

1 **GROWTH DIFFERENTIATION FACTOR-15 AND THE**
2 **ASSOCIATION BETWEEN TYPE 2 DIABETES AND**
3 **LIVER FIBROSIS IN NAFLD**

4 Running title: GDF-15 links type 2 diabetes and liver fibrosis

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56 **Abbreviations**

57 **NAFLD** - Non-alcoholic fatty liver disease

58 **NASH** - Non-alcoholic steatohepatitis

59 **T2DM** - Type 2 diabetes mellitus

60 **CVD** - Cardiovascular disease

61 **CKD** - Chronic kidney disease

62 **GDF-15** - Growth differentiation factor-15

63 **MIC-1** - Macrophage inhibitory cytokine-1

64 **TGFβ** - Transforming growth factor-beta

65 **VCTE** - Vibration-controlled transient elastography

66 **ELF** - Enhanced liver fibrosis

67 **APRI** - AST to platelet ratio index

68 **FIB-4** - Fibrosis-4

69 **kPa** - Kilo-pascals

70 **HbA1c** - Haemoglobin A1c

71 **INSYTE** - Investigation of Synbiotic Treatment in NAFLD

72 **DEXA** - Dual-energy x-ray absorptiometry

73 **HDL** - High-density lipoprotein

74 **ALT** - Alanine aminotransferase

75 **AST** - Aspartate transaminase

76 **hs-CRP** - High-sensitivity C-reactive protein

77 **TNFα** - Tumour necrosis factor alpha

78 **IL** - Interleukin
79 **LPS** - Lipopolysaccharide
80 **e-GFR** - Estimated-glomerular filtrations rate
81 **GIP** - Gastric inhibitory polypeptide
82 **GLP-1** - Glucagon-like-peptide-1
83 **PYY** - Peptide-YY
84 **PP** - Pancreatic peptide
85 **¹H-MRS** - Proton magnetic resonance spectroscopy
86 **IQR** - Inter-quartile range
87 **ROC** - Receiver operating characteristic
88 **AUC** - Area under the curve
89 **CI** - confidence interval
90 **AUROC** - Area under the receiver operating characteristic curve
91 **PPV** - Positive predictive value
92 **NPV** - Negative predictive value
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121 **Abstract:**

122 **Background:** Type 2 diabetes mellitus (T2DM) is a strong risk factor for liver fibrosis in non-alcoholic fatty
123 liver disease (NAFLD). It remains uncertain why T2DM increases the risk of liver fibrosis. It has been
124 suggested that growth differentiation factor-15 (GDF-15) concentrations increase the risk of liver fibrosis.
125 We aimed to investigate a) if GDF-15 concentrations were associated with liver fibrosis and involved in the
126 relationship between T2DM and liver fibrosis and b) what factors linked with T2DM are associated with
127 increased GDF-15 concentrations.

128 **Methods:** Ninety-nine patients with NAFLD (61% men, 42.4% T2DM) were studied. Serum GDF-15
129 concentrations were measured by electro-chemiluminescence immunoassay. Vibration-controlled transient
130 elastography (VCTE)-validated thresholds were used to assess liver fibrosis. Regression modelling, receiver
131 operator characteristic curve analysis and Sobel test statistics were used to test associations, risk predictors
132 and the involvement of GDF-15 in the relationship between T2DM and liver fibrosis respectively.

133 **Results:** Patients with NAFLD and T2DM (n=42) had higher serum GDF-15 concentrations [mean(SD):
134 1271.0(902.1) vs. 640.3(332.5) pg/ml, $p<0.0001$], and a higher proportion had VCTE assessed \geq F2 fibrosis
135 (48.8 vs. 23.2%, $p=0.01$) than those without T2DM. GDF-15 was independently associated with liver
136 fibrosis ($p=0.001$), and GDF-15 was the most important single factor predicting \geq F2 or \geq F3 fibrosis (\geq F2
137 fibrosis AUROC 0.75, (95%CI 0.63-0.86), $p<0.001$, with sensitivity, specificity, positive predictive (PPV)
138 and negative predictive (NPV) values of 56.3%, 86.9%, 69.2% and 79.1% respectively). GDF-15 was
139 involved in the association between T2DM and \geq F2 fibrosis (Sobel test statistic 2.90, $p=0.004$). Other factors
140 associated with T2DM explained 60% of the variance in GDF-15 concentrations, ($p<0.0001$). HbA1c
141 concentrations alone explained 30% of the variance ($p<0.0001$).

142 **Conclusions:** GDF-15 concentrations are a predictor of liver fibrosis and potentially involved in the
143 association between T2DM and liver fibrosis in NAFLD. HbA1c concentrations explain a large proportion of
144 the variance in GDF-15 concentrations.

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146 **Keywords:** Non-alcoholic fatty liver disease, Growth differentiation factor-15, Macrophage inhibitory
147 cytokine-1, Type-2 diabetes mellitus, HbA1c, liver fibrosis.

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150 1. Introduction

151 Non-alcoholic liver disease (NAFLD) is a ‘multisystem’ disease that increases the risk of type 2 diabetes
152 mellitus (T2DM), cardiovascular disease (CVD) [1], and chronic kidney disease (CKD) [2]. Bi-directional
153 relationships exist between NAFLD and T2DM and not only is NAFLD an independent risk factor for
154 incident T2DM, but when both diseases co-exist, T2DM increases the risk of faster progression of NAFLD
155 to advanced fibrosis, cirrhosis and hepatocellular carcinoma [2-4]. However, the factors involved in the
156 association between T2DM and increased risk of liver fibrosis in patients with NAFLD are not fully
157 understood.

158 Growth differentiation factor-15 (GDF-15), also known as macrophage inhibitory cytokine (MIC)-1, is a
159 stress-inducible cytokine that can be ubiquitously expressed [5]. Circulating GDF-15 concentrations are
160 increased in patients with T2DM [6, 7], and are separately reported to associate with obesity [8], liver
161 disease severity [9, 10], CVD [11] and CKD [12]. A recent multicentre transcriptomic study demonstrated
162 that hepatic GDF-15 expression was positively associated with NAFLD severity and GDF-15 expression was
163 significantly higher in patients with advanced liver fibrosis [10]. In support of these findings a previous study
164 measured serum GDF-15 concentrations in patients (in an Asian population) with both T2DM and NAFLD
165 and showed that GDF-15 concentrations were higher in those with T2DM and advanced liver fibrosis [13].
166 Furthermore, growing evidence suggests that GDF-15 may have profibrogenic effects within the liver and
167 other tissues [13-15]. Taken together, these studies suggest that increased GDF-15 concentrations may
168 increase the risk of liver fibrosis. However, it is not known whether circulating GDF-15 concentrations are
169 potentially involved in the known relationship between T2DM and liver fibrosis in patients with NAFLD.

