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ORIGINAL ARTICLE

Limited impact of impaired awareness of hypoglycaemia and severe hypoglycaemia on the inflammatory profile of people with type 1 diabetes

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Abstract

Aim: To investigate whether a history of severe hypoglycaemia (SH) or the associated presence of impaired awareness of hypoglycaemia (IAH) is characterized by a pro-inflammatory profile in people with type 1 diabetes.

Research design and methods: We measured circulating inflammatory markers and pro- and anti-inflammatory cytokine production after *ex vivo* stimulation of peripheral blood mononuclear cells (PBMCs) in a well-characterized cohort of individuals with type 1 diabetes (n = 239) and in people without diabetes (n = 56). Data were corrected for confounders by using multivariate linear regression models.

Results: People with type 1 diabetes had higher circulating concentrations of high-sensitivity C-reactive protein (hs-CRP; 0.91 [0.36–2.25] vs. 0.52 [0.20–0.98] pg/mL, $P < 0.001$ and interleukin-18-binding protein (IL-18BP; 1746 [1304–2112] vs. 1381 [1191–1807] pg/mL; $P = 0.001$) than those without diabetes. In multivariate analysis, only higher hs-CRP concentrations persisted. Neither circulating immune cells nor *ex vivo* cytokine levels produced by PBMCs in response to an extensive panel of stimuli differed in groups defined by awareness state or a history of SH, apart from

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elevated IL-18BP in people with, versus those without, history of SH (1524 [1227–1903] vs. 1913 [1459–2408] pg/mL; $P < 0.001$).

Conclusions: IAH or history of SH in people with type 1 diabetes was not associated with altered inflammatory profiles, arguing against chronically elevated inflammatory activity mediating the increased cardiovascular risk associated with hypoglycaemia. The finding of higher circulating concentrations of IL-18BP in individuals with a history of SH requires further investigation.

KEYWORDS

cardiovascular disease, cohort study, diabetes complications, hypoglycaemia, type 1 diabetes

1 | INTRODUCTION

Hypoglycaemia is a frequent complication of insulin treatment in people with type 1 diabetes and a barrier to achieving optimal glycaemic control.[1] Adults with type 1 diabetes experience, on average, two hypoglycaemic events per week and annually one episode of severe hypoglycaemia (SH),[2] defined as an event requiring external assistance because of cognitive decline.[3] Recurrent hypoglycaemia can lead to impaired awareness of hypoglycaemia (IAH), defined as diminished ability to perceive the onset of hypoglycaemia,[4] resulting in a sixfold greater risk of SH.[5] The prevalence of IAH in people with long-standing type 1 diabetes has been estimated at 20% to 30%.[5]

People with type 1 diabetes are at higher risk of cardiovascular disease and mortality compared to those without diabetes.[6–8] Among people with type 1 diabetes, recurrent (severe) hypoglycaemia is independently associated with accelerated atherosclerosis as well as an increased risk of cardiovascular events and all-cause mortality.[7,9–11] Type 1 diabetes has been characterized by chronic low-grade inflammation.[12] Chronic inflammation plays a critical role in the pathogenesis of atherosclerosis with monocyte-derived macrophages contributing to plaque instability[13,14] and recent suggestions of cardiovascular benefit after anti-inflammatory treatment.[15] We and others have shown that acute hypoglycaemia leads to increased numbers of leukocytes, particularly due to increased monocyte, lymphocyte and neutrophil counts,[16,17] and induction of pro-inflammatory functional changes in immune cells in people with and without type 1 diabetes.[18] Importantly, we showed that hypoglycaemia-induced monocytosis is mainly driven by increases in non-classic monocytes, which produce more pro-inflammatory cytokines and contribute more to the development of cardiovascular disease than other monocyte subsets.[17]

Since hypoglycaemia occurs particularly frequently in people with type 1 diabetes and IAH or with a recent history of SH, we hypothesized that these people would be characterized by a pro-inflammatory profile. To test this hypothesis, we extensively measured circulating inflammatory markers and anti- and pro-inflammatory cytokine production after *ex vivo* stimulation of peripheral blood mononuclear cells (PBMCs) in a large group of carefully characterized people with type 1 diabetes, and compared the results between those with and without IAH or a history of SH.

2 | MATERIALS AND METHODS

2.1 | Study population

Studies were performed in a cohort of people with type 1 diabetes, selected from the outpatient diabetes clinic of the Radboud University Medical Centre, the Netherlands. Inclusion criteria were a diagnosis of type 1 diabetes (based on clinical criteria with or without anti-glutamic acid decarboxylase positivity) and age above 18 years. Pregnant women were excluded. Participants were not allowed to use antibiotics in the 4 weeks before inclusion, and tests were rescheduled if the participant had fever in the week before inclusion.

