

RESEARCH

Macrophage activation marker sCD163 correlates with accelerated lipolysis following LPS exposure: a human-randomised clinical trial

Nikolaj Rittig¹, Mads Svart¹, Niels Jessen², Niels Møller¹, Holger J Møller³ and Henning Grønbaek⁴

¹Department of Internal Medicine and Endocrinology (MEA) and Medical Research Laboratory, Aarhus University Hospital, Aarhus C, Denmark

²Research Laboratory for Biochemical Pathology, Institute for Clinical Medicine, Aarhus University Hospital, Aarhus C, Denmark

³Department of Clinical Biochemistry Aarhus University Hospital, Aarhus C, Denmark

⁴Department of Hepatology and Gastroenterology, Aarhus University Hospital, Aarhus C, Denmark

Correspondence should be addressed to N Rittig: nikolaj.rittig@clin.au.dk

Abstract

Background: Macrophage activation determined by levels of soluble sCD163 is associated with obesity, insulin resistance, diabetes mellitus type 2 (DM2) and non-alcoholic fatty liver disease (NAFLD). This suggests that macrophage activation is involved in the pathogenesis of conditions characterised by adaptations in the lipid metabolism. Since sCD163 is shed to serum by inflammatory signals including lipopolysaccharides (LPS, endotoxin), we investigated sCD163 and correlations with lipid metabolism following LPS exposure.

Methods: Eight healthy male subjects were investigated on two separate occasions: (i) following an LPS exposure and (ii) following saline exposure. Each study day consisted of a four-hour non-insulin-stimulated period followed by a two-hour hyperinsulinemic euglycemic clamp period. A ³H-palmitate tracer was used to calculate the rate of appearance ($Ra_{\text{palmitate}}$). Blood samples were consecutively obtained throughout each study day. Abdominal subcutaneous adipose tissue was obtained for western blotting.

Results: We observed a significant two-fold increase in plasma sCD163 levels following LPS exposure ($P < 0.001$), and sCD163 concentrations correlated positively with the plasma concentration of free fatty acids, $Ra_{\text{palmitate}}$, lipid oxidation rates and phosphorylation of the hormone-sensitive lipase at serine 660 in adipose tissue ($P < 0.05$, all). Furthermore, sCD163 concentrations correlated positively with plasma concentrations of cortisol, glucagon, tumour necrosis factor (TNF)- α , interleukin (IL)-6 and IL-10 ($P < 0.05$, all).

Conclusion: We observed a strong correlation between sCD163 and stimulation of lipolysis and fat oxidation following LPS exposure. These findings support preexisting theory that inflammation and macrophage activation play a significant role in lipid metabolic adaptations under conditions such as obesity, DM2 and NAFLD.

Key Words

- ▶ sCD163
- ▶ endotoxin/LPS
- ▶ lipid metabolism
- ▶ insulin resistance
- ▶ inflammation

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Introduction

Macrophage activation is considered an important part of the pathogenesis of several diseases. Hence, the macrophage activation marker-soluble CD163 (sCD163) is of high interest and has already been associated with diseases such as obesity, sepsis, insulin resistance,

diabetes mellitus type 2 (DM2) and non-alcoholic fatty liver disease (NAFLD) (1, 2, 3, 4, 5, 6). These diseases are also associated with changes in the lipid metabolism, accelerated lipolysis and increased circulating free fatty acid (FFA) levels (7, 8, 9).

Administration of LPS to healthy human volunteers is a well-established model simulating the initial steps of sepsis (10). The model excels itself by producing a homogeneous and uniform inflammatory response, which is attractive in clinical studies as it reduces inter-individual differences and ensures a uniform inflammatory response in the hours following LPS. The LPS molecule is the major virulence factor of Gram-negative bacteria and binds to trans-membrane receptors named Toll-like receptors (TLR), which are expressed on the surface of innate immune cells (e.g. macrophages). Attachment of LPS to the TLR initiates intracellular signalling cascades that ultimately lead to the transcription and release of a broad range of pro- and anti-inflammatory cytokines, e.g. TNF- α (11, 12). Furthermore, the same mechanism leads to shedding of sCD163, which can be measured in the circulation as a robust marker of macrophage activation (13, 14). We have previously shown how lipopolysaccharide (LPS) infusion causes increased lipolysis rates and insulin resistance in humans (15, 16) and others have shown how LPS exposure causes elevated concentrations of sCD163 (13, 17). However, the association between lipolysis and sCD163 has not yet been investigated.

