Cytokeratin-18 and enhanced liver fibrosis scores in type 1 and type 2 diabetes and effects of two different insulins

Arun Sanyal,[1] Kenneth Cusi,[2] Mark L Hartman,[3] Shuyu Zhang,[3] Edward J Bastyr,[3,4] Juliana M Bue-Valleskey,[3] Annette M Chang,[3] Axel Haupt,[3] Scott J Jacober,[3] Robert J Konrad,[3] Qianyi Zhang,[3] Byron J Hoogwerf[3]

[1]Virginia Commonwealth University Medical Center, Richmond, Virginia, USA
[2]Division of Endocrinology, Diabetes and Metabolism, University of Florida, Gainesville, Florida, USA
[3]Lilly Corporate Center, Eli Lilly and Company, Indianapolis, Indiana, USA
[4]Division of Endocrinology & Metabolism, Indiana University School of Medicine, Indianapolis, Indiana, USA

Correspondence to
Dr Byron J Hoogwerf, Lilly Corporate Center, Eli Lilly and Company, Indianapolis, IN 46285,USA; byronhoogwerf@gmail.com

Accepted 23 October 2017

ABSTRACT

Data on cytokeratin-18 (K-18) and enhanced liver fibrosis (ELF) score in insulin-treated diabetes patients with non-alcoholic fatty liver disease (NAFLD) are limited. This study analyzed phase III data comparing basal insulin peglispro (BIL) and insulin glargine in type 1 (T1D), and type 2 diabetes (T2D) (insulin-naïve and insulin-treated). Alanine aminotransferase (ALT), K-18, ELF scores and liver fat content (LFC), measured by MRI, were obtained longitudinally. Baseline K-18 (U/L) was higher in T2D (range: 207–247) than T1D (range: 148–183), correlated with ALT in all populations (r (range) 0.264–0.637, p<0.05), but with LFC only in T2D (r (range) 0.474–0.586, p<0.05). K-18 increased significantly from baseline in BIL-treated, but not glargine-treated patients. Change from baseline (CFB) K-18 was significantly correlated with CFB in ALT in BIL-treated T2D populations. Baseline ELF scores were higher in T2D (range: 9.12–9.20) than T1D (range: 8.24–8.36), correlated with ALT in T1D only (0.209, p<0.05), and not correlated with LFC in any population. ELF scores increased significantly from baseline in BIL-treated but not glargine-treated patients. There were no correlations between CFB in LFC and ELF score at week 52 in any treatment group/population. In all BIL-treated populations, CFB in ALT and CFB in ELF score at week 52 were positively correlated. These data characterize associations of K-18 and ELF score with ALT and LFC in insulin-treated patients with T1D and T2D. Hepatopreferential insulins may be associated with increased K-18 and ELF scores but mechanisms and clinical significance are unknown. ClinicalTrials.gov identifiers are NCT01481779, NCT01435616, NCT01454284 and NCT01582451.

Significance of this study

What is already known about this subject?

- Cytokeratin-18 (K-18) and enhanced liver fibrosis (ELF) scores have been used to assess disease severity and outcomes in patients with liver disease.
- Studies of K-18 and ELF scores come largely from patients with hepatitis, non-alcoholic steatohepatitis and other inflammatory diseases, but little is known about the value of these biomarkers in patients with diabetes.
- Diabetes is often associated with more liver fat and hepatic fibrosis, but differences between type 1 diabetes (T1D) and type 2 diabetes (T2D), or effects of insulin treatment are unknown.

What are the new findings?

- This is the largest data set on K-18 and ELF scores in patients with T1D and T2D treated with insulin.
- K-18 and ELF scores were higher in T2D than T1D; K-18 and ELF scores increased with basal insulin peglispro (BIL), but not insulin glargine in both T1D and T2D.
- In BIL-treated patients with T2D, changes from baseline in K-18 and ELF scores correlated with changes in ALT.
- In BIL-treated patients with T1D and T2D, changes from baseline in K-18 correlated with changes in liver fat.
- These data characterize the relationships among liver fat, commonly used liver biomarkers and these novel liver biomarkers in insulin-treated patients with T1D and T2D; no comparably large and complete data set has been previously published.

To cite: Sanyal A, Cusi K, Hartman ML, *et al*. *J Investig Med* Published Online First: [*please include* Day Month Year]. doi:10.1136/jim-2017-000609

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is prevalent in patients with type 2 diabetes (T2D).[1 2] T2D is associated with increased risk to develop cirrhosis in NAFLD.[3] Potential contributions or protective roles of glucose-lowering therapies on NAFLD progression is under investigation.[4] The role of insulin therapy in development or progression of NAFLD is not well characterized. Insulin effects on hepatic triglyceride accumulation and de novo lipogenesis are confounded by associations with free fatty acid (FFA) flux and insulin resistance.[5 6] Rates of hepatic lipogenesis in rat

Significance of this study

How might these results change the focus of research or clinical practice?

- Hepatopreferential insulins, such as BIL, may be associated with increased K-18 and ELF scores, but mechanisms and clinical significance are unknown.
- When novel biomarkers are used in clinical practice, clinicians need to know the utility of such biomarkers in the patient cohort in whom they were obtained. These data help demonstrate both the value and limitations of these biomarkers in insulin-treated patients with diabetes.
- There are increasing research data on interventions to reduce non-alcoholic fatty liver disease and non-alcoholic steatohepatitis. These data will inform the future use of these novel biomarkers with such interventions in patients with diabetes.

models increased with FFA flux independent of increased plasma insulin concentrations and intrahepatic insulin signaling.[6]

Recently, we reported that patients with type 1 diabetes (T1D) and T2D treated with a hepatopreferential insulin due to reduced peripheral action (basal insulin peglispro (BIL)) have higher liver fat content (LFC) and alanine aminotransferase (ALT) than those treated with insulin glargine. No severe drug-induced liver injury was apparent with BIL treatment. The changes in LFC differed among the T1D and T2D populations. In insulin-naïve patients with T2D, LFC decreased with glargine but was unchanged with BIL. In patients with T1D and T2D previously treated with basal insulin, LFC was unchanged with glargine but increased with BIL.[7] LFC decreases in insulin-naïve patients with T2D treated with glargine were similar to those reported by Tang *et al* in insulin-naïve patients with T2D.[8] The clinical implications of different effects of BIL and glargine on LFC and ALT are uncertain. The potential impact of insulin on other biomarkers of possible liver cell apoptosis and fibrosis, or other key features of NAFLD, has not been reported.

Evaluation of the progression of NAFLD to non-alcoholic steatohepatitis (NASH) and cirrhosis requires histological assessment of a liver biopsy. Liver biopsy is limited by its invasive nature leading to efforts to develop non-invasive methods for disease assessment. MR assessment of hepatic steatosis is sensitive and corresponds to histological assessment.[9] MR-based methods do not currently provide an assessment of liver injury. Cytokeratin-18 (K-18) is a fragment of cellular cytokeratin released following apoptosis.[10 11] Circulating K-18 levels have been extensively evaluated as a biomarker for NASH[10–17] and track changes in ALT in NASH clinical trials.[15] K-18 has been associated with biopsy-determined fibrosis in a carefully studied pediatric cohort.[17] Enhanced liver fibrosis (ELF) score is a panel of circulating biomarkers used to evaluate hepatic fibrosis in NAFLD,[18 19] and is sensitive to change over time.[20] Much of the data on K-18[21–23] and ELF scores[14 24–26] have come from patients with liver disease other than NAFLD (eg, hepatitis C). There are limited data about K-18 and ELF scores in patients with diabetes with NAFLD, and even less data in insulin-treated patients with diabetes, especially longitudinal data on effects of insulin treatment.

Herein, we provide information on these biomarkers in patients with T1D and T2D treated with two different basal insulin regimens: glargine and BIL.[27] These patients are a cohort from the phase III BIL development program.[27] LFC and liver enzyme data for this cohort have been published.[7 28] Because of changes observed in ALT and LFC,[7] stored samples were analyzed for K-18 (M30 fragment) and ELF score. Data on these two biomarkers are the focus of this report and all analyses should be considered exploratory. Objectives of this paper were to evaluate: baseline K-18 and ELF scores in large T1D and T2D cohorts; relationships of K-18 and ELF to ALT and LFC; longitudinal impact of glargine versus BIL on these measures. We discuss these findings in the context of other studies that have evaluated K-18 and ELF scores in patients with liver disease.

METHODS

Participants

Data from four phase III BIL studies were analyzed.[29–32] These analyses present results from three populations: T1D,[29 30] insulin-naïve patients with T2D[31] and previously insulin-treated patients with T2D.[32] (Clinicaltrials.gov identifiers: NCT01481779; NCT01435616; NCT01454284; NCT01582451). Data from T1D studies were integrated. Due to differences in the two T2D populations (insulin-naïve vs insulin-treated) data from these cohorts were analyzed separately. Study durations ranged from 52 to 78 weeks. All studies were conducted in accordance with the International Conference on Harmonization Guidelines for Good Clinical Practice and the Declaration of Helsinki. All patients signed informed consent documents, and the protocols and consent documents were approved by local ethical review boards prior to study initiation.

Patients with obvious signs, symptoms or diagnosis of liver disease (excluding NAFLD), or liver enzyme elevations >2.5X upper limit of normal (ULN) for ALT or aspartate aminotransferase (AST), or ≥2X ULN for total bilirubin were excluded. Comprehensive information on these populations has been published.[7 28–32]

Study procedures

LFC was evaluated by MRI in subsets of patients in the studies[29–32] as previously described.[7] We considered 6% to be the ULN for LFC measured by MRI, based on a published study reporting the 95th percentile for LFC was 5.56% in a large population of individuals with no risk factors for hepatic steatosis assessed by MR spectroscopy.[33]

For ALT, the ULN was determined using age-specific and gender-specific reference ranges by Covance (Princeton, New Jersey, USA). Serum caspase-cleaved K-18 fragment levels were measured in EDTA plasma using the M30-Apoptosense ELISA (PEVIVA AB, Stockholm, Sweden) in assays conducted by Pacific Biomarkers (Seattle, Washington, USA). ELF score components (tissue inhibitor of matrix metalloproteinase (TIMP-1), hyaluronic acid (HA), aminoterminal peptide of procollagen III (P3NP)) were measured in serum using the ADVIA Centaur immunoassay system (Siemens Healthcare Diagnostics, Tarrytown, New York, USA) using

Table 1 Baseline demographics and characteristics*

	T1D integrated		T2D insulin-naïve		T2D previously treated with insulin	
	Glargine (n=64)	BIL (n=118)	Glargine (n=59)	BIL (n=117)	Glargine (n=53)	BIL (n=110)
Age, years	37.4±12.2	40.1±12.3	58.4±10.1	59.4±9.6	62.1±10.2	61.6±8.6
Male, n (%)	40 (62.5)	67 (56.8)	36 (61.0)	72 (61.5)	31 (58.5)	66 (60.0)
Weight, kg	79.1±17.8	79.0±14.8	93.9±19.5	94.8±17.8	93.5±16.9	93.0±16.0
Body mass index, kg/m^2	26.4±4.0	26.7±3.6	32.5±5.4	32.9±5.1	32.2±5.1	32.0±5.0
Diabetes duration, years	15.4±10.7	17.7±12.2	11.6±7.0	12.0±7.1	12.6±7.4	12.6±6.7
Lipid-lowering medication, n (%)	10 (15.6)	24 (20.3)	41 (69.5)	84 (71.8)	37 (69.8)	76 (69.1)
HbA1c, %	7.9±1.1	7.9±1.2	8.3±1.1	8.3±0.9	7.4±0.8	7.4±0.8
K-18, U/L†	183.0±15.8	148.2±11.6	246.9±27.8	223.8±19.3	219.2±23.9	207.1±16.1
ELF score†	8.24±0.10	8.36±0.07	9.14±0.11	9.14±0.08	9.12±0.10	9.20±0.07
ALT, IU/L	20±11	21±11	31±21	30±15	26±12	26±13
AST, IU/L	21±7	22±9	26±13	25±12	24±10	23±8
LFC (%)†	3.41±0.41	3.04±0.30	12.73±1.14	13.25±0.81	9.96±1.09	10.39±0.75
Triglycerides, mg/dL	1.01±0.60	1.06±0.66	1.70±0.74	1.96±1.07	1.75±0.89	1.77±0.87
LDL-cholesterol, mg/dL	2.79±0.64	2.74±0.77	2.30±0.70	2.28±0.90	2.37±0.79	2.43±0.86
HDL-cholesterol, mg/dL	1.57±0.42	1.61±0.39	1.20±0.27	1.20±0.32	1.18±0.33	1.21±0.3

No statistically significant treatment differences in any of the listed baseline parameters within each population.
Data are mean±SD except where indicated.
*Adapted from Cusi *et al*.[7]
†Least squares mean±SE.
n, number of patients randomized; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BIL, basal insulin peglispro; ELF, enhanced liver fibrosis; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; K-18, cytokeratin-18; LDL, low-density lipoprotein; LFC, liver fat content; T1D, type 1 diabetes; T2D, type 2 diabetes.

reagents from a single lot in assays conducted by Covance. ELF score was calculated as: ELF=2.278+0.851×ln(HA in ng/mL)+0.751×ln(P3NP in ng/mL)+0.394×ln(TIMP-1 ng/mL).[34]

Statistical analyses

Analyses were conducted on the full analysis set population, which had both baseline and ≥1 postbaseline MRI scan for LFC. For treatment comparisons for baseline characteristics, Fisher's exact test was used for categorical and two-sample t-tests for continuous outcomes. A mixed-model repeated measures model was used to analyze continuous variables collected at multiple post-treatment time-points. Spearman's correlation analyses were performed to assess relationships among biomarkers (K-18 and ELF score), LFC and ALT.

Proportions of patients meeting the following prespecified criteria were summarized: K-18 change from baseline (CFB) ≥50 U/L, K-18 CFB ≥100 U/L, K-18 CFB ≥100 U/L with K-18 level ≥600 U/L, ELF score CFB ≥1 and ELF score CFB ≥2. Cut-off values were defined prior to availability of results by the authors based on literature review to identify proportions of patients with potentially clinically meaningful changes.[12 15 35–41] Treatment comparisons were performed using Fisher's exact test (for populations from a single study) and Cochran-Mantel-Haenszel test (for integrated analysis populations) with study as a stratification factor.

Analyses were performed for each study population separately using SAS V.9.1 or higher (SAS Institute, Cary, North Carolina, USA). All tests were conducted at a two-sided α level of 0.05.

RESULTS

Baseline characteristics

Baseline characteristics were similar among treatment groups for all three populations (table 1). A more complete list of baseline characteristics for these populations has been published.[7 28]

Cytokeratin-18

Baseline K-18 was nominally lower in T1D versus T2D (table 1). There were no statistically significant CFB in K-18 over time in glargine-treated patients in all populations (figure 1). In contrast, there were significant increases from baseline K-18 in BIL-treated patients at multiple time-points, and significant between-treatment differences at multiple time-points in all populations (figure 1).

Significantly more BIL-treated versus glargine-treated patients had K-18 elevations ≥50 U/L in T1D at weeks 26 and 52, in insulin-naïve T2D at weeks 52 and 78 and in previously insulin-treated T2D at weeks 26 and 52 (table 2). Similarly, significantly more BIL-treated vs glargine-treated patients had K-18 elevations ≥100 U/L in T1D at week 26, and in both T2D populations at weeks 26 and 52 (table 2). Finally, significantly more BIL-treated versus glargine-treated patients had K-18 elevations ≥100 U/L to ≥600 U/L in previously insulin-treated T2D patients at week 26 (table 2).

Baseline LFC and baseline K-18 were positively correlated in both T2D populations but not in T1D (table 3). Baseline

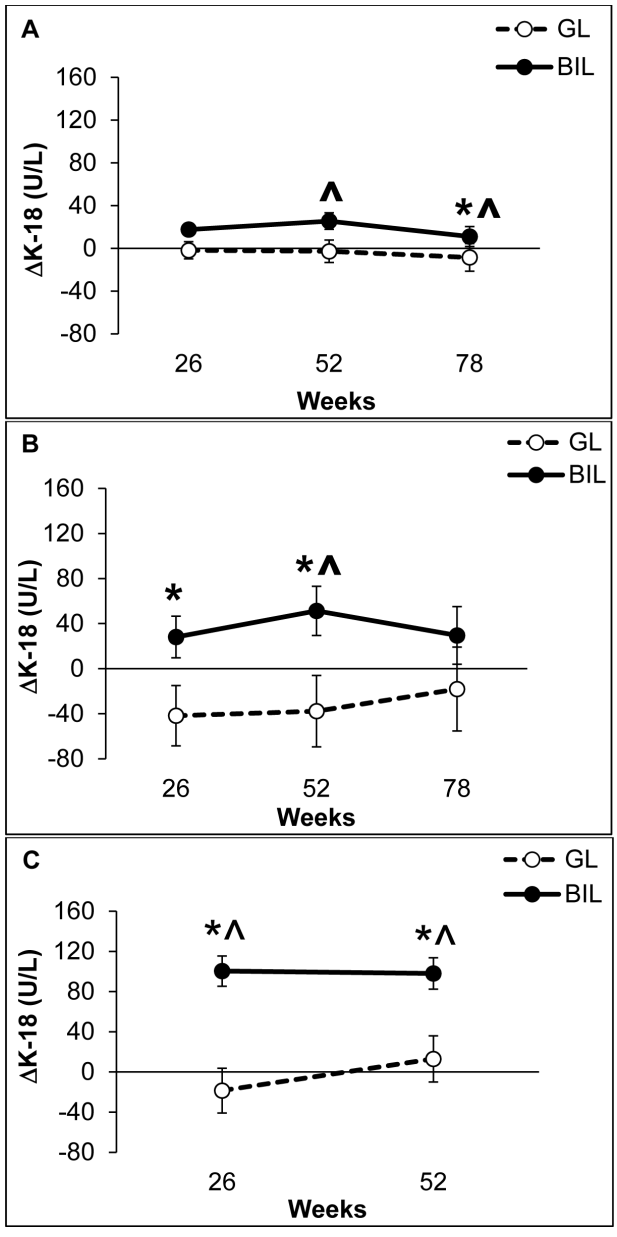

Figure 1 Plasma keratin-18 M30 fragment (U/L). (A) Type 1 diabetes. (B) Type 2 diabetes, insulin-naïve. (C) Type 2 diabetes, previously treated with insulin. Data are least squares mean±SE for the change from baseline. *p<0.05 for between-treatment comparisons; ^p<0.05 for within-treatment comparisons. BIL, basal insulin peglispro; GL, insulin glargine.

ALT and baseline K-18 were positively correlated in all populations. In BIL-treated patients in all populations, and in glargine-treated patients in the T2D insulin-naïve population, change in LFC and change in K-18 at week 52 were positively correlated. In BIL-treated and glargine-treated patients in both T2D populations, change in ALT and change in K-18 at week 52 were positively correlated (table 3).

ELF score

Baseline ELF scores were nominally lower in T1D versus T2D (table 1). There were no significant increases from baseline in ELF score over time in glargine-treated patients in all populations (figure 2). In contrast, there were significant increases from baseline ELF score in BIL-treated patients at multiple time-points in all populations, and significant between-treatment differences at both weeks 26 and 52 in previously insulin-treated patients with T2D (figure 2).

Baseline LFC and baseline ELF score were not correlated in any population (table 3). Baseline ALT and baseline ELF score were positively correlated in T1D only (table 3). In all populations, there were no correlations between change in LFC and change in ELF score at week 52 in any treatment group (table 3). In BIL-treated patients only, in all populations, change in ALT and change in ELF score at week 52 were positively correlated (table 3). Whereas there were nominally more BIL patients with ELF score CFB ≥1, there were no statistically significant treatment differences in any population at any time-point in proportions of patients achieving an ELF score change ≥1 (table 4). In all populations, no patients in either treatment group had a change of ELF score ≥2 at any time point (data not shown).

DISCUSSION

This study provides new information on K-18 and ELF scores in several different insulin-treated diabetes cohorts, including T1D[29 30] in whom there are currently no published data, and two distinct T2D cohorts—insulin-naïve[31] and previously insulin-treated.[32] There are only limited data on effects of insulin on these biomarkers, and no data on effects of a hepatopreferential insulin. Longitudinal data in a large group of patients with diabetes has not been previously reported. Even in the absence of hard clinical liver outcomes or liver biopsies, this dataset adds to the understanding of K-18 and ELF score as biomarkers of liver disease in insulin-treated patients with diabetes. Herein, we discuss key findings from these studies and put these data into the context of current literature.

BIL has different effects on LFC than glargine.[7] In insulin-naïve patients with T2D, glargine reduced LFC while BIL treatment was associated with no change. In previously insulin-treated patients with T2D, glargine had no effect while LFC increased with BIL.[7] Similar results were seen in serum triglycerides, suggesting differences in lipid flux between the two insulins and different patient types.[42]

Several studies have demonstrated a relationship between K-18 and liver injury in NAFLD.[10–17] Circulating K-18 represents a fragment of the molecule that is released from apoptotic cells. In NASH, there is increased apoptosis and thus an increase in K-18 provides presumptive evidence of an increased likelihood of tissue injury and steatohepatitis. In the current analyses, baseline K-18 was higher in T2D than T1D (table 1). Mean values of ~200 U/L observed in the T2D populations are similar to the 'normal' and 'not NASH' (simple steatosis) groups in liver biopsy study by Wieckowska *et al*,[11] lower than baseline values in non-diabetes patients with NAFLD in the PIVENS trial (>400 U/L)[15] and in the study by Chan *et al* (>300 U/L).[43] We estimated the proportion of patients who might have steatohepatitis, based on a K-18 cut-off value of ≥250 U/L[35] at baseline, to be 24%, 41% and 46% for T1D, T2D insulin-treated and T2D insulin-naïve, respectively. At baseline K-18 values

Table 2 Proportion of patients achieving plasma K-18 change ≥50 and ≥100 U/L

	26 Weeks			52 Weeks			78 Weeks		
	GL	BIL	P Value*	GL	BIL	P Value*	GL	BIL	P Value*
Type 1 diabetes									
N	60	114		58	103		38	74	
K-18 change ≥50 U/L	6 (10.0)	28 (24.6)	0.031	7 (12.1)	28 (27.2)	0.024	5 (13.2)	12 (16.2)	0.671
K-18 change ≥100 U/L	1 (1.7)	14 (12.3)	0.017	2 (3.4)	13 (12.6)	0.062	3 (7.9)	9 (12.2)	0.491
K-18 change ≥100 U/L and K-18 ≥600 U/L	0 (0.0)	0 (0.0)	–	0 (0.0)	2 (1.9)	0.318	1 (2.6)	1 (1.4)	0.630
Type 2 diabetes, insulin-naïve									
N	51	106		48	99		18	38	
K-18 change ≥50 U/L	6 (11.8)	27 (25.5)	0.060	7 (14.6)	30 (30.3)	0.044	2 (11.1)	17 (44.7)	0.016
K-18 change ≥100 U/L	3 (5.9)	20 (18.9)	0.032	1 (2.1)	20 (20.2)	0.002	1 (5.6)	11 (28.9)	0.079
K-18 change ≥100 U/L and K-18 ≥600 U/L	1 (2.0)	5 (4.7)	0.665	0 (0.0)	5 (5.1)	0.173	1 (5.6)	2 (5.3)	>0.999
Type 2 diabetes, previously treated with insulin									
N	47	103		45	97		–	–	–
K-18 change ≥50 U/L	4 (8.5)	51 (49.5)	<0.001	12 (26.7)	48 (49.5)	0.011	–	–	–
K-18 change ≥100 U/L	1 (2.1)	36 (35.0)	<0.001	7 (15.6)	35 (36.1)	0.017	–	–	–
K-18 change ≥100 U/L and K-18 ≥600 U/L	0 (0.0)	11 (10.7)	0.018	1 (2.2)	11 (11.3)	0.104	–	–	–

Data are presented as n (%).
*P values are for between-treatment comparisons at each time-point.
N, number of patients randomized; BIL, basal insulin peglispro; GL, insulin glargine; K-18, cytokeratin-18; T1D, type 1 diabetes; T2D, type 2 diabetes.

positively correlated with ALT in all three cohorts, but with LFC only in T2D, consistent with previous reports.[36]

After 52 weeks of BIL treatment, mean K-18 increased significantly in all populations. The magnitude of the mean increase in K-18 from baseline with BIL was greatest in patients with T2D who switched from another basal insulin to BIL (98.0 U/L), least in T1D (25.5 U/L) and intermediate in insulin-naïve T2D (51.3 U/L). Similarly, increases in K-18 with BIL treatment to ≥600 U/L with at least a 100 U/L CFB were more common in previously insulin-treated T2D (11.3%) than insulin-naïve patients with T2D (5.1%); such changes were observed in only ∼2% of glargine-treated patients with T2D. Changes in K-18 and ALT were significantly correlated in both BIL-treated T2D populations. Increases in K-18 and ALT were observed with BIL in insulin-naïve patients with T2D despite there being no statistically significant CFB in LFC.[7 31] Increases in both K-18 and ALT with BIL were greater in previously insulin-treated patients with T2D in whom statistically significant increases in LFC (∼5%) were observed.[7 32] As previously reported,[7]

Table 3 Correlations between K-18 and ELF score with LFC and with ALT

		Baseline LFC versus baseline factor	Baseline ALT versus baseline factor		Change in LFC versus change in factor at week 52	Change in ALT and change in factor at week 52
	Factor	r	r	Treatment	r	r
Type 1 diabetes						
	K-18	0.077	0.264*	GL	0.007	0.235
				BIL	0.362*	0.050
	ELF	0.126	0.209*	GL	−0.106	0.175
				BIL	−0.036	0.545*
Type 2 diabetes, insulin-naïve						
	K-18	0.474*	0.637*	GL	0.375*	0.623*
				BIL	0.374*	0.437*
	ELF	−0.138	0.021	GL	−0.162	−0.178
				BIL	0.153	0.298*
Type 2 diabetes, previously treated with insulin						
	K-18	0.586*	0.539*	GL	0.235	0.296*
				BIL	0.380*	0.657*
	ELF	−0.010	0.098	GL	−0.138	0.047
				BIL	0.160	0.358*

*P<0.05.
r, Spearman's correlation coefficient; BIL, basal insulin peglispro; GL, insulin glargine; K-18, cytokeratin-18; ELF, enhanced liver fibrosis score; LFC, liver fat content; T1D, type 1 diabetes; T2D, type 2 diabetes.

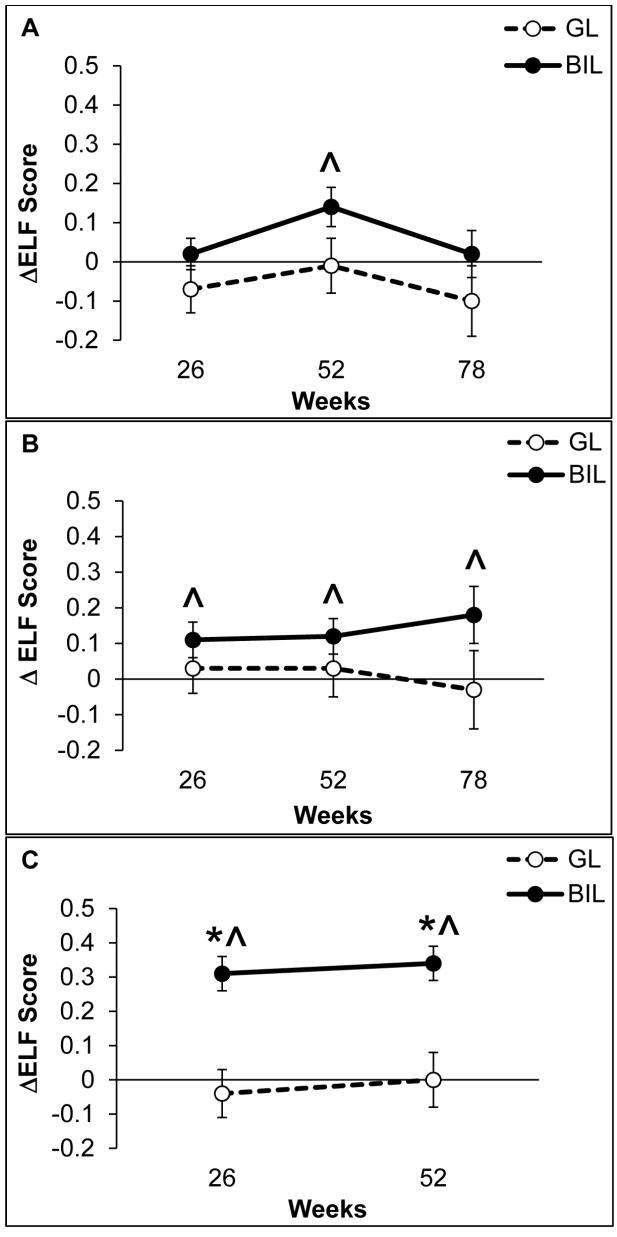

Figure 2 Enhanced liver fibrosis (ELF) score over the course of the study. (A) Type 1 diabetes. (B) Type 2 diabetes, insulin-naïve. (C) Type 2 diabetes, previously treated with insulin. Data are least squares mean±SE for the change from baseline. *p<0.05 for between-treatment comparisons; ^p<0.05 for within-treatment comparisons. BIL, basal insulin peglispro; GL, insulin glargine.

higher LFC with BIL versus glargine treatment may reflect the fact that BIL suppresses lipolysis to a lesser degree than glargine.[44] Switching from an insulin that potently suppresses lipolysis (glargine) to one with a weaker effect on lipolysis (BIL) may result in increased flux of FFA to the liver that are esterified into hepatic triglyceride. In the absence of withdrawal from a conventionally acting basal insulin, the weaker antilipolytic effect of BIL does not appear to increase LFC.[7] It is possible that even in the absence of increased LFC, increased FFA flux to the liver may cause some hepatocyte apoptosis as suggested by increased K-18 and ALT in BIL-treated insulin-naïve patients with T2D. In insulin-resistant patients with NAFLD, increased flux of FFA to the liver has been proposed as a mechanism for activation of inflammatory pathways that may promote cellular dysfunction and lipoapoptosis, leading to the development of NASH.[45] However, a direct effect of insulin on inflammatory pathways in the liver is not supported by rat studies in which intraperitoneal insulin was associated with less of an inflammatory response as measured by less macrophage infiltration and lower levels of reactive oxygen species than subcutaneous insulin.[46]

Chalasani *et al*[3] summarized the utility of K-18 in their 2012 Practice Guidelines, by characterizing K-18 as a 'promising biomarker for identifying steatohepatitis' but added 'it is premature to recommend in routine clinical practice'. They noted that although many studies reported associations between elevated K-18 levels and NASH,[3] the wide variety of proposed cut-points and differences in sensitivity and specificity limited the ability of K-18 to clearly discriminate NASH from NAFLD. The meta-analysis of nine studies by Musso *et al* in which K-18 was used to identify NASH reported cut-offs between 121.6 and 479 U/L.[47] The Edinburgh study in patients with T2D[48] analyzed variables associated with K-18. Median (range) K-18 values (102 (29–933) U/L) were lower than in the current report (table 2). They also reported higher K-18 values in patients with hepatic steatosis and increased serum triglycerides. Whether the higher K-18 values in patients with T2D in the current study are a function of longer disease duration, effects of insulin or differences in study cohorts is uncertain.