170 Additional factors associated with T2DM have also been proposed to explain why T2DM is a risk factor for
171 liver fibrosis. These include insulin resistance, altered adipokine concentrations [16] and altered gut
172 microbiota composition [17]. Moreover, it has also been suggested that oral hypoglycaemic agents, such as
173 metformin, can affect circulating GDF-15 concentrations [18, 19]. Whether these factors explain the increase
174 in GDF-15 concentration in patients with T2DM and NAFLD remains uncertain. Therefore we aimed to test
175 a) if GDF-15 concentrations were a predictor of liver fibrosis and potentially involved in the association
176 between T2DM and liver fibrosis and b) what factors linked with T2DM are independently associated with,
177 and explain the variance in GDF-15 concentrations, in patients with NAFLD.

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179 **2. MATERIALS AND METHODS**

180 A total of 99 predominantly Northern European patients with NAFLD (age range of 20-77 years) were
181 studied to perform this secondary analysis of baseline characteristics of patients recruited to the INSYTE
182 (**I**nvestigation of **S**ynbiotic **T**reatment in NAFLD) trial (www.clinicaltrials.gov registered number
183 NCT01680640). These patients were recruited as described in detail previously [20, 21]. The trial design was
184 approved by the Southampton and South West Hampshire research ethics committee (12/SC/0614). All
185 patients gave their written informed consent.

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187 **2.1 Inclusion and exclusion criteria**

188 The inclusion and exclusion criteria for the INSYTE trial have been previously described in detail [20, 21].
189 Briefly, participants were aged >18 years with a diagnosis of NAFLD confirmed in secondary care, with
190 evidence of hepatic steatosis confirmed by via proton magnetic resonance spectroscopy (¹H-MRS) at
191 recruitment.

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193 **2.2 Anthropometric and biochemical measurements**

194 Anthropometric and biochemical measurements were collected as previously described [20, 21]. Body
195 composition was assessed by dual-energy x-ray absorptiometry (DEXA). Blood pressure was measured
196 using a Marquette Dash 300 monitor (GE Healthcare, Little Chalfont, Bucks, UK) as previously described
197 [21]. Handgrip strength was measured using a Jamar hand Dynamometer with participants seated and their
198 arms rested on the chair arms – data are presented as grip strength (kg). Fasting glucose, haemoglobin A1c
199 (HbA1c), fasting insulin, total cholesterol, high-density lipoprotein (HDL)-cholesterol, triglycerides, alanine
200 aminotransferase (ALT), aspartate aminotransferase (AST), adiponectin, leptin, high-sensitivity C-reactive
201 protein (hs-CRP), tumour necrosis factor- α (TNF α), interleukin (IL)-6, IL-8 and IL-10 concentrations were
202 measured in serum samples using commercially available kits according to the manufacturer's instructions.
203 Serum lipopolysaccharide (LPS) concentrations were measured as previously described [20]. Concentrations
204 of GDF-15, leptin and adiponectin were measured in fasting serum samples by the Cambridge Biochemical
205 Assay Laboratory, University of Cambridge. Serum GDF-15 quantification was done with antibodies and
206 standards from R&D Systems (R&D Systems - catalogue number DY957) and in accordance with

207 manufacturer's instructions and as previously described [18]. Estimated glomerular filtration rate (e-GFR)
208 was measured using the CKD-Epidemiology Collaboration (CKD-EPI) study equation [22]. Satiety
209 hormones, such as plasma ghrelin, gastric inhibitory polypeptide (GIP), glucagon-like peptide (GLP-1),
210 peptide YY (PYY) and pancreatic peptide (PP) concentrations were also measured as previously described
211 [21] using the MILLIPLEX®MAP Human Metabolic Hormone Panels Kit.

212

213 **2.3 Liver fat and vibration-controlled transient elastography measurements**

214 Liver fat and VCTE-derived kPa measurements were collected as previously described [20, 21]. In all
215 participants, the quantification of intra-hepatic fat content was undertaken via proton magnetic resonance
216 spectroscopy (¹H-MRS) (See supplementary material for method). Liver VCTE-derived kPa measurements
217 were assessed as a clinically recognised proxy measure of liver elasticity using the Echosens (Waltham, MA)
218 Fibroscan® by a trained clinician (ES). Results are expressed as the medians (IQRs) in kilo-pascals (kPa).
219 Liver VCTE-derived kPa measurements of ≥ 8.2 kPa and ≥ 9.7 kPa were used as validated proxy thresholds
220 for identification \geq F2 and \geq F3 fibrosis with the former having a AUROC of 0.77 (95%CI; 0.72, 0.82) for the
221 prediction of \geq F2 fibrosis (sensitivity and specificity = 71% and 70% respectively) as recently reported [23].
222 The technical description and examination procedures for liver VCTE-kPa measurements have also been
223 previously described [24].

224

225 **2.4 Appetite, hunger, and satiety assessment**

226 Assessment of patient appetite, hunger and satiety was done as previously described [20, 21]. See
227 supplementary material for further description of this methodology.

228

229 **2.5 Gut microbiota analyses – DNA extraction, sequencing and bioinformatics**

230 Gut microbiota DNA extraction from faecal samples, 16S amplicon sequencing and bioinformatic analyses
231 were performed as previously described [20, 21]. See supplementary material for further details of this
232 methodology.

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236 **2.6 Statistical analysis**

237 Data were analysed using Statistical Package for the Social Sciences (SPSS) Version 26.0 (New York, USA).

238 Data were tested for normality using the Shapiro-Wilk and Kolmogorov Smirnov tests and are presented as

239 means (SD) for normally distributed variables and medians and inter-quartile ranges (IQRs) for non-

240 normally distributed variables. Comparisons of continuous variables between groups were performed with

241 the unpaired Student t-test for normally distributed variables and the Mann-Whitney U test for non-normally

242 distributed variables, and differences in proportions were investigated using the chi-squared-test or the

243 Fisher's exact test as appropriate. Univariable associations between variables were investigated using the

244 Pearson's correlation for normally distributed or Spearman's rank correlation for non-normally distributed

245 variables. To test for the independence of associations between explanatory factors and serum GDF-15

246 concentrations, VCTE-measured liver kPa measurements or additional liver fibrosis biomarkers, factors were

247 entered into a multivariable linear regression model with either: a) logarithmically transformed GDF-15

248 concentrations; b) logarithmically transformed liver kPa measurements; c) Enhanced liver fibrosis (ELF)

249 scores; d) logarithmically transformed Fibrosis-4 (FIB-4) scores; e) logarithmically transformed AST to

250 platelet ratio index (APRI) scores or f) hepatic mitochondrial function (HMF) (as determined by the 13C-

251 ketoisocaproate breath test [13C-KICA BT]) as the outcome variable. Regression models were run with all

252 explanatory factors, or stepwise, to investigate the proportion of total variance in serum GDF-15

253 concentrations that could be explained by each individual explanatory factor. Binary logistic regression

254 modelling was used to investigate whether serum GDF-15 concentrations and/or T2DM status were

255 independently associated with \geq F2 and \geq F3 fibrosis (as indicated by the validated VCTE measurement of

256 \geq 8.2 and \geq 9.7 kPa respectively), to identify whether other liver fibrosis biomarkers were associated with \geq F2

257 and \geq F3 fibrosis and also to identify factors that were independently associated with high (\geq 1193.7 pg/ml)

258 serum GDF-15 concentrations. Goodness of fit for the models was tested with Hosmer-Lemeshow tests.

259 Sobel test statistics and p values were calculated to test the potential involvement of GDF-15 concentrations

260 in the association between T2DM and either \geq F2, or \geq F3 fibrosis separately according to [25]. Receiver-

261 operating characteristic (ROC) curve analysis for GDF-15 or HbA1c was performed to estimate areas under

262 the receiver-operating characteristic curves (AUROCs), as well as to estimate the best cut-off values

263 (Youden's index) to predict \geq F2 and \geq F3 fibrosis, or high serum GDF-15 concentrations. See supplementary

264 material for methods used for the statistical analysis of the gut microbiota. Where data were not available on
265 all 99 participants, the number of subjects included in the analysis for which there was complete data are
266 presented in the relevant table or figure legend.
267

268 RESULTS

269

270 3.1 Characteristics of participants

271 The mean (SD) age of the 99 patients with NAFLD (61 men, 38 women) included in the study was 50.9
272 (12.8) years. **Table 1** shows the characteristics of patients, stratified by T2DM status. In patients with
273 NAFLD and T2DM, liver VCTE-derived kPa measurements were significantly higher and there was a
274 greater prevalence of \geq F2 and \geq F3 liver fibrosis, (according to the previously validated VCTE thresholds of
275 \geq 8.2 kPa and \geq 9.7 kPa respectively) compared to counterparts without T2DM. Furthermore, FIB-4 scores
276 were significantly higher in patients with both NAFLD and T2DM. Fasting glucose and HbA1c
277 concentrations were also higher in patients with NAFLD and T2DM, whereas fasting insulin concentrations
278 were not different between groups. Twenty nine (69%) patients with T2DM were receiving metformin
279 treatment. In patients with NAFLD and T2DM, age, HOMA-IR, IL-8 and hs-CRP were also higher than in
280 those without T2DM whereas HMF was lower in patients with both NAFLD and T2DM. Notably, serum
281 GDF-15 concentrations were markedly higher in patients with NAFLD and T2DM than in those without
282 T2DM (**Table 1** and **Figure 1a**). Regarding gut microbiota composition, the relative abundance of the
283 Enterobacteriaceae family was greater in patients with NAFLD and T2DM than in those without T2DM
284 ($p=0.001$, data not shown). In patients with NAFLD and T2DM, fasting ghrelin concentrations were lower
285 and fasting GLP-1 concentrations were higher, compared to patients without T2DM (**Supplementary table**
286 **1**). Furthermore, following a breakfast challenge, ghrelin AUC was lower and GLP-1, PP and PYY AUCs
287 were higher in patients with NAFLD and T2DM, than in their counterparts without T2DM (**Supplementary**
288 **table 1**). However, neither fasted nor AUC values, for reported hunger, fullness, and satiety, were
289 significantly different in patients with or without T2DM (**Supplementary table 1**).

290

291 3.2 Serum GDF-15 concentrations predict liver fibrosis and are potentially involved in the association 292 between T2DM and liver fibrosis

293 In univariable analysis, higher serum GDF-15 concentrations were associated with higher liver VCTE-
294 derived kPa measurements (**Figure 1b**), and serum GDF-15 concentrations were higher in patients with \geq F2
295 fibrosis (**Figure 1c**). Both APRI and FIB-4 test scores were positively associated with the presence of \geq F2

296 and \geq F3 fibrosis independently of age and sex (**Supplementary table 2**). Similarly, serum GDF-15 was
297 significantly and positively associated with APRI, FIB-4 and ELF test scores (**Table 2**). Serum GDF-15
298 concentrations were associated with higher concentrations of fasting glucose (**Table 2**) and HbA1c (**Figure**
299 **1d**) ($r = 0.60$, and $r = 0.62$, respectively, $p < 0.00001$ for both). Serum GDF-15 concentrations were also
300 positively associated with age, BMI, total body fat percentage, HOMA-IR, AST, IL-6, IL-8, hs-CRP
301 concentrations and negatively with e-GFR, HMF and handgrip strength (**Table 2**).