Results of circulating inflammatory markers were compared to a cohort of 56 controls, who were recruited simultaneously. The control group consisted of healthy, mostly young, people with no abnormalities in the medical history or drug use; absence of diabetes was based on self-report (Table 1). Both cohorts are part of the Human Functional Genomics Project (HFGP).[19,20] Ethical approval for the study was obtained from the institutional review board of the Radboud University Medical Centre (NL54214.091.15, 2015-1930 and NL42561.091.12, 2012-550). Participant inclusion and experiments were conducted according to the principles expressed in the Declaration of Helsinki. All participants gave written informed consent before participation.

2.2 | Clinical characteristics

All participants with type 1 diabetes were asked to complete the clamp-validated modified Dutch version of the Clarke questionnaire, in which a score of three or more out of five indicates IAH.[21] SH was defined in accordance with the definition of the American Diabetes Association Working Group on Hypoglycemia, as an event requiring assistance of another person to actively administer carbohydrate, glucagon or other resuscitative actions, in the last year (ie, the year before entering the study).[1] Clinical characteristics, including age, sex, body

TABLE 1 Characteristics of study participants

	IAH		History of SH		Healthy controls (n = 56)
	IAH group (n = 72)	Non-IAH group (n = 167)	SH group (n = 119)	Non-SH group (n = 120)	
Age, years	57.2 ± 14.1*	49.7 ± 16.5*	54.8 ± 16.1*	49.1 ± 15.8*	39.2 ± 17.4†
Males, n (%)	43 (60)	87 (52)	69 (58)	61 (51)	34 (61)
BMI, kg/m^2	25.1 ± 3.5	26.2 ± 4.7	25.6 ± 4.1	26.1 ± 4.6	23.9 ± 3.2†
Systolic blood pressure, mmHg	134 ± 20	131 ± 16	132 ± 18	132 ± 16	—
Diastolic blood pressure, mmHg	73 ± 11	73 ± 10	72 ± 10	73 ± 10	—
Current smoking, n (%)	8 (11)	18 (11)	16 (13)	10 (8)	5 (9)
Current alcohol use, n (%)	45 (63)	124 (74)	83 (70)	86 (72)	46 (82)
Age at diabetes onset, years	23 ± 13	24 ± 14	24 ± 14	22 ± 13	—
Diabetes duration, years	35 ± 16*	26 ± 15*	31 ± 16	27 ± 15	—
HbA1c, mmol/mol (%)	63 ± 15 (7.9 ± 3.5)	64 ± 15 (8.0 ± 3.5)	64 ± 14 (8.0 ± 3.4)	64 ± 15 (8.0 ± 3.5)	—
Insulin dose, IU/kg/d	0.56 [0.45–0.75]	0.57 [0.43–0.69]	0.56 [0.44–0.74]	0.57 [0.43–0.70]	—
Microvascular complications, n (%)	61 (85)*	98 (59)*	88 (74)	71 (59)	—
Macrovascular complications, n (%)	18 (25)*	22 (13)*	28 (24)*	12 (10)*	—

Note: Data are presented as mean ± SD, median (interquartile range) or number (%), as appropriate.

Abbreviations: BMI, body mass index; HbA1c, glycated haemoglobin; IAH, impaired awareness of hypoglycaemia; SH, severe hypoglycaemia.

*$P < 0.05$ between subgroups (IAH vs. non-IAH, SH vs. non-SH).

†$P < 0.05$ vs. type 1 diabetes group.

mass index (BMI), blood pressure, current smoking, alcohol use, age at diabetes diagnosis, duration of diabetes, glycated haemoglobin, insulin dose, presence of microvascular complications (ie, retinopathy, nephropathy, neuropathy) and macrovascular complications (ie, coronary heart disease, stroke, peripheral arterial disease) were obtained from questionnaires and clinical records.

2.3 | Isolation of peripheral mononuclear cells

Between February 2016 and June 2017, all participants were seen at the outpatient clinic of the Radboud University Medical Centre, Nijmegen, the Netherlands. Venous blood was collected into 10-mL EDTA tubes (Vacutainer system; Becton Dickinson, Franklin Lakes, New Jersey) between 8:00 and 11:00 AM. PBMCs were isolated by Ficoll-Paque density gradient centrifugation (GE Healthcare, Amersham, UK). PBMCs were washed twice with cold phosphate-buffered saline and suspended in Roswell Park Memorial Institute (RPMI) 1640 Dutch-modified culture medium (Gibco/Invitrogen, Breda, the Netherlands), supplemented with 50 mg/L gentamycin (Centraform), 1 mM pyruvate (Gibco/Invitrogen) and 2 mM L-glutamine (Gibco/Invitrogen). Cells were counted and cell composition was evaluated on a Sysmex XN-450 Hematology Analyser (Sysmex Corp., Kobe, Japan).

