *p<0.05.

Cohort #	Description	Gender	Deaths (V/Cd)	$CdCl_2$ dose and duration	Diet (Exposure/ Washout)	Body weight at GSIS (g, V/Cd)	AUC insulin during GSIS (ng/ml*min, V/Cd)	AUC glucose during GSIS (mg/dL*min, V/Cd)	Islet Cd (nmol/g protein, V/Cd)	Islet Zn (nmol/g protein, V/Cd)
1	Islet Cd kinetics	m	1/0	1mM, 19.6 weeks	Regular Chow/ TD180240	28.7±0.7/27.1±0.7 (pre-washout)	22.6±2.7/ 17.7±1.4 (pre-washout)	55430±2581/ 38835±4598* (pre-washout)	UD/96.6±11.6	19833±724/ 19745±1902
2	Effects of Cd accumulation on islet function in males	m	0/0	1mM, 19 weeks	TD88137/ TD88137	53.8±1.4/50.3±1.2	88.9±10.1/57.3±6.6*	44417±2514/ 35375±6125	UD/53.6±8.6	25270±1434 /24874±2645
3	Replicate of cohort 2	m	3/2	1mM, 19.6 weeks	TD88137/ TD88137	46.6±1.8/46.1±2.1	71.0±18.6/53.7±10.6	33803±2119/ 46365±2795*	2.5±1.9/ 114.3±31.2	49403±2278 /50552±1788
4	Effects of Cd accumulation on islet function in males with lower Cd dose	m	0/2 (fight wounds)	0.5mM, 16.9 weeks	Regular Chow/ TD88137	44.0±1.8/41.4±1.5	N/A	N/A	N/A	N/A
5	Effects of Cd accumulation on islet function in females	f	3/2	1mM, 17.9 weeks	Regular Chow/ TD180240	44.7±1.6/40.1±3.5	62.1±6.4/ 52.1±6.1	43544±3704/ 42745±4865	33.6±15.1/ 119.3±13.1	22337±2293 /23171±2216

9	Effects of Cd accumulation on islet function in females with lower Cd dose	f	4/2	0.5mM, 18 weeks	TD180240/ TD88137	43.7±3.8/47.8±1.1	64.0±4.3/49.6±5.3	41713±4768/ 51546±4033	UD/30.4±3.1	27330±1970 /30641±2609

N/A = not applicable, UD=undetectable.

610 Suppl Table 2: Linear regression analysis between islet Cd content, islet Zn content, glucose AUC and insulin AUC in

611 males alone (n=33), females alone (n=29) and combined male and female mice (n=62).

Gender	Predictor	Parameter Estimate	LCL Mean	UCL Mean	Pr > |t|
Males and females	**Gender**	-12.93	-30.22	4.37	0.149
	Islet Cd	-0.09	-0.20	0.03	0.137
	Islet Zn	0.0003	-24×10^{-5}	0.0009	0.257
	Age	1.27	0.65	1.90	<0.001
	AUC glucose	-37×10^{-5}	-92×10^{-5}	0.0002	0.196
Males	**Islet Cd**	-0.19	-0.37	-0.02	0.042
	Islet Zn	0.0007	-46×10^{-6}	0.001	0.077
	Age	1.33	0.61	2.06	0.001
	AUC glucose	-2×10^{-4}	-0.001	0.0006	0.634
Females	**Islet Cd**	-0.006	-0.15	0.14	0.931
	Islet Zn	-34×10^{-5}	-0.001	0.0007	0.529
	Age	0.49	-1.96	2.94	0.699
	AUC glucose	-56×10^{-5}	-0.001	0.0001	0.111

612 LCL: lower control limits, UCL: upper control limits, Pr > |t|: t-distribution two-tailed p-value.

613

614 **References**
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