302 Multivariable linear regression modelling was undertaken to investigate which factors were independently
303 associated with liver VCTE-derived kPa measurements as the outcome variable. In a regression model where
304 GDF-15, age, sex, total body fat, T2DM status, e-GFR and AST were entered as putative key explanatory
305 variables and liver kPa measurement as the outcome, only serum GDF-15 concentrations were independently
306 associated with liver kPa measurement [unstandardised β coefficient = 0.35 (95%CI 0.15- 0.56), $p = 0.001$
307 (model fit $R^2 = 0.261$; $p = 0.001$)] (**Supplementary table 3**). In this regression model, GDF-15 concentrations
308 alone explained 21% of the total variance in liver VCTE-derived kPa value. Intriguingly, upon removal of
309 GDF-15 from this model, T2DM status became independently associated with liver VCTE-derived kPa
310 measurement [β coefficient = 0.09 (0.18-0.17), $p = 0.015$], whereas age, sex, total body fat, e-GFR and AST
311 were not associated with liver VCTE-derived kPa measurement (model fit $R^2 = 0.152$; $p = 0.03$). Serum GDF-
312 15 concentrations were also positively and independently associated with ELF, FIB-4 and APRI scores and,
313 according to stepwise analysis, contributed the most towards the total variance of each liver fibrosis
314 biomarker (**Supplementary table 3**). Serum GDF-15 concentrations were not independently associated with
315 HMF in a model with the same combination of explanatory variables (data not shown).

316 Since we found that serum GDF-15 concentrations were independently associated with liver VCTE-derived
317 kPa measurements, and alone explained 21% of the variance in liver VCTE-derived kPa measurements, we
318 next tested whether GDF-15 concentrations and/or T2DM status could predict \geq F2 fibrosis, as determined by
319 the validated VCTE threshold of ≥ 8.2 kPa [23]. In a model that did not include serum GDF-15
320 concentrations, T2DM status was associated with \geq F2 fibrosis (**Table 3 – model 1**). However, when both
321 T2DM status and serum GDF-15 concentrations were added as covariates and \geq F2 fibrosis status was the
322 outcome, only GDF-15 concentration was associated with \geq F2 fibrosis (**Table 3 – model 2**). Furthermore, in
323 a fully adjusted model where T2DM, GDF-15 concentrations, age, sex, total body fat percentage, eGFR and

324 AST concentrations were entered as key covariates (identified from multivariable linear regression
325 modelling see **Supplementary table 3**) and \geq F2 fibrosis status was the outcome variable, only serum GDF-
326 15 concentrations were associated with \geq F2 fibrosis (**Table 3 – model 3**). Goodness of fit for the models was
327 tested with Hosmer-Lemeshow tests. A model that only included GDF-15 concentrations as the explanatory
328 variable showed excellent goodness of fit (chi squared statistic = 2.71, $p = 0.95$). Additionally, as HOMA-IR
329 was significantly higher in NAFLD patients with T2DM compared to those without T2DM and insulin
330 resistance may be an important factor in the relationship between T2DM and liver fibrosis, we explored
331 whether GDF-15 concentrations were associated with liver fibrosis severity, independently of HOMA-IR. In
332 a model where \geq F2 fibrosis status was the outcome and HOMA-IR, T2DM status and GDF-15
333 concentrations were the explanatory variables, only GDF-15 concentration (and not HOMA-IR or T2DM)
334 was associated with \geq F2 fibrosis [OR = 1.002 (1.001-1.003), $p = 0.004$, for each 1 pg/ml of GDF-15].
335 Furthermore, we found strikingly similar results for \geq F3 fibrosis status (data not shown but available from
336 the authors).

337 Given this result, we performed ROC curve analysis to assess the ability of GDF-15 concentrations to predict
338 the presence of \geq F2 fibrosis and to identify an optimal GDF-15 concentration cut-off for predicting \geq F2
339 fibrosis. Accordingly, the AUROC for the prediction of \geq F2 fibrosis was 0.75 (95%CI; 0.63-0.86, $p < 0.001$).
340 The Youden index (optimal cut-off) GDF-15 concentration was 1193.7 pg/ml with sensitivity, specificity,
341 positive predictive value (PPV) and negative predictive value (NPV) of 56.3%, 86.9%, 69.2% and 79.1%
342 respectively (**Figure 2**). We then repeated the ROC curve analysis using the higher validated liver kPa
343 threshold for \geq F3 fibrosis (≥ 9.7 kPa), as the binary outcome. As shown in **Supplementary figure 1**, these
344 results were remarkably similar to those obtained for \geq F2 fibrosis [AUROC 0.762 (95%CI 0.64-0.89),
345 $p < 0.0001$, optimal cut-off of serum GDF-15 1193.7 pg/ml; sensitivity 63.6%, specificity 83.1%, PPV 53.8%
346 and NPV 88.1%]. In order to assess whether circulating concentrations of GDF-15 were potentially involved
347 in the relationship between T2DM and either \geq F2 fibrosis or \geq F3 fibrosis, we next calculated Sobel test
348 statistics and p -values. These data suggested that GDF-15 was potentially involved in the associations
349 between T2DM and \geq F2 fibrosis as well as between T2DM and \geq F3 fibrosis (Sobel test statistics 2.90,
350 $p = 0.004$; and 2.71, $p = 0.007$, for \geq F2 fibrosis and \geq F3 fibrosis respectively).

351

352 **3.3 HbA1c levels are independently associated with, and can predict high GDF-15 concentrations in**
353 **patients with NAFLD**

354 Given that very little is known regarding the potential regulatory factors of elevated GDF-15 in patients with
355 NAFLD and T2DM, we next looked to identify the factors associated with T2DM that were independently
356 associated with GDF-15 concentrations. GDF-15 concentrations were higher in patients treated with
357 metformin, compared to those not receiving metformin ($p < 0.0001$) (**Figure 3**). In univariable analyses, we
358 did not find any significant associations between participant-reported measures of satiety and/or plasma
359 concentrations of satiety hormones with GDF-15 concentrations (**Supplementary table 4**). Similarly, none
360 of the measures of satiety (AUC values) were associated with GDF-15 concentrations except for GLP-1
361 AUC, which was positively associated with GDF-15 concentrations ($r = 0.30$, $p = 0.003$). However, this
362 association was no longer significant after controlling for metformin use ($r = 0.06$, $p = 0.57$). Within the faecal
363 microbiota, there was a greater relative abundance of the Enterobacteriaceae family in the high vs. the low
364 GDF-15 concentration tertile ($q = 0.003$). Similarly, according to univariable correlation analyses, serum
365 GDF-15 concentrations were associated with abundance of Enterobacteriaceae family ($r = 0.52$, $p < 0.0001$) in
366 faecal samples. However, in multivariable regression modelling, this family of bacteria was not
367 independently associated with GDF-15 concentrations (data not shown).

368 In regression modelling, the explanatory factors that were independently (all $p = 0.01$ or less) associated with
369 higher GDF-15 concentrations were as follows: higher HbA1c, older age, higher AST, metformin treatment,
370 higher hs-CRP and lower e-GFR (model fit $R^2 = 0.60$, $p < 0.00001$) (**Table 4**). Collectively, these factors
371 together explained 60% of the total variance in GDF-15 concentrations. None of the measures of appetite,
372 hunger and/or satiety were independently associated with GDF-15 concentrations (data not shown). As we
373 were able to explain a substantial proportion of the total variance in GDF-15 concentrations within the
374 present cohort, we undertook stepwise linear regression modelling to investigate the proportion of the
375 variance in GDF-15 concentrations that could be explained by each of the aforementioned independent
376 factors. In doing so, we found HbA1c alone explained 29.6% of the total variance in GDF-15 concentrations
377 (**Table 4, model 1**). The addition of age (**model 2**) led to a statistically significant increase in R^2 of 0.102
378 ($p < 0.001$), AST an increase in R^2 of 0.083 ($p < 0.001$) (**model 3**), metformin use an increase in R^2 of 0.062
379 ($p = 0.001$) (**model 4**), hs-CRP an increase in R^2 of 0.03 ($p = 0.013$) (**model 5**) and e-GFR an increase in R^2 of

380 0.028 (p=0.015) (**model 6**). Thus, the addition of each of these independent factors explained a further 10.2%
381 (age), 8.3% (AST), 6.2% (metformin treatment), 3.0% (hs-CRP) and 2.8% (e-GFR), respectively, compared
382 to the 29.6% of the total variance in GDF-15 concentrations, explained by HbA1c alone.

383 We next tested whether increased HbA1c concentration was associated with a high GDF-15 concentration
384 using a GDF-15 threshold of ≥ 1193.7 pg/ml that we had identified as the optimal cut-off for the prediction of
385 \geq F2 fibrosis (**Figure 2**). We carried out binary logistic regression modelling where, in the first model, only
386 HbA1c concentration was entered as a covariate and GDF-15 concentrations were entered as the binary
387 outcome [< 1193.7 pg/ml (n = 72) vs. ≥ 1193.7 pg/ml (n=27)]. In this regression model, higher HbA1c was
388 associated GDF-15 concentrations (OR, 1.07; 95%CI, 1.0-2.0; p<0.0001) (**Supplementary table 5 – model**
389 **1**). In the final adjusted model where HbA1c, age, metformin treatment, hs-CRP, AST and e-GFR were
390 entered as covariates, and GDF-15 concentrations were entered as the binary outcome, higher HbA1c,
391 metformin use, higher hs-CRP, higher AST and lower e-GFR were all independently associated with higher
392 GDF-15 concentrations (**model 2**). Next, we carried out a ROC curve analysis to assess whether HbA1c
393 predicted high GDF-15 concentrations. The AUROC for the prediction of GDF-15 concentrations was 0.83
394 (95%CI; 0.75-0.91) and the optimal cut-off HbA1c concentration was 42.5 mmol/mol (sensitivity,
395 specificity, PPV and NPV) were 85.2%, 76.4%, 57.5% and 93.2%, respectively) for the prediction of GDF-
396 15 concentrations (**Figure 4**).

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400 3. DISCUSSION

401 The novel findings in this study are that in patients with NAFLD, HbA1c concentrations explain a large
402 proportion (~30%) of the variance in GDF-15 concentrations and that circulating concentrations of GDF-15
403 may be involved in the known association between T2DM and liver fibrosis. This study provides the most
404 in-depth investigation of factors independently associated with serum GDF-15 concentrations in patients
405 with, and without, T2DM who have NAFLD, and also demonstrates that older age, higher HbA1c, higher
406 AST, higher hs-CRP, lower e-GFR and metformin use (but not gut microbiota composition, adipokines or
407 measures of satiety) were all independently associated with higher serum GDF-15 concentrations.
408 Collectively, these factors explained a large proportion (60%) of the total variance in GDF-15
409 concentrations. Moreover, ROC curve analysis also confirmed that HbA1c was a good predictor of high
410 GDF-15 concentrations.

411 Our findings that a serum GDF-15 concentration of 1193.7 pg/ml was the optimal threshold for predicting
412 \geq F3 fibrosis are consistent with other recent work carried out in an Asian NAFLD cohort where a serum
413 GDF-15 concentration of 1520 pg/ml was found to be the optimal threshold for predicting histologically
414 proven advanced fibrosis (\geq F3 liver fibrosis) [13]. Furthermore, data from a recent large multicentre
415 transcriptomics study identified hepatic GDF-15 expression as a key factor strongly and positively associated
416 with liver fibrosis severity in patients with NAFLD [10]. In addition to liver fibrosis, our findings that GDF-
417 15 concentrations were significantly increased in patients with NAFLD and T2DM compared to those
418 without T2DM, are consistent with various previous studies indicating that GDF-15 concentrations are
419 increased in patients with T2DM [7, 26, 27].

420 Importantly, a strength of our study is the significant proportion of patients with T2DM and NAFLD
421 (42.4%) and the wide range of HbA1c concentrations (27.0 to 95.0 mmol/mol). Considering the chronic
422 nature of NAFLD and that GDF-15 expression is stress-inducible, it is likely that a range of factors
423 commonly associated with T2DM and/or liver fibrosis are also associated with increasing GDF-15
424 concentrations. Considering this, we investigated multiple factors and found that, increased HbA1c
425 concentrations were most strongly associated with increased GDF-15 concentrations and that HbA1c
426 concentrations were a good predictor of high (\geq 1193.7 pg/ml) GDF-15 concentrations with an AUROC of
427 0.83 (95%CI; 0.75-0.91). Interestingly, the optimal cut-off of HbA1c concentration for predicting high GDF-

428 15 concentrations was 42.5 mmol/mol, which is remarkably similar to the threshold for diagnosing pre-
429 diabetes in patients. In addition to this, we found that HbA1c concentrations explained a large proportion
430 (~30%) of the total variance in circulating GDF-15 and were a good predictor of high GDF-15
431 concentrations, supporting our findings that GDF-15 concentrations may be involved in the relationship
432 between T2DM and liver fibrosis. These findings could suggest that chronic hyperglycaemia has role in
433 increasing the circulating concentrations of GDF-15 in patients with both NAFLD and T2DM. Interestingly,
434 administration of a high glucose load resulted in a rise in serum GDF-15 concentrations in both non-obese
435 and obese individuals suggesting a potential direct role of hyperglycaemia on increased circulating GDF-15
436 concentrations [28, 29]. Conversely, given the increased NAFLD severity observed in patients with
437 coexisting T2DM, it is also likely that an increased hepatic expression of GDF-15, due to hepatic
438 inflammation and/or fibrosis and exacerbated by the presence of T2DM, also contributes to elevations in
439 circulating concentrations of GDF-15. Furthermore, whilst a growing body of evidence suggests that GDF-
440 15 may have a pro-fibrogenic role within the liver [13, 14], others have found GDF-15 to be protective and
441 to ameliorate NASH and other metabolic disorders in mice [30, 31]. Consequently, further work should be
442 carried out to elucidate the functional role of GDF-15 in liver fibrosis in NAFLD. Additionally, research
443 should look to explore the potentially additive effects of hyperglycaemia, and other factors involved in the
444 T2DM milieu, on the expression and circulating concentrations of GDF-15.

445 Recent pre-clinical and clinical studies of T2DM suggest that GDF-15 expression is also increased by oral
446 metformin treatment and that the beneficial effects of metformin on weight loss (and associated
447 hyperglycaemia) may be mediated by metformin-induced GDF-15 acting centrally to suppress appetite [18,
448 19]. In our study we show for the first time that metformin treatment is associated with higher GDF-15
449 concentrations in patients with T2DM and NAFLD, and this association is independent of potential
450 confounding factors. However, in contrast to HbA1c, we found that metformin treatment explained very little
451 of the total variance in GDF-15 concentrations within our cohort (6% vs ~30% for HbA1c). Similarly, we
452 found that of the investigated inflammatory markers, only increased hs-CRP was independently associated
453 with increased GDF-15 concentrations. However, similar to metformin treatment, hs-CRP only explained a
454 small proportion (3%) of the total variance in GDF-15 concentrations. Furthermore, we found that changes in

455 the faecal microbiota, circulating LPS and adipokine concentrations and patient-reported appetite, hunger
456 and/or satiety were not independently associated with serum GDF-15 concentrations.

457 Our study has some limitations. Firstly, we used the validated VCTE-derived threshold of ≥ 8.2 kPa and ≥ 9.7
458 kPa as proxies for the identification of patients with $\geq F2$ and $\geq F3$ fibrosis respectively [23], instead of a liver
459 histology diagnosed fibrosis. That said, growing evidence indicates that liver VCTE has good diagnostic
460 accuracy for the identification of liver fibrosis in patients with NAFLD [32]. Furthermore, a recent large
461 study validated the use of a liver VCTE threshold of ≥ 8.2 kPa and ≥ 9.7 kPa as good diagnostic thresholds for
462 identifying $\geq F2$ (AUROC; 0.77, 95%CI; 0.72-0.82) and $\geq F3$ (AUROC; 0.80, 95%CI; 0.75-0.84) fibrosis
463 validated by histology [23]. Our study also utilised a relatively small cohort and further work should also be
464 carried out in larger cohorts with access to liver biopsy data to further investigate the role of circulating
465 GDF-15 in the relationship between T2DM and liver fibrosis. Whilst evidence does suggest that GDF-15
466 may have a pro-fibrogenic role within the liver and we found that HbA1c explains almost 30% of the
467 variance in GDF-15 concentrations, our findings showing that GDF-15 may be involved in the known
468 association between T2DM and liver fibrosis should be interpreted with caution. With the current study
469 design, we are unable to address causation and we suggest that further work is required to explore the
470 functional role of GDF-15 in the known association between T2DM and liver fibrosis in patients with
471 NAFLD. We also did not collect data on metformin treatment dosage or duration of treatment within the
472 current cohort and we are not able to investigate whether dose-or time-dependent effects exist between
473 metformin use and serum GDF-15 concentrations.

474 In conclusion, in patients with NAFLD and T2DM, GDF-15 concentrations predicted both $\geq F2$ and $\geq F3$ liver
475 VCTE determined fibrosis, and GDF-15 concentrations may be involved in the association between T2DM
476 and liver fibrosis in NAFLD. Furthermore, we explained a large proportion (~60%) of the variance in GDF-
477 15 concentrations and found that HbA1c alone explained almost 30% of that variance. Further investigations
478 are warranted to establish the causal or consequential role of GDF-15 in liver fibrosis and to further explore
479 the potential implementation of circulating GDF-15 as a biomarker for liver fibrosis in patients with NAFLD
480 and T2DM.

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483 **CRedit Authorship Contributions:**

484 **Josh Bilson**, BSc(Hons), MRes, PhD candidate (Formal analysis: Lead; Writing - original draft:
485 Lead; Writing - review & editing: Lead; Data curation: supporting). **Eleonora Scorletti**, MD, PhD
486 (Formal analysis: supporting, Data curation: supporting; Investigation: supporting) **Laure B.**
487 **Bindels**, PhD (Formal analysis: supporting; Data curation: supporting). **Paul R Afolabi**, PhD
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491 Validation: Supporting; Writing – original draft: Supporting; Writing – review & editing:
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496 Supporting; Visualization: Supporting; Writing – original draft: Supporting; Writing – review &
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601 **Figure/Table legends**

602 **Figure 1 – Differences in serum GDF-15 concentrations by type 2 diabetes status, predicted liver**
603 **fibrosis severity and scatter plots showing the association between serum GDF-15 concentrations and**
604 **both haemoglobin A1c and liver stiffness measurements (as assessed by vibration-controlled transient**
605 **elastography [VCTE]).**

606 **A)** shows the differences in serum GDF-15 concentrations (logarithmically transformed) between NAFLD
607 patients with and without coexisting type 2 diabetes. **B)** shows the scatter plot for the association of serum
608 GDF-15 concentrations with liver VCTE measurements (kPa). **C)** shows the differences in serum GDF-15
609 concentrations between NAFLD patients with <F2 or ≥F2 fibrosis according to the validated VCTE
610 measurement threshold of ≥ 8.2 kPa as a proxy for the identification of ≥F2 fibrosis. **D)** shows the scatter
611 plot for the association of serum GDF-15 with HbA1c concentrations. Data are presented as mean ± SD.
612 Associations are Spearman’s rank correlation coefficients. Sample size **A** and **D** n=99; **B** and **C** n=93.

613 Abbreviations: VCTE; vibration-controlled transient elastography, GDF-15; growth differentiation factor-15
614 and T2DM; type 2 diabetes mellitus

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616 **Figure 2 – Receiver-operating characteristic curve of serum GDF-15 concentrations for ≥F2 fibrosis**
617 **(≥8.2 kPa as measured by VCTE).** Sample size n = 93.

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619 **Figure 3 – Differences in serum GDF-15 concentrations in patients with NAFLD not receiving vs.**
620 **receiving metformin treatment.** Data are presented as means ± SD.

621

622 **Figure 4 – Receiver-operating characteristic curve of HbA1c concentrations for a high serum GDF-15**
623 **concentration.** State variable was serum GDF-15 concentrations < 1193.7 pg/ml vs. ≥ 1193.7 pg/ml (0 and 1
624 respectively). Sample size n = 99.

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629 **Table 1 – Characteristics of patients with NAFLD stratified by pre-existing type 2 diabetes status.**

630 Data presented as means ± SDs or medians (inter-quartile ranges).

631 † Cross-tab. Pearson chi-squared test. & data was only available for no T2DM n = 52 and T2DM n = 41 ^s data
632 was only available for no T2DM n = 52 and T2DM n = 32, # data was only available for; no T2DM n = 50
633 and T2DM n = 38.

634 Abbreviations: T2DM, Type 2 diabetes mellitus, BMI: body mass index, DEXA: dual energy x-ray
635 absorptiometry, HOMA-IR: Homeostatic model assessment of insulin resistance, HDL: high-density
636 lipoprotein, AST: aspartate aminotransferase, ALT: alanine transaminase, MRS: magnetic resonance
637 spectroscopy, 13C-KICA BT: 13C-ketoisocaproate breath test, VCTE: vibration-controlled transient
638 elastography, APRI: AST to platelet Ratio Index, FIB-4: Fibrosis-4, ELF: Enhanced Liver Fibrosis GDF-15;
639 growth differentiation factor-15, TNFα: tumour necrosis factor-α, IL: interleukin, hs-CRP; high-sensitivity
640 C-reactive protein, LPS; Lipopolysaccharide, e-GFR: estimated glomerular filtration rate.

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645 **Table 2 – Univariable linear associations with serum GDF-15 concentrations.**

646 Sample size, n=99, ‡Samples size n=93, &Sample size 82, # sample size 88

647 †Spearman’s rank correlation coefficients.

648 Abbreviations: BMI: body mass index, DEXA: dual energy x-ray absorptiometry, HDL: high-density
649 lipoprotein, AST: aspartate aminotransferase, ALT: alanine transaminase, MRS: magnetic resonance
650 spectroscopy, 13C-KICA BT: 13C-ketoisocaproate breath test, VCTE: vibration-controlled transient
651 elastography, APRI: AST to platelet ratio index, FIB-4: Fibrosis-4, ELF: enhanced liver fibrosis, GDF-15:
652 growth differentiation factor-15, TNF α : tumour necrosis factor- α , IL: interleukin, hs-CRP: high-sensitivity
653 C-reactive protein, e-GFR: estimated glomerular filtration rate.

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656 **Table 3 – Binary logistic regression analysis showing that only serum GDF-15 concentrations and**
657 **T2DM status were significant independent predictors of a predicted liver fibrosis severity of \geq F2 (as**
658 **measured by VCTE).**

659 Model 1 only contains T2DM status. ^aModel is adjusted for age, sex, total body fat percentage, e-GFR and
660 AST concentrations. Note - GDF-15 ORs are for each 1pg/ml of GDF-15. Dependent variable was liver
661 VCTE measurements <8.2 kPa vs. \geq 8.2 kPa (0 and 1 respectively) as a proxy threshold for the identification
662 of \geq F2 fibrosis. Sample size n=93.

663 Abbreviations: T2DM, type 2 diabetes mellitus, AST: aspartate aminotransferase, VCTE: vibration-
664 controlled transient elastography, GDF-15; growth differentiation factor-15 and e-GFR: estimated
665 glomerular filtration

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668 **Table 4 – Multivariable linear regression models explaining variance in serum GDF-15 concentrations.**

669 Sample size, n=99. In all regression models, the dependent variable was the logarithmically transformed
670 serum GDF-15 concentrations (pg/ml).

671 NB: R-square (R^2 or the coefficient of determination) is a statistical measure in a regression model that
672 determines the proportion of variance in the dependent variable that can be explained by the independent
673 variables.

674 Abbreviations: AST; aspartate aminotransferase, hs-CRP; high-sensitivity C-reactive protein, eGFR;
675 estimated glomerular filtration rate and CI; confidence interval.

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684 **Table 1 – Characteristics of patients with NAFLD stratified by pre-existing type 2 diabetes status.**

Variables	Without T2DM (n = 57)	With T2DM (n = 42)	685	
			p-value	686
Age (yrs)	48.7 ± 14.2	53.8 ± 10.1	0.04	687
Sex (male) (n,%) †	38 (66.7%)	23 (54.8%)	0.23	
Smoking history (no) (n, %) †	53 (93%)	34 (81%)	0.12	688
Systolic blood pressure (mmHg)	133.7 (19.5)	134.4 (27.5)	0.8	689
Diastolic blood pressure (mmHg)	74.4 ± 8.3	73.9 ± 10.7	0.82	
BMI (kg/m ²)	32.3 (6.4)	34.9 (6.8)	0.06	690
DEXA lean body mass (kg)	64.5 ± 13.2	62 ± 10.6	0.25	691
DEXA total body fat (%)	33.8 (10.2)	36.7 (12.2)	0.22	
Handgrip strength (kg)	36.7 (25.8)	31.2 (19.5)	0.05	692
Fasting glucose (mmol/l)	5.3 (1.0)	8.1 (3.3)	<0.0001	693
Haemoglobin A1c (mmol/mol)	35.0 (5.5)	59.5 (23)	<0.0001	
Fasting insulin (mIU/L) [§]	14.2 (9.7)	13.7 (9.0)	0.94	694
HOMA-IR ^{&}	3.5 (2.7)	5.4 (5.1)	<0.001	695
Metformin use (yes) (n, %) †	0 (0%)	29 (69%)	<0.0001	
Triglycerides (mmol/l)	1.8 (1)	1.8 (1.2)	0.27	696
Total cholesterol (mmol/l)	5.2 (1.4)	4.4 (1.3)	0.001	697
HDL cholesterol (mmol/l)	1.2 (0.4)	1.2 (0.3)	0.61	
AST (IU/l)	34.0 (22.0)	38.0 (31.5)	0.54	698
ALT (IU/l)	56.0 (45.0)	59.0 (41.8)	0.8	699
MRS-measured liver fat (%)	23.7 (34.8)	27.0 (24.1)	0.871	
13C-KICA BT (cPDR over 1h-%)	14.5 ± 3.7	12.6 ± 3.2	0.008	700
Liver VCTE (kPa) ^{&}	6.0 (3.1)	8.0 (4.8)	0.01	701
Liver VCTE ≥ 8.2 kPa, (yes) (%)†	12 (23.2%)	20 (48.8%)	0.01	
Liver VCTE ≥ 9.7 kPa, (yes) (%)†	7 (13.5%)	15 (36.6%)	0.009	702
APRI ^{&}	0.4 (0.3)	0.4 (0.5)	0.44	703
FIB-4 ^{&}	0.9 (1.2)	1.2 (1.1)	0.02	
ELF [#]	6.9 ± 0.4	7.0 ± 0.3	0.06	704
GDF-15 (pg/ml)	640.3 (332.5)	1271.0 (902.1)	<0.0001	705
Adiponectin (µg/ml)	4.9 (2.4)	3.8 (2.6)	0.31	
Leptin (ng/ml) [#]	20.0 (32.9)	24.4 (31.2)	0.77	706
TNFα (pg/ml) [#]	12.9 (5.5)	10.4 (4.1)	0.15	707
IL-6 (pg/ml)	2.6 (1.6)	2.6 (2.0)	0.26	
IL-8 (pg/ml)	13.8 (7.2)	17.8 (10.5)	0.01	708
IL-10 (pg/ml)	0.8 (0.4)	0.7 (0.4)	0.89	709
hs-CRP (mg/l)	2.0 (3.0)	4.0 (5.3)	0.003	
LPS (EU/ml)	0.2 (0.1)	0.1 (0.1)	0.51	710
e-GFR (ml/min/1.73 m ²)	90.0 (12.3)	90.0 (9.8)	0.68	711

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718 **Table 2 – Univariable linear associations with serum GDF-15 concentrations.**

719	Variables	Correlation coefficient(s)	p-value
720	Age (yrs)	0.44	<0.00001
721	Systolic blood pressure (mmHg)	0.05	0.65
	Diastolic blood pressure (mmHg)	0.06	0.56
722	BMI (kg/m ²) †	0.29	0.003
	DEXA lean body mass (kg)	-0.15	0.16
723	DEXA total body fat (%)	0.22	0.03
724	Handgrip strength (kg)	-0.31	0.002
	Fasting glucose (mmol/l) †	0.60	<0.00001
725	Haemoglobin A1c (mmol/mol) †	0.62	<0.00001
	Fasting insulin (mIU/L) ^{&}	0.11	0.32
726	HOMA-IR ^{&}	0.41	<0.0001
	Triglycerides (mmol/l) †	0.20	0.84
727	Total cholesterol (mmol/l) †	-0.29	0.003
728	HDL cholesterol (mmol/l) †	-0.01	0.94
	AST (IU/l) †	0.29	0.003
729	ALT (IU/l) †	0.13	0.19
	MRS-measured liver fat (%) †	-0.10	0.35
730	¹³ C-KICA BT (cPDR over 1h-%) †	-0.38	<0.001
731	Liver VCTE (kPa) †‡	0.41	<0.0001
	APRI †‡	0.28	0.007
732	FIB-4 †‡	0.53	<0.00001
	ELF † [#]	0.53	<0.00001
733	Adiponectin (µg/ml) [#]	-0.02	0.85
734	Leptin (ng/ml) [#]	0.16	0.14
	TNFα (pg/ml) †	0.06	0.57
735	IL-6 (pg/ml) †	0.25	0.02
	IL-8 (pg/ml) †	0.33	0.001
736	IL-10 (pg/ml) †	0.19	0.07
	hs-CRP (mg/l) †	0.32	0.001
737	LPS (EU/ml)	0.12	0.25
738	e-GFR (ml/min/1.73 m ²) †	-0.24	0.018

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751 **Table 3 – Binary logistic regression analysis showing that only serum GDF-15 concentrations and**
752 **T2DM status were significant independent predictors of a predicted liver fibrosis severity of \geq F2 (as**
753 **measured by VCTE).**

Variables	OR (95%CI)	<i>P</i> -value
Model 1		
T2DM status	3.18 (1.3-7.72)	0.01
Model 2		
T2DM status	1.16 (0.39-3.48)	0.79
Serum GDF-15 (pg/ml)	1.002 (1.001-1.003)	0.001
Model 3^a		
T2DM status	1.30 (0.4-4.2)	0.72
Serum GDF-15 (pg/ml)	1.002 (1.001-1.003)	0.006

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785 **Table 4 – Multivariable linear regression models explaining variance in serum GDF-15 concentrations.**

Independent variables	R-square (R²) of regression model	R² change	p-value
Model 1	0.296	0.296	<0.00001
HbA1c (mmol/mol)			
Model 2	0.397	0.102	<0.001
HbA1c (mmol/mol) and age (yrs)			
Model 3	0.480	0.083	<0.001
HbA1c (mmol/mol), age (yrs) and AST (IU/l)			
Model 4	0.542	0.062	0.001
HbA1c (mmol/mol), age (yrs), AST (IU/l) and metformin use (yes)			
Model 5	0.572	0.030	0.013
HbA1c (mmol/mol), age (yrs), AST (IU/l), metformin use (yes) and hs-CRP (mg/l)			
Model 6	0.60	0.028	0.015
HbA1c (mmol/mol), age (yrs), AST (IU/l), metformin use (yes), hs-CRP (mg/l) and e-GFR (ml/min/1.73 m ²)			

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