2.4 | Stimulation experiments

Cells (5×10^5 PBMCs per well) were added to round-bottom 96-well plates (Greiner, Monroe, USA) and were stimulated at 37°C and 5% CO_2. Cells were stimulated with the TLR-4 ligand lipopolysaccharide (LPS)100 ng/mL (*Escherichia coli* LPS O55:B5, Sigma, St Louis, Missouri) and the TLR-2 ligand Pam3Cys 10 μg/mL (EMC Microcollections, Tübingen, Germany). After 24 hours' stimulation, supernatants were stored in −20°C until used for cytokine measurements (R&D Duoset ELISA Systems, Minneapolis, Minnesota).

2.5 | Laboratory measurements

Leukocyte counts and differentiation were measured in whole blood samples on a Sysmex XN-450 Hematology Analyser (Sysmex Corp., Kobe, Japan). In unstimulated EDTA plasma samples, we measured high-sensitivity C-reactive protein (hs-CRP), interleukin (IL)-18 and IL-18-binding protein (IL-18BP; R&D Duoset ELISA Systems). IL-1β, IL-6, TNF-α, IL-1 receptor antagonist (IL-1Ra) and IL-10 (R&D Duoset ELISA Systems) were measured in the stimulated supernatants. All cytokines were measured according to the manufacturer's instructions. Per ELISA plate, three control samples were used, all in duplicate. The control samples were pooled samples from multiple blood donors. Samples were diluted on the ELISA plates. All dilutions were based on pilot experiments performed before the actual ELISA measurements. Detection limits used for cytokine measurements are shown in the Supporting Information (Table S1).

2.6 | Statistical analysis

Baseline characteristics are expressed as mean (±SD) or median (interquartile range [IQR]) or number (%), as appropriate. Differences in baseline characteristics between people with or without type 1 diabetes and, in the type 1 diabetes group, between those with or without IAH and with or without a history of SH, respectively, were compared using independent Student *t*-tests or Mann–Whitney *U*-tests, as appropriate. To compare categorical data, chi-squared tests were used. A P value < 0.05 was considered statistically significant.

Multiple linear regression models were used to correct for potential baseline confounders. We applied two models. In model 1, we corrected for age and sex. In model 2, we additionally adjusted for BMI, smoking, macrovascular complications and microvascular complications. Regression coefficients were exponentiated to present geometric means along with 95% confidence intervals. We also analysed the association between the inflammation variables and the number of severe hypoglycaemic events in the last year using a linear regression model. Correlations between inflammatory markers with each other and between inflammatory markers and geographic variables were calculated using Spearman correlation analysis.

To meet model assumptions, inflammatory markers were logarithmically transformed. Assumption of normality and homoscedasticity were assessed by residual plots. Results were corrected for multiple testing with Bonferroni correction. Consequently, a P value < 0.003 was considered statistically significant and a P value < 0.05 was taken to indicate a tendency for difference. Inflammatory markers are presented as median (IQR) unless stated otherwise.

All statistical analyses were performed with SPSS version 25 software (IBM Corp., Armonk, New York) or with the R Project for Statistical Computing, version 3.6.2 (Vienna, Austria). Graphs were made with PRISM version 8.0 (GraphPad Software, La Jolla, California).

3 | RESULTS

After exclusion of four participants with missing Clarke scores, 239 people with type 1 diabetes were analysed. Among this group, 72 participants (30%) were classified as having IAH. Participants with IAH were older, had longer diabetes duration and were more likely to have a history of microvascular and macrovascular complications compared to those without IAH (Table 1). Similarly, participants with SH in the past year (n = 119, 50%) were older and more likely to have a history of macrovascular complications than those without SH. There were no differences in the level of glycaemic control between any of the subgroups.

People with type 1 diabetes had higher circulating concentrations of hs-CRP (0.91 [0.36–2.25] vs. 0.52 [0.20–0.98] pg/mL; P < 0.001) and IL-18BP (1746 [1304–2112] vs. 1381 [1191–1807] pg/mL; P = 0.001) than people without diabetes, whereas circulating concentrations of IL-18 did not differ (147 [122–189] vs.145 [115–193] pg/mL; P = 0.654). After correction for baseline variables, the difference in hs-CRP levels, but not IL-18BP, remained significant (Table S2).

There was also a tendency towards higher leukocyte counts in people with type 1 diabetes (5.7 [5.0–7.1] $\cdot 10^9$/L vs. 5.2[4.5–6.3] $\cdot 10^9$/L; $P = 0.011$).