The regulation of lipolysis is complex and has been reviewed by others in detail (18, 19). Concisely, inflammatory cytokines (especially TNF- α) and hormones (especially epinephrine) stimulate lipolysis through the actions of adipose triglyceride lipase and hormone-sensitive lipase (HSL), which are rate-limiting enzymes in the conversion of triglycerides into glycerol and FFA. Increased rates of lipolysis elevate circulating FFA concentrations and stimulate ketogenesis in the liver, and conversion of excessive FFA into ketone bodies, e.g. β -hydroxybutyrate (BHB) (20). The association between sCD163 and lipid metabolic adaptations is elusive.

The primary aim of this study was to confirm that LPS causes an increase in plasma sCD163 and to investigate if the increase in plasma sCD163 correlates with increased lipid metabolism, inflammatory cytokines and hormones following LPS exposure. We hypothesised that macrophage activation measured by sCD163 is involved in lipid metabolism and insulin resistance after LPS exposure.

Materials and methods

Subjects and design

Data originate from a human-randomised controlled crossover study from which results regarding protein and lipid metabolism have been published (15, 16). The study

design included eight healthy lean male test subjects who underwent three experimental interventions:

1. Saline (placebo).
2. LPS administration (LPS).
3. LPS and amino acid administration (LPS+A).

In this paper, we only present data from study arms (i) and (ii) because amino acid supplementation is beyond the scope of this paper.

The primary investigator enrolled all test subjects using a computerised programme to randomise interventions. Four of the test subjects received LPS during their first trial followed by placebo during their second trial and the other four test subjects received placebo first followed by LPS. During each trial, intravenous catheters were inserted in both cubital veins and one in a dorsal hand vein in order to give intravenous infusions and collect blood samples. Study days were separated with at least three weeks. Both study days consisted of a four-hour non-insulin-stimulated period followed by a two-hour hyperinsulinemic euglycemic clamp period.

Ethics

The Danish Ethical Committee approved the study (1-10-71-410-12) and registered at clinicaltrials.gov (NCT01705782). Both written and oral consents were obtained before inclusion of subjects in the trial. The study was conducted in accordance with the principles stated in the Declaration of Helsinki.

Lipopolysaccharide (LPS)

During intervention (ii) a bolus (1 ng/kg or 10 U/kg) of *Escherichia coli* endotoxin (10,000 USP Endotoxin, lot HOK354; The United States Pharmacopeial Convention, Inc., Rockville, Maryland) was given at time=0min and was followed by a 10-mL saline infusion. During placebo conditions, a 10-mL saline infusion was given at time=0min. Test subjects were blinded but were able to distinguish trial days due to significant clinical symptoms following LPS exposure.

Hyperinsulinemic euglycemic clamp

Insulin was infused at a rate of 1.0U/kg/min during the insulin-stimulated period (time=240–360min, Insulin Actrapid; Novo-Nordisk, Copenhagen, Denmark). Plasma glucose concentrations were measured with a 10-min interval and variable rates of 20% glucose infusion were used to clamp plasma glucose concentrations at 5mmol/L. The

glucose infusion rate (GIR) was then used as a measure of insulin sensitivity. Data are presented as the change in GIR between placebo and LPS conditions ($\Delta\text{GIR} = \text{GIR}_{\text{placebo}} - \text{GIR}_{\text{LPS}}$) to leave out inter-individual variability.

Blood and tissue sample analysis

Blood samples were stored at -20°C and analysed in the same assay after all participants had completed all trials. Serum concentrations of cortisol (ELISA, DRG Cortisol Enzyme Immunoassay Kit, Germany), glucagon (EMD Millipore's Glucagon Radioimmunoassay (RIA) Kit), lactate and glucose (YSI 2300 model Stat Plus, Bie & Berntsen), β -hydroxybutyrate (BHB) (hydrophilic interaction liquid chromatography tandem mass spectrometry (HILIC-MS/MS)) and FFA (*in vitro* enzymatic colorimetric method assay NEFA-HR(2), Wako Chemicals GmbH, Germany) were analysed with the above standing methods and were all performed in accordance with the manufacturer's recommendations. Serum concentrations of tumour necrosis factor alpha (TNF- α), interleukin (IL)-6 and IL-10 were measured in a magnetic Bio-Plex Pro Human Chemokine Assay (Bio-Rad). sCD163 (BEP-2000 ELISA analyzer, Dade Behring, Marburg, