Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Letter to the Editor: Do biomarkers of COVID-19 severity simply reflect a stress response in type 2 diabetes: Biomarker response to hypoglycemia☆,☆☆

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To the Editor

Type 2 diabetes (T2D) is associated with high risk for acquiring SARS-Cov-2 infection, severe disease, acute respiratory distress syndrome and increased mortality [1,2] A recent publication reported five predictive COVID-19 severity markers that included CCL17 which was expressed in low levels in severe/critical patients at an early phase of infection; IFNλ, IL6, IP10, and CXCL9 values surged and then dropped suddenly before the development of severe disease requiring oxygen support [3]. This suggested that CCL17 may be a useful first triage marker and the other reported proteins may help to predict severe disease onset [3].

However, it is unknown whether T2D patients already reflect this pattern of biomarkers at baseline, indicative of a stress response, and whether they change further in response to a hypoglycemic insult, or whether these protein biomarkers are COVID-19 infection specific.

Previous reports have demonstrated that hypoglycemia has also been associated with worse outcomes in hospitalized SARS infected patients. A retrospective analysis looking at the relationship between fasting blood glucose levels with death, in patients with SARS, showed that both hyperglycemia and hypoglycemia were independent predictors for death in SARS [4]. Case reports from Turkey demonstrated that 3 COVID-19 patients undergoing hemodialysis who were treated with hydroxychloroquine (HCQ) developed hypoglycemia after HCQ treatment for COVID-19 [5]. Moreover, an Indian study of patients with T2D revealed that the COVID-19 lockdown has been associated with increased risk of hypoglycemia in patients with T2DM, especially those receiving sulfonylureas (SU), insulin and/or HCQ, and especially in patients with associated co-morbidities [6]. Therefore, based on the above evidence, we hypothesized that an 'induced hypoglycemic condition' might be an ideal stress condition to elucidate the 'immuno-biomarkers' for severe outcome in COVID-19. To address this, analysis of these cytokines was undertaken in T2D and controls subjected to severe hypoglycemic stress.

A case-control parallel study in T2D ($n = 23$) and controls (n = 23) was performed in the Diabetes Centre at Hull Royal Infirmary as previously described [7] (Table 1). "All subjects were Caucasian, aged 40–70 years. The duration of diabetes was <10 years and all T2D subjects were on a stable dose of medication (metformin, statin and/or angiotensin converting enzyme inhibitor/angiotensin receptor blocker) over the prior 3 months. For those with T2D, no medications for glycemic control except metformin was allowed, HbA1c levels were <10% (86 mmol/mol)], and none had either hypoglycemic unawareness or hypoglycemia within a 3-month period. In the control group, diabetes was excluded with an oral glucose tolerance test. All subjects had a body mass index (BMI) between 18 and 49 kg/m^2, and all had normal renal and hepatic biochemical indices and no prior history of cancer nor any contraindication to insulin infusion to achieve hypoglycemia (ischemic heart disease, epilepsy, seizure history, drop attacks, history of adrenal insufficiency and treated hypothyroidism). All participants provided written informed consent. The trial was approved by the North West-Greater Manchester East Research Ethics Committee (REC number:16/NW/0518), registered at www.clinicaltrials.gov (NCT03102801) and conducted according to the Declaration of Helsinki."

Subjects underwent hyperinsulinemic-clamp-induced hypoglycemia (<40 mg/dl) with blood sampling at baseline, hypoglycemia and post-hypoglycemia [8]; Somascan proteomic analysis of cytokine-related proteins was undertaken.

Data handling and statistical analysis has been described before [7]. "Initial Relative Fluorescent Units (RFUs) were obtained from microarray intensity images using the Agilent Feature Extraction Software (Agilent, Santa Clara, CA). Raw RFUs were normalized and calibrated using the software pipeline provided by SomaLogic. Statistical analyses were performed on $\log_2$ RFU values using R version 3.5.2 (R Foundation for Statistical Computing, Vienna, Austria) including base R package. For the proteomic analysis we fitted an intercept-free general linear model as a function of a subgroup (i.e. condition:timepoint), while taking the patient ID as a random effect using the R package limma. Subsequently, we computed the p value for two contrasts: baseline to hypoglycemia for both T2D and controls, and false discovery rate (FDR) corrected at a value of <0.05 as the cutoff for significance."