The ELF score is derived from P3NP, HA and TIMP-1, which all play a role in fibrogenesis and tissue remodeling. Several studies have demonstrated that the ELF score correlates with fibrosis stage assessed by liver biopsy in several liver diseases[49 50] and elevated ELF scores have been associated with clinical outcomes.[39] Most of these data were in patients with hepatitis C. There are less data on patients with NAFLD, especially patients with diabetes. It is therefore reasonable to assume that an increase in ELF score represents increased fibrosis, although there are only limited longitudinal data on the sensitivity of the ELF score to change with fibrosis progression. In the current study, baseline ELF scores were higher in T2D than T1D (table 1). Mean ELF scores in our T2D populations fall well within ranges associated with increased risk for clinical outcomes. Parkes *et al* reported that ELF scores between 8.34 and 10.425 were associated with more clinical outcomes than lower values.[39] In the ELF scores from nine studies by Xie *et al*, ELF scores between 8.5 and 10.18 discriminated patients with fibrosis scores >2.[50] The Edinburgh study reported ELF scores in patients with T2D. Mean scores were nominally higher in patients on insulin versus diet or oral agents, with values comparable to those in the current study.[48] Fagan *et al* studied ELF scores with biopsy data. ELF scores ≥9.8 had a sensitivity of 74.4% and specificity 92.4% for detecting advanced fibrosis.[49] In the current study, the estimated percentage of patients with baseline ELF score ≥9.8 is 3%, 19% and 21% for T1D, T2D insulin-treated and T2D insulin-naïve, respectively. Thus, mean scores for all time periods in T2D in the current study (range: 9.10–9.52) fall within ranges associated with increased fibrosis and clinical risk. Mean ELF scores in T1D were consistently lower in our study. The current data will

Table 4 Proportion of patients achieving ELF score changes ≥1

	26 Weeks			52 Weeks			78 Weeks		
	GL	BIL	p Value*	GL	BIL	p Value*	GL	BIL	p Value*
Type 1 diabetes									
N	60	114		58	105		38	76	
ELF score change ≥1	0 (0.0)	5 (4.4)	0.081	1 (1.7)	9 (8.6)	0.089	1 (2.6)	4 (5.3)	0.520
Type 2 diabetes, insulin-naïve									
N	51	106		47	99		19	39	
ELF score change ≥1	1 (2.0)	10 (9.4)	0.105	2 (4.3)	6 (6.1)	>0.999	0 (0.0)	4 (10.3)	0.292
Type 2 diabetes, previously treated with insulin									
N	48	104		47	101		–	–	–
ELF score change ≥1	1 (2.1)	9 (8.7)	0.172	3 (6.4)	14 (13.9)	0.269	–	–	–

Data are presented as n (%).
No patients in any group at any time-point had an ELF score change ≥2.
*P values are for between-treatment comparisons at each time-point.
N, number of patients randomized; BIL, basal insulin peglispro; GL, insulin glargine; K-18, cytokeratin-18; ELF, enhanced liver fibrosis score; LFC, liver fat content; T1D, type 1 diabetes; T2D, type 2 diabetes.

help inform future studies involving patients with diabetes with NAFLD.

There are potential confounders to the ELF scores, especially in diabetes. In addition to effects of age, gender and time of day reported by Lichtinghagen *et al* in healthy subjects,[38] Mine *et al* reported that mean serum HA was twofold higher in patients with diabetes than non-diabetes controls and significant correlations of HA with fasting plasma glucose, HbA1c, glycated albumin, triglycerides and body mass index (all r ≥0.7, all p<0.0001).[51] When ELF scores are used in studies that include patients with diabetes, these variables should be considered.

Strengths of this study include serial data collection in well-characterized insulin-treated cohorts that included routine liver enzyme determinations and measures of LFC over 52–78 weeks. The post hoc analyses of both K-18 and the components of the ELF score were assayed using a single machine and assay materials from a single lot to minimize any assay-related variation. Limitations include absence of liver biopsy data or clinical outcomes. The smaller treatment differences with ELF score compared with K-18 are potentially consistent with the longer time frames required for development of hepatic fibrosis[52] or with an absence of any effect of BIL on hepatic fibrosis. Considerations related to K-18 and ELF scores and any effects on liver pathology remain hypothetical. Absence of non insulin-treated controls limits the ability to assess the 'natural history' of these biomarkers in similar patients with diabetes. Thus, it is uncertain whether these increases in LFC, ALT, K-18 and ELF score reflect a higher risk of steatohepatitis with BIL treatment.

Information on the intersection of liver enzymes and novel soluble biomarkers in insulin-treated patients with diabetes is very limited. Data comparing effects of a conventional insulin and a hepatopreferential insulin on these biomarkers have not been previously reported. The current data characterize associations of K-18 and ELF score with ALT and LFC over time in patients with T1D and T2D treated with insulin. In particular, these data suggest that hepatopreferential insulins may be associated with increased K-18 and ELF scores in comparison to glargine, although the mechanisms and clinical significance are unknown. Future trials that include serial biopsy data, longitudinal clinical outcome data or perhaps additional imaging measures, such as MR elastography, will help to refine understanding of the value of these biomarkers in evaluation of NAFLD in patients with diabetes.

Acknowledgements Writing support was provided by Michelle Carey, PhD, of INC Research/inVentiv Health Clinical.

Contributors AS, KC, MLH, SZ and BJH contributed to data analysis and interpretation, and in reviewing and editing the manuscript. SZ and QZ designed and conducted the statistical analyses, and participated in the interpretation of the results and writing the manuscript. AMC, EJB, SJJ and JMB-V contributed to the design and medical oversight of trials, data analysis and interpretation and reviewing and editing of the manuscript. AH contributed to design of the MRI studies, data analysis and interpretation, and in reviewing and editing the manuscript. All authors approved the final version of the article, including the authorship list.

Funding The studies and writing of this paper were funded in full by Eli Lilly and Company.

Competing interests AS has stock options in Genfit, is President of Sanyal Biotechnologies, has served as a consultant to Merck, Eli Lilly and Company, Bristol Myers, Novartis, AbbVie, AstraZeneca, Gilead, Intercept, Genfit, Zafgen, Enanta, Immuron, Galmed, Nitto Denko, Durect, Ikaria, Echosens and Salix. His institution receives grant support from Intercept, Merck, AstraZeneca, Bristol Myers and Gilead. EJB was an employee and shareholder of Eli Lilly and Company during the trials and results analyses; he is currently a consultant to Viacyte. MLH, SZ, JMB-V, AMC, AH, SJJ, RJK, QZ and BJH are employees and stockholders of Eli Lilly and Company.

Patient consent Obtained.

Ethics approval The data from the four studies came from the larger data sets of multicenter and international studies. Thus, there were multiple ethics committees across sites and countries involved for each of these four studies. We believe there are too many to list individually in this manuscript. Each of the four studies from which these data derive have been previously published (and are referenced in this manuscript) and the role of the ethical review boards in each study is noted in the primary manuscripts.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1 Doycheva I, Cui J, Nguyen P, *et al*. Non-invasive screening of diabetics in primary care for NAFLD and advanced fibrosis by MRI and MRE. *Aliment Pharmacol Ther* 2016;43:83–95.

2 Bril F, Cusi K. Nonalcoholic fatty liver disease: the new complication of type 2 diabetes mellitus. *Endocrinol Metab Clin North Am* 2016;45:765–81.
3 Chalasani N, Younossi Z, Lavine JE, *et al*. The diagnosis and management of non-alcoholic fatty liver disease: practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. *Hepatology* 2012;55:2005–23.
4 Than NN, Newsome PN. A concise review of non-alcoholic fatty liver disease. *Atherosclerosis* 2015;239:192–202.
5 Gill JM, Sattar N. Hepatic VLDL overproduction: is hyperinsulinemia or insulin resistance the culprit? *J Clin Endocrinol Metab* 2011;96:2032–4.
6 Vatner DF, Majumdar SK, Kumashiro N, *et al*. Insulin-independent regulation of hepatic triglyceride synthesis by fatty acids. *Proc Natl Acad Sci* 2015;112:1143–8.
7 Cusi K, Sanyal AJ, Zhang S, *et al*. Different effects of basal insulin peglispro and insulin glargine on liver enzymes and liver fat content in patients with type 1 and type 2 diabetes. *Diabetes Obes Metab* 2016;18(Suppl 2):50–8.
8 Tang A, Rabasa-Lhoret R, Castel H, *et al*. Effects of insulin glargine and liraglutide therapy on liver fat as measured by magnetic resonance in patients with type 2 diabetes: a randomized trial. *Diabetes Care* 2015;38:1339–46.
9 Permutt Z, Le TA, Peterson MR, *et al*. Correlation between liver histology and novel magnetic resonance imaging in adult patients with non-alcoholic fatty liver disease - MRI accurately quantifies hepatic steatosis in NAFLD. *Aliment Pharmacol Ther* 2012;36:22–9.
10 Tamimi TI, Elgouhari HM, Alkhouri N, *et al*. An apoptosis panel for nonalcoholic steatohepatitis diagnosis. *J Hepatol* 2011;54:1224–9.
11 Wieckowska A, Zein NN, Yerian LM, *et al*. In vivo assessment of liver cell apoptosis as a novel biomarker of disease severity in nonalcoholic fatty liver disease. *Hepatology* 2006;44:27–33.
12 Feldstein AE, Wieckowska A, Lopez AR, *et al*. Cytokeratin-18 fragment levels as noninvasive biomarkers for nonalcoholic steatohepatitis: a multicenter validation study. *Hepatology* 2009;50:1072–8.
13 Feldstein AE, Alkhouri N, De Vito R, *et al*. Serum cytokeratin-18 fragment levels are useful biomarkers for nonalcoholic steatohepatitis in children. *Am J Gastroenterol* 2013;108:1526–31.
14 Mayo MJ, Parkes J, Adams-Huet B, *et al*. Prediction of clinical outcomes in primary biliary cirrhosis by serum enhanced liver fibrosis assay. *Hepatology* 2008;48:1549–57.
15 Vuppalanchi R, Jain AK, Deppe R, *et al*. Relationship between changes in serum levels of keratin 18 and changes in liver histology in children and adults with nonalcoholic fatty liver disease. *Clin Gastroenterol Hepatol* 2014;12:2121–30.
16 Yilmaz Y, Dolar E, Ulukaya E, *et al*. Soluble forms of extracellular cytokeratin 18 may differentiate simple steatosis from nonalcoholic steatohepatitis. *World J Gastroenterol* 2007;13:837–44.
17 Mandelia C, Collyer E, Mansoor S, *et al*. Plasma Cytokeratin-18 level as a novel biomarker for liver fibrosis in children with nonalcoholic fatty liver disease. *J Pediatr Gastroenterol Nutr* 2016;63:181–7.
18 Guha IN, Parkes J, Roderick P, *et al*. Noninvasive markers of fibrosis in nonalcoholic fatty liver disease: Validating the European Liver Fibrosis Panel and exploring simple markers. *Hepatology* 2008;47:455–60.
19 Friedrich-Rust M, Rosenberg W, Parkes J, *et al*. Comparison of ELF, FibroTest and FibroScan for the non-invasive assessment of liver fibrosis. *BMC Gastroenterol* 2010;10:103.
20 Mudaliar S, Henry RR, Sanyal AJ, *et al*. Efficacy and safety of the farnesoid X receptor agonist obeticholic acid in patients with type 2 diabetes and nonalcoholic fatty liver disease. *Gastroenterology* 2013;145:574–82.
21 Fernandes FF, Ferraz ML, Andrade LE, *et al*. Enhanced liver fibrosis panel as a predictor of liver fibrosis in chronic hepatitis C patients. *J Clin Gastroenterol* 2015;49:235–41.
22 Guéchot J, Trocmé C, Renversez JC, *et al*. Independent validation of the Enhanced Liver Fibrosis (ELF) score in the ANRS HC EP 23 Fibrostar cohort of patients with chronic hepatitis C. *Clin Chem Lab Med* 2012;50:693–9.
23 Kwok R, Tse YK, Wong GL, *et al*. Systematic review with meta-analysis: non-invasive assessment of non-alcoholic fatty liver disease--the role of transient elastography and plasma cytokeratin-18 fragments. *Aliment Pharmacol Ther* 2014;39:254–69.
24 Irvine KM, Wockner LF, Shanker M, *et al*. The enhanced liver fibrosis score is associated with clinical outcomes and disease progression in patients with chronic liver disease. *Liver Int* 2016;36:370–7.
25 Kim BK, Kim HS, Park JY, *et al*. Prospective validation of ELF test in comparison with Fibroscan and FibroTest to predict liver fibrosis in Asian subjects with chronic hepatitis B. *PLoS One* 2012;7:e41964.
26 Swanson S, Ma Y, Scherzer R, *et al*. Association of HIV, Hepatitis C Virus, and Liver fibrosis severity with the enhanced liver fibrosis score. *J Infect Dis* 2016;213:1079–86.
27 Jacober SJ, Prince MJ, Beals JM, *et al*. Basal insulin peglispro: overview of a novel long-acting insulin with reduced peripheral effect resulting in a hepato-preferential action. *Diabetes Obes Metab* 2016;18(Suppl 2):3–16.
28 Cusi K, Sanyal AJ, Zhang S, *et al*. NAFLD prevalence and its metabolic associations in patients with type 1 diabetes and type 2 diabetes. *Diabetes Obes Metab* 2017;19:1630–4.
29 Garg S, Dreyer M, Jinnouchi H, *et al*. A randomized clinical trial comparing basal insulin peglispro and insulin glargine, in combination with prandial insulin lispro, in patients with type 1 diabetes: IMAGINE 1. *Diabetes Obes Metab* 2016;18(Suppl 2):25–33.
30 Bergenstal RM, Lunt H, Franek E, *et al*. Randomized, double-blind clinical trial comparing basal insulin peglispro and insulin glargine, in combination with prandial insulin lispro, in patients with type 1 diabetes: IMAGINE 3. *Diabetes Obes Metab* 2016;18:1081–8.
31 Davies MJ, Russell-Jones D, Selam JL, *et al*. Basal insulin peglispro versus insulin glargine in insulin-naïve type 2 diabetes: IMAGINE 2 randomized trial. *Diabetes Obes Metab* 2016;18:1055–64.
32 Buse JB, Rodbard HW, Trescoli Serrano C, *et al*. Randomized clinical trial comparing basal insulin peglispro and insulin glargine in patients with type 2 diabetes previously treated with basal insulin: IMAGINE 5. *Diabetes Care* 2016;39:92–100.
33 Szczepaniak LS, Nurenberg P, Leonard D, *et al*. Magnetic resonance spectroscopy to measure hepatic triglyceride content: prevalence of hepatic steatosis in the general population. *Am J Physiol Endocrinol Metab* 2005;288:E462–E468.
34 Siemens. ADVIA Centaur Enhanced Liver Fibrosis (ELF) test specifications. 2016 https://static.healthcare.siemens.com/siemens_hwem-hwem_ssxa_websites-context-root/wcm/idc/groups/public/@global/documents/download/mdaw/mtc2/~edisp/xc1_elf_spec_sheet-00046572.pdf (accessed 2 Nov 2016).
35 Chen J, Zhu Y, Zheng Q, *et al*. Serum cytokeratin-18 in the diagnosis of non-alcoholic steatohepatitis: a meta-analysis. *Hepatol Res* 2014;44:854–62.
36 Cusi K, Chang Z, Harrison S, *et al*. Limited value of plasma cytokeratin-18 as a biomarker for NASH and fibrosis in patients with non-alcoholic fatty liver disease. *J Hepatol* 2014;60:167–74.
37 Lavine JE, Schwimmer JB, Van Natta ML, *et al*. Effect of vitamin E or metformin for treatment of nonalcoholic fatty liver disease in children and adolescents: the TONIC randomized controlled trial. *JAMA* 2011;305:1659–68.
38 Lichtinghagen R, Pietsch D, Bantel H, *et al*. The Enhanced Liver Fibrosis (ELF) score: normal values, influence factors and proposed cut-off values. *J Hepatol* 2013;59:236–42.
39 Parkes J, Roderick P, Harris S, *et al*. Enhanced liver fibrosis test can predict clinical outcomes in patients with chronic liver disease. *Gut* 2010;59:1245–51.
40 Sanyal AJ, Chalasani N, Kowdley KV, *et al*. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N Engl J Med* 2010;362:1675–85.
41 Shen J, Chan HL, Wong GL, *et al*. Non-invasive diagnosis of non-alcoholic steatohepatitis by combined serum biomarkers. *J Hepatol* 2012;56:1363–70.
42 Ginsberg H, Cariou B, Orchard T, *et al*. Lipid changes during basal insulin peglispro, insulin glargine, or NPH treatment in six IMAGINE trials. *Diabetes Obes Metab* 2016;18:1089–92.
43 Chan WK, Sthaneshwar P, Nik Mustapha NR, *et al*. Limited utility of plasma M30 in discriminating non-alcoholic steatohepatitis from steatosis--a comparison with routine biochemical markers. *PLoS One* 2014;9:e105903.
44 Mudaliar S, Henry RR, Ciaraldi TP, *et al*. Reduced peripheral activity leading to hepato-preferential action of basal insulin peglispro compared with insulin glargine in patients with type 1 diabetes. *Diabetes Obes Metab* 2016;18(Suppl 2):17–24.
45 Cusi K. Role of obesity and lipotoxicity in the development of nonalcoholic steatohepatitis: pathophysiology and clinical implications. *Gastroenterology* 2012;142:711–25.
46 Dal S, Jeandidier N, Schaschkow A, *et al*. Portal or subcutaneous insulin infusion: efficacy and impact on liver inflammation. *Fundam Clin Pharmacol* 2015;29:488–98.
47 Musso G, Gambino R, Cassader M, *et al*. Meta-analysis: natural history of non-alcoholic fatty liver disease (NAFLD) and diagnostic accuracy of non-invasive tests for liver disease severity. *Ann Med* 2011;43:617–49.
48 Morling JR, Fallowfield JA, Williamson RM, *et al*. Non-invasive hepatic biomarkers (ELF and CK18) in people with type 2 diabetes: the Edinburgh type 2 diabetes study. *Liver Int* 2014;34:1267–77.
49 Fagan KJ, Pretorius CJ, Horsfall LU, *et al*. ELF score ≥9.8 indicates advanced hepatic fibrosis and is influenced by age, steatosis and histological activity. *Liver Int* 2015;35:1673–81.
50 Xie Q, Zhou X, Huang P, *et al*. The performance of enhanced liver fibrosis (ELF) test for the staging of liver fibrosis: a meta-analysis. *PLoS One* 2014;9:e92772.

51 Mine S, Okada Y, Kawahara C, *et al*. Serum hyaluronan concentration as a marker of angiopathy in patients with diabetes mellitus. *Endocr J* 2006;53:761–6.

52 Singh S, Allen AM, Wang Z, *et al*. Fibrosis progression in nonalcoholic fatty liver vs nonalcoholic steatohepatitis: a systematic review and meta-analysis of paired-biopsy studies. *Clin Gastroenterol Hepatol* 2015;13:643–54.