In the diabetes group, there were no differences in levels of hs-CRP or IL-18 or leukocyte counts between people with or without IAH. There was a tendency towards higher concentrations of IL-18BP in individuals with IAH compared to those with normal awareness ($P = 0.016$; Figure 1). This difference disappeared after correction for baseline differences in the fully adjusted model (Table 2). We found no differences in the *ex vivo* production of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α and the anti-inflammatory cytokines IL-10 and IL-1Ra by PBMCs in response to different TLR ligands between people with and without IAH (Figure 2 and Figure S1).

The findings were broadly similar when comparing data in subgroups defined by history of SH in the last year. Indeed, there were no differences in hs-CRP or IL-18 concentrations or leukocyte counts between subgroups with or without history of SH, except for higher IL-18BP concentrations in the SH subgroup (Figure 1), which remained significantly different after correction for baseline differences in the fully adjusted model. There were also no differences

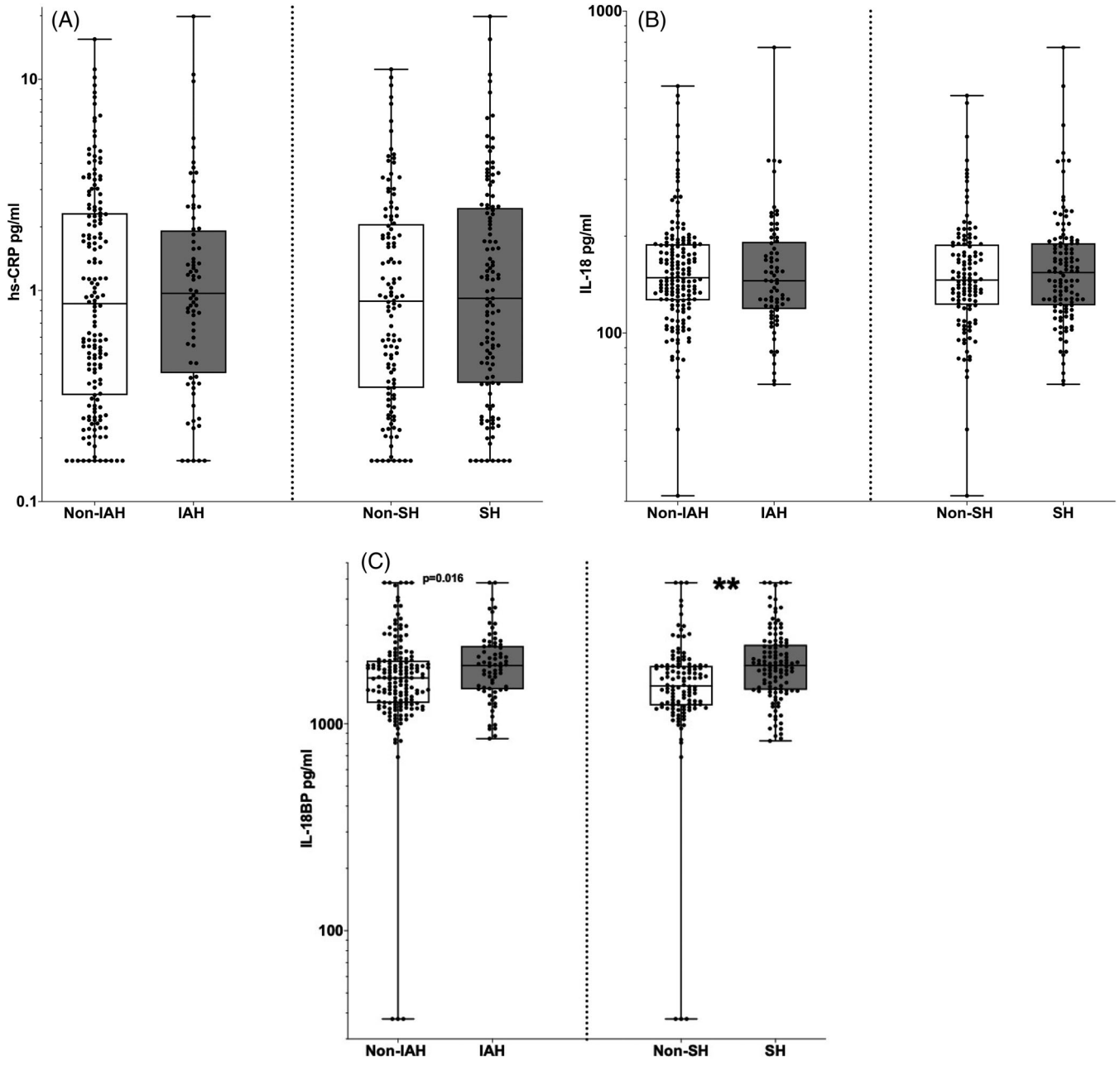

FIGURE 1 Circulating inflammatory markers in people with type 1 diabetes, stratified by awareness state and history of severe hypoglycaemia (SH). Circulating levels of **A,** high-sensitivity C-reactive protein (hs-CRP), **B,** interleukin (IL)-18 and **C,** IL-18-binding protein (IL-18BP) in pg/mL are shown for the type 1 diabetes subgroups defined by presence (grey) or absence (white) of impaired awareness of hypoglycaemia (IAH) or recent history of SH, respectively. $^{**}P < 0.003$

TABLE 2 Linear regression models for inflammatory markers in in people with type 1 diabetes, stratified by awareness state and history of severe hypoglycaemia

	IAH vs. non-IAH group		SH vs. non-SH group	
	Exponentiated regression coefficients (95% CI)	*P*	Exponentiated regression coefficients (95% CI)	*P*
hs-CRP				
Model 1	1.039 (0.746, 1.448)	0.819	1.073 (0.799, 1.441)	0.638
Model 2	1.097 (1.255, 1.510)	0.568	1.092 (0.814, 1.464)	0.558
IL-18				
Model 1	0.946 (0.840, 1.065)	0.357	1.023 (0.922, 1.135)	0.661
Model 2	0.923 (0.815, 1.045)	0.204	1.009 (0.899, 1.131)	0.880
IL-18BP				
Model 1	1.077 (0.912, 1.270)	0.381	1.228 (1.065, 1.415)	0.005
Model 2	1.022 (0.862, 1.212)	0.804	1.212 (1.038, 1.415)	0.015
Leukocytes				
Model 1	1.156 (0.592, 2.261)	0.669	1.220 (0.654, 2.277)	0.531
Model 2	1.030 (0.830, 1.278)	0.787	0.951 (0.528, 1.714)	0.867
LPS 100 nanogram-IL-1β				
Model 1	1.008 (0.820, 1.239)	0.939	1.181 (0.980, 1.420)	0.079
Model 2	1.030 (0.830, 1.278)	0.787	1.204 (0.989, 1.467)	0.064
Pam3Cys-IL-1β				
Model 1	1.092 (0.958, 1.245)	0.189	1.067 (0.946, 1.204)	0.291
Model 2	1.080 (0.944, 1.235)	0.262	1.061 (0.937, 1.201)	0.349
LPS 100 nanogram-IL-6				
Model 1	1.007 (0.847, 1.197)	0.937	1.063 (0.908, 1.245)	0.449
Model 2	1.042 (0.870, 1.249)	0.652	1.077 (0.912, 1.271)	0.382
Pam3Cys-IL-6				
Model 1	0.980 (0.855, 1.124)	0.777	1.016 (0.891, 1.158)	0.809
Model 2	0.968 (1.191, 1.114)	0.642	1.012 (0.889, 1.154)	0.853
LPS 100 nanogram-TNF-α				
Model 1	0.961 (0.787, 1.172)	0.693	1.049 (0.876, 1.256)	0.600
Model 2	0.958 (0.781, 1.175)	0.680	1.054 (0.874, 1.273)	0.578
Pam3Cys-TNF-α				
Model 1	0.948 (0.776, 1.158)	0.600	1.078 (0.899, 1.292)	0.416
Model 2	0.925 (0.754, 1.135)	0.455	1.090 (0.901, 1.317)	0.373
LPS 100 nanogram-IL-10				
Model 1	1.015 (0.840, 1.227)	0.873	0.933 (0.785, 1.111)	0.437
Model 2	1.075 (0.883, 1.307)	0.468	1.040 (0.868, 1.246)	0.671
Pam3Cys-IL-10				
Model 1	0.999 (0.828, 1.206)	0.994	0.896 (0.755, 1.064)	0.210
Model 2	1.026 (0.843, 1.250)	0.797	0.944 (0.787, 1.132)	0.532
LPS 100 nanogram-IL-1Ra				
Model 1	1.104 (0.968, 1.261)	0.140	0.992 (0.879, 1.120)	0.896
Model 2	1.123 (0.978, 1.289)	0.099	1.030 (0.907, 1.171)	0.640
Pam3Cys-IL-1Ra				
Model 1	1.055 (0.931, 1.196)	0.393	0.951 (0.847, 1.066)	0.388
Model 2	1.045 (0.917, 1.191)	0.511	0.964 (0.854, 1.088)	0.550

Note: Regression coefficients were exponentiated to present geometric means along with 95% CIs. Model 1: correction for age and sex (non-IAH and non-SH, respectively, as reference group). Model 2 (non-IAH and non-SH, respectively, as reference group): correction for age, sex, BMI, smoking, macrovascular complications and microvascular complications.

Abbreviations: BMI, body mass index; CI, confidence interval; IAH, impaired awareness of hypoglycaemia; IL, interleukin; Ra, receptor antagonist; SH, severe hypoglycaemia; LPS, lipopolysaccharide; TNF-α, tumour necrosis factor-α.

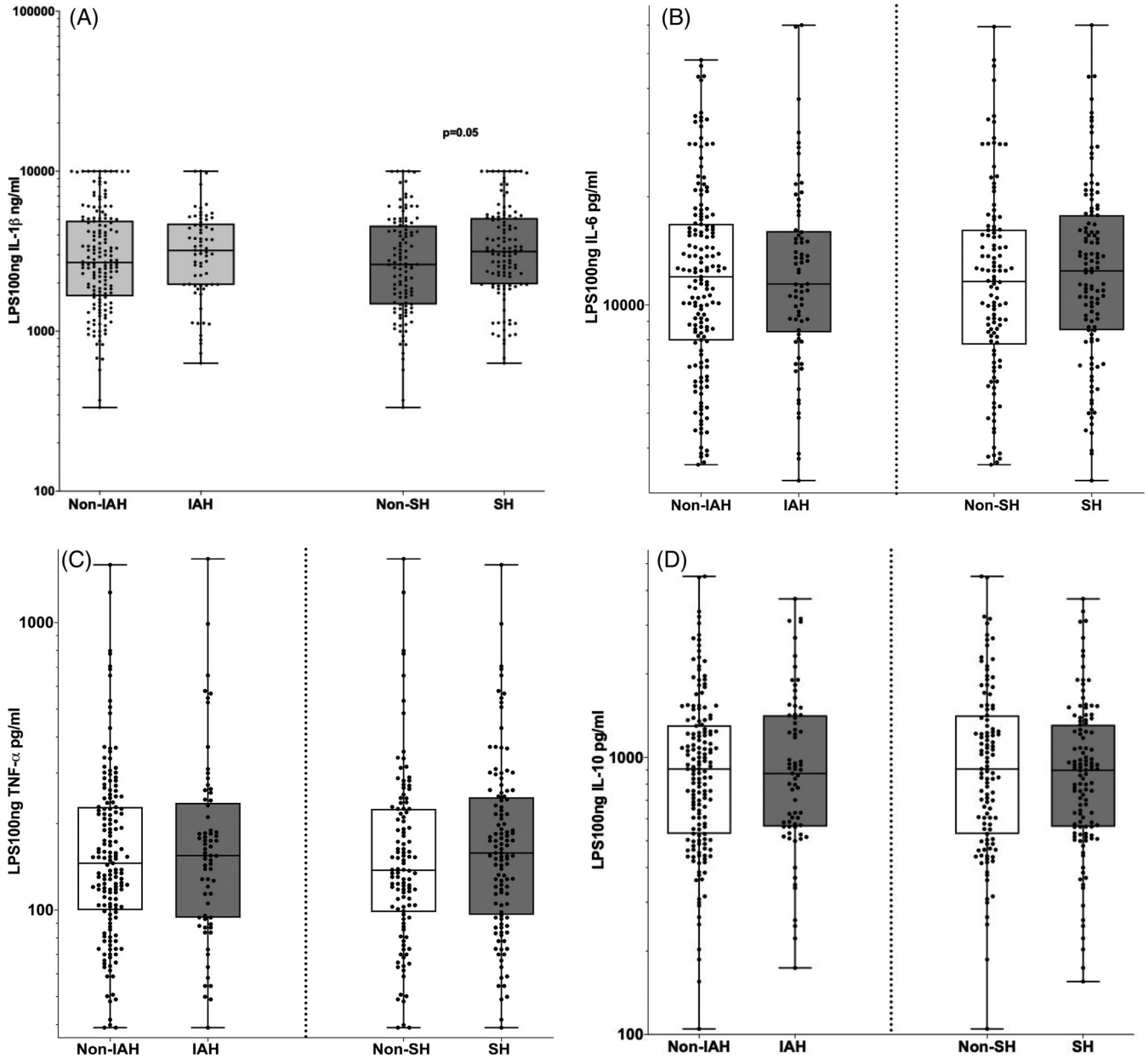

FIGURE 2 *Ex vivo* lipopolysaccharide (LPS) 100 ng-stimulated cytokine production by peripheral blood mononuclear cells in response to TLR-4 agonist in people with type 1 diabetes, stratified by awareness state and history of severe hypoglycaemia (SH). *Ex vivo* LPS 100 ng-stimulated **A,** interleukin (IL)-1β, **B,** IL-6, **C,** tumour necrosis factor-α (TNF-α), **D**, IL-10 and **E**, IL-1 receptor antagonist (IL-1Ra) are shown for the type 1 diabetes subgroup defined by presence (grey) or absence (white) of impaired awareness of hypoglycaemia (IAH) or recent history of SH, respectively

between those with and without a history of SH in *ex vivo*-stimulated cytokine production in response to different TLR ligands (Figure 2 and Figure S1).