There are no studies detailing the changes in cytokine proteins in response to hypoglycaemia on which to base a power calculation. Sample size for pilot studies has been reviewed by Birkett and Day [9]. They concluded that a minimum of 20 degrees-of-freedom was required to estimate effect size and variability. Hence, we needed to analyse the samples from a minimum of 20 patients per group. Data trends were visually evaluated for each parameter and non-parametric tests were applied on data that violated the assumptions of normality when tested using the Kolmogorov-Smirnov Test. Comparison between groups was performed at each timepoint using Student's t-test. A p-value of <0.05 was considered statistically significant. Within-group comparisons are as follows: changes from baseline, and from hypoglycemia, to each subsequent timepoint were compared using Student's t-test. The sample size was too small to adjust for baseline covariates. Statistical analysis was performed using Graphpad Prism (San Diego, CA, USA)."

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Table 1
Demographic and clinical characteristics of the study participants.

Baseline	Type 2 diabetes (n = 23)	Controls (*n* = 22)	p-Value
Age (years)	62 ± 7	55 ± 10	<0.0001
Sex (M/F)	12/11	10/12	0.77
Weight (kg)	90.9 ± 11.4	79.0 ± 8.5	<0.0001
Height (cm)	167 ± 14	169 ± 5	0.64
BMI (kg/m^2)	32 ± 4	28 ± 3	<0.0001
Systolic BP (mmHg)	131 ± 8	122 ± 8	0.001
Diastolic BP (mmHg)	81 ± 7	75 ± 6	0.003
Duration of diabetes (years)	4.5 ± 2.9	N/A	
Insulin (uIU/ml)	47.5 ± 86	9.8 ± 8.1	0.001
HbA1c (mmol/mol)	52.6 ± 10.9	37.4 ± 2.2	<0.0001
HbA1c (%)	6.8 ± 1.0	5.6 ± 0.2	<0.0001
Total cholesterol (mmol/l)	4.2 ± 1.0	4.8 ± 0.7	0.014
Triglyceride (mmol/l)	1.7 ± 0.7	1.3 ± 0.6	0.055
HDL-cholesterol (mmol/l)	1.1 ± 0.3	1.5 ± 0.4	0.001
LDL-cholesterol (mmol/l)	2.2 ± 0.8	2.7 ± 0.8	0.051
CRP (mg/l)	3.1 ± 2.8	5.3 ± 11.0	0.66

Data are presented as mean ± 1SD.
BMI: Body mass index, BP: Blood pressure, HDL-cholesterol: High density lipoprotein cholesterol, LDL-cholesterol: Low density lipoprotein cholesterol, CRP: C-reactive protein. HbA1c: Haemoglobin A1c.

Cytokine profiles before, during, and post-hypoglycemia (time course 0.5 to 4-h and 24-h) are shown in Fig. 1. Baseline CCL17 levels were higher in T2D ($p < 0.05$), fell significantly post-hypoglycemia and remained lower at 24-h ($p < 0.05$), changes not seen in controls (Fig. 1A). CXCL9 was lower at baseline in T2D ($p < 0.05$) with a fall at 2-h post-hypoglycemia ($p < 0.05$); CXCL9 levels in controls were unaffected by hypoglycemia (Fig. 1B). Baseline Il-6 did not differ between T2D and controls and showed an increase at 4-h in both T2D ($p < 0.005$) and controls ($p < 0.05$) (Fig. 1C). Baseline IFNλ1 did not differ between T2D and controls, but controls showed a fall 2-h post-hypoglycemia ($p < 0.05$), a change not seen in T2D (Fig. 1D). CXCL10 levels did not differ in controls and T2D at hypoglycemia but were lower at each comparable timepoint in T2D (Fig. 1E).

This study shows that CCL17 and CXCL9 fell in response to hypoglycemic stress in T2D, whilst IL-6 showed an increase and CXCL10 and IFNλ were unaffected post-hypoglycemia. This suggests that the very low level of CCL17 seen in severe COVID-19 infection may relate to the severity of the stress response for which T2D patients are more predisposed; therefore, an early fall in CCL17 and CXCL9 in T2D patients with moderate symptoms may predict early pulmonary progression. This also suggests that CCL17, an inflammatory-regulated cytokine, is also a more immediate stress parameter, with changes seen 1-h post-hypoglycemia. CXCL9 is produced by monocytes with dysregulation being reported in SARS-CoV-2 infection [10,11]; a transient fall was seen only in T2D 2-h post-hypoglycemia, suggesting a stress-induced element, whilst levels at all timepoints were lower than controls, suggesting that the peak and fall described [3] may be less apparent in T2D. Similarly, CXCL10 (IP-10), induced by IFNλ plasmacytoid dendritic cells and dysregulated in COVID-19 [10,11], was lower in T2D at all timepoints and did not respond to hypoglycemia, suggesting that any initial increase would be less apparent in T2D and that it is modulated by factors other than stress. IL-6, a cytokine reflecting hyperinflammatory endothelial dysfunction in COVID-19 [11], showed a comparable response in T2D and controls. IFNλ, a molecule released from immune cells, may be important in development of severe/critical symptoms; a fall post-hypoglycemia was seen in controls, but

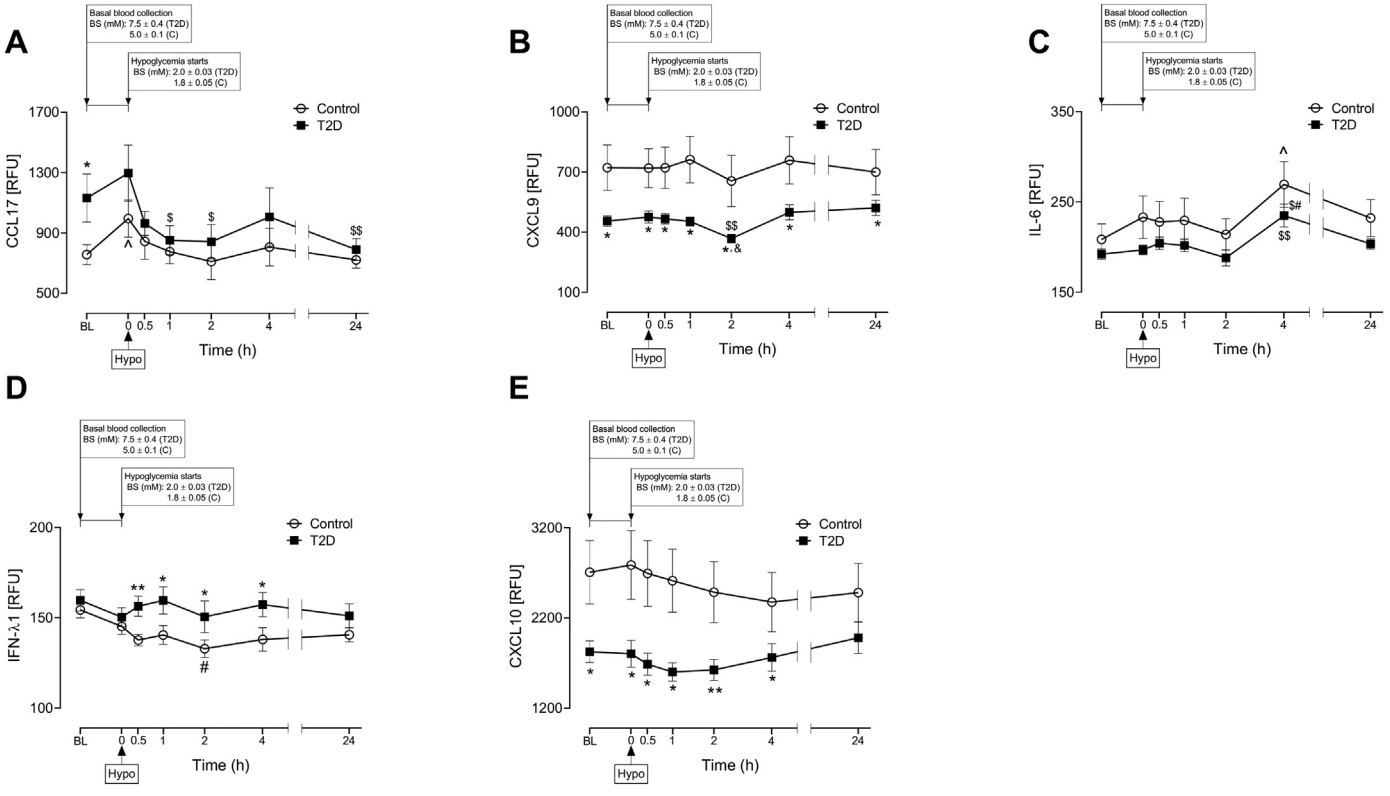

Fig. 1. Comparison of circulatory levels of cytokines reported as biomarkers of incipient severe COVID-19 disease in plasma before, during and after iatrogenic induction of hypoglycemia. Blood sampling was performed at baseline (BL), at hypoglycemia (0 min) and post-hypoglycemia (30 min, 1-h, 2-h, 4-h and 24-h) for controls (white circles) and for T2D (black squares). At baseline (BL), blood sugar (BS) was 7.5 ± 0.4 mM (for T2D) and 5.0 ± 0.1 mM (for control, C). At point of hypoglycemia, blood sugar (BS) was 2.0 ± 0.03 mM (for T2D) and 1.8 ± 0.05 mM (for controls). Proteomic (Somalogic) analysis of amyloid-related proteins was undertaken for Chemokine (C-C motif) ligand 17 [*CCL17*] (A), Chemokine (C-X-C motif) ligand 9 [*CXCL9*] (B), Interleukin 6 [IL-6] (C), Chemokine (C-X-C motif) ligand 10 [*CXCL10*] (D), Interferon lambda 1 [IFNλ1] (E). Statistics: (*, $p < 0.05$ or **, $p < 0.01$, control vs T2D); (^, $p < 0.05$, control baseline vs control hypoglycemia); (#, $p < 0.05$, control hypoglycemia vs control post-hypoglycemia timepoints); ($, $p < 0.05$, $$, $p < 0.01$, T2D hypoglycemia vs T2D post-hypoglycemia timepoints); (&, $p < 0.05$, T2D baseline vs T2D post-hypoglycemia timepoints); (^, $p < 0.05$, T2D hypoglycemia vs T2D post-hypoglycemia timepoints); (#%, $p < 0.01$, control baseline vs control post-hypoglycemia timepoints). RFU, relative fluorescent units.

levels remained unchanged in T2D, suggesting that factors other than stress are important for its modulation in COVID-19 disease.

The underlying pathophysiology of action of chemokines (for example, CCL17 or CXCL9) is not fully understood. However, since the level of IL-6 was increased in response to hypoglycemia, it is tempting to speculate that they exert their function through IL-6-GP130 signaling pathways (either IL-6 binds to the membrane bound IL-6 receptor [mIL-6R] (*cis-signaling*) or IL-6 binds to the soluble IL-6 receptor [sIL-6R] (*trans-signaling*) and they thereby form complexes with GP130, the universal signal-transducing receptor of all IL-6 family cytokines [12,13]. Moreover, it is also evident that IL-6 directs T cell recruitment by regulating the secretion of local chemokines including CXCL10 and CCL17 [14].

Strengths of this study are that the T2D subjects included all had short disease duration and were relatively treatment naïve. The major study limitation is the small subject numbers and, with more subjects, even greater differences in plasma levels of cytokine-related proteins may have emerged. Notwithstanding, it should be noted that these individuals were subjected to a severe hypoglycemic episode, during which significant changes in protein levels would have become apparent. Although the T2D subjects were older and more obese, this should not have altered the levels of protein expression. Another limitation is that the collection of peripheral blood samples only extended to the 24-h time point, and an extended period would better profile the time course of post-hypoglycemia protein changes.

In conclusion, an early decline in CCL17 may be indicative of an increased stress response. Therefore, there is translational potential for CCL17 as a biomarker in high-risk COVID-19 infected patients, as it may predict the onset of deteriorating COVID-19 disease.

Ethics approval and consent to participate

The study was approved by the North West-Greater Manchester East Research Ethics Committee (REC number:16/NW/0518), registered at www.clinicaltrials.gov (NCT03102801) and conducted according to the Declaration of Helsinki. All study participants signed an informed consent form prior to participation.

Consent for publication

All authors gave their consent for publication.

Availability of data and materials

All the data for this study will be made available upon reasonable request to the corresponding author.

Author contributions

ASMM and AEB analyzed the data and wrote the manuscript. AAQ contributed to study design, performed experiments, collected, analyzed, and interpreted data and edited the manuscript. TS supervised clinical studies and edited the manuscript. SLA contributed to study design, data interpretation and the writing of the manuscript. All authors reviewed and approved the final version of the manuscript. Alexandra E Butler is the guarantor of this work.

Declaration of competing interest

No authors have any conflict of interest or competing interests to declare.

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