Also, comparing the IL-18/IL-18BP ratio in different subgroups revealed no differences in IL-18/IL-18BP ratio between participants with diabetes and healthy controls; neither were there differences in the subgroups defined by awareness status or history of SH in the last year. Correction for confounders in the models did not change these results (Table S5).

In the entire diabetes cohort, the number of SH events reported in the past year averaged 1.4 ± 2.4 events, with 21.9% of the group reporting two or more events (Table S4). There were no statistically significant associations between the number of SH events and circulating levels of inflammatory markers or *ex vivo*-stimulated cytokine production in response to different TLR ligands, except for a trend towards higher IL-18BP levels with increasing number of SH events ($P = 0.042$; data not shown).

In sensitivity analyses, results were essentially similar after exclusion of people with a Clarke score of 2, which classifies neither for

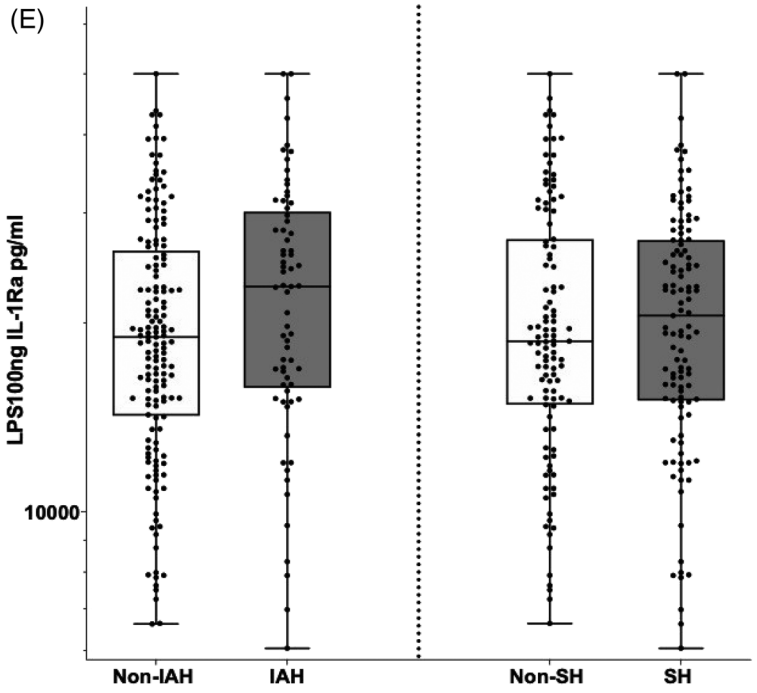

FIGURE 2 (Continued)

IAH or normal awareness, or of people with SH who did not require medical external assistance (Table S3).

We found correlations between demographic variables and various cytokines. For example, there was a significant correlation between IL-18BP and age, gender, systolic blood pressure, diabetes duration, micro- and macrovascular complication and history of SH. We also found correlations between IL-18BP and hs-CRP and IL-18 (Figure S2).

4 | DISCUSSION

This study confirms that circulating levels of inflammatory markers are increased in people with type 1 diabetes. The major new finding is that the presence of IAH or a history of SH in the last year, even when recurrent, has little impact on the inflammatory profile of people with diabetes. Indeed, this was true for both circulating immune factors and for the *ex vivo* production of cytokines after stimulation of innate immune cells. Yet, serum concentrations of IL-18BP were consistently higher in people with a history of SH compared to those without SH. Altogether these results do not support increased inflammatory activity as an important factor mediating the increased cardiovascular risk associated with hypoglycaemia.

Our data, suggestive of chronic low-grade inflammation in individuals with type 1 diabetes, are in agreement with earlier studies.[22,23] Few studies have investigated the impact of (recurrent) hypoglycaemia on chronic inflammation. In people with type 1 diabetes, Kiec-Wilk et al[24] found an association between the number of self-reported hypoglycaemia events over a period of 1 week and the level of circulating inflammatory and endothelial dysfunction markers. Also, higher concentrations of circulating inflammatory markers have been reported in people with type 2 diabetes and a history of recurrent (severe) hypoglycaemia as compared to those without such a history.[25] These studies were relatively small and usually investigated only a few circulating cytokines. However, the immune system is a complex and interactive network of specialized cells, lymphoid organs, circulating humoral factors and cytokines, with considerable inter-individual variation in composition and response to stimuli.[26] Therefore, apart from the larger sample size and more careful phenotyping, we applied a more comprehensive set of inflammatory measurements that included *ex vivo* stimulation of cytokine production. *Ex vivo* stimulation of isolated subtypes allows standardization of experimental conditions and provides detailed information regarding functional responses to various stimuli to quantify inflammatory activity and capacity.[19]

We previously reported that acute hypoglycaemia causes a rise in circulating immune cells and induction of pro-inflammatory changes in adults with type 1 diabetes or without diabetes.[18] We thus reasoned that repetitive exposure to hypoglycaemia, which is common in IAH and people with a recent history of SH, would result in a chronically elevated inflammatory profile, similar to individuals with atherosclerosis.[14,27] Our data do not support this hypothesis. There may be several explanations for our findings. First, the sympathoadrenal response to hypoglycaemia is suppressed in people with IAH,[28,29] and possibly also in those with SH. Since epinephrine importantly contributes to the inflammatory response,[16,18] a suppressed hormone response may fail to stimulate the immune system. Second, it is possible that hypoglycaemia-induced stimulation of the inflammatory system quickly returns to baseline after restoration of normoglycaemia without any long-term effects.

Third, the link between (severe) hypoglycaemia and cardiovascular disease in type 1 diabetes may be explained by pathways other than the inflammatory markers examined here, such as oxidative stress,[6] haemodynamic or haemorheological changes,[9,10] pro-thrombotic factors,[30,31] alteration in vasomotor balance and endothelial dysfunction. Nevertheless, for most of these potential pathways it remains to be determined whether the effect of acute hypoglycaemia persists over time. Fourth, the chronically elevated inflammatory state in people with diabetes has been suggested to induce tolerance, resulting in attenuated cytokine responses to stimulation.[30] Finally, residual confounding, such as frailty,[10] may increase the risk of hypoglycaemia and of cardiovascular disease, but independently of each other.[31]

We found elevated concentrations of IL-18BP, but not of IL-18, particularly in the SH subgroup. IL-18BP acts as an anti-inflammatory cytokine as it binds IL-18, a pro-inflammatory cytokine that has pathophysiological roles in several inflammatory conditions, including atherosclerosis.[32,33] By binding IL-18, IL-18BP protects tissues against uncontrolled pro-inflammatory activity of IL-18 and has been shown to slow the progression of atherosclerosis.[34] In people with type 1 diabetes, higher concentrations of both IL-18 and IL-18BP have been associated with poor glycaemic control,[35–37] whereas IL-18 has also been associated with markers of endothelial dysfunction.[37] Given these data, it seems more likely that IL-18BP elevation reflects a protective response to neutralize pro-inflammatory activity rather than it being a direct mediator between hypoglycaemia and cardiovascular disease, although this requires further investigation.

A strength of the present study is its large sample size, providing our study of inflammatory markers in relation to IAH and SH with sufficient robustness. Indeed, a retrospective power calculation for all *ex vivo* cytokines (including IL-18 and IL-18BP) showed that we had a power of 85% to 100% to find differences of 5% and a power of 100% to find differences of 10% between the non-IAH and IAH subgroups, respectively; findings for the non-SH and SH subgroups were similar. Furthermore, we measured different circulating markers and performed *ex vivo* experiments with both pro-inflammatory and anti-inflammatory markers.

A limitation of the study is that information on SH was collected retrospectively rather than prospectively, which could have led to non-differential misclassification bias and recall bias, especially for SH events dating back longer or occurring more frequently. Although self-reported recall is sufficiently accurate to differentiate between presence or absence of a history of SH, this may be less so for the number of such events.[38] Furthermore, we did not collect data on glucose levels when blood was sampled. Although it seems unlikely that participants would have been hypoglycaemic at that moment, the occurrence of (modest) hyperglycaemia cannot be completely excluded. Also, people did not receive instructions to avoid hypoglycaemia 24 hours prior to blood sampling. However, since patients with IAH or history of SH are at higher risk of hypoglycaemia, it is unlikely that this would have changed the results. We have examined the activity of circulating immune cells using an *ex vivo* approach. To what extent these effects reflect the *in vivo* situation at the tissue level cannot be derived from the present study.

In conclusion, the inflammatory profile of people with type 1 diabetes and IAH or a recent history of SH generally does not differ from that in those without these conditions, as measured by circulating inflammatory factors and *ex vivo* stimulation of innate immune cells. The higher circulating levels of IL-18BP in participants with a history of SH compared to those without such a history requires further investigation. Altogether, our results suggest that mechanisms other than those mediated by the inflammatory parameters studied here are needed to explain the relationship between (severe) hypoglycaemia and cardiovascular disease.

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CONFLICTS OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

N.A. analysed the data and wrote the first draft of the manuscript. A.W.M.J. collected all the clinical data, recruited the participants and performed laboratory measurements with help from M.J., W.v.d.H. and L.V.d.W. R.t.H. assisted during data processing. B.E.D.G. and C.J. T. designed the study. R.S., A.v.G. and C.J.T. obtained funding. All authors discussed the results and provided input for and commented on the manuscript at all stages. Guarantors: Namam Ali and Bastiaan E. De Galan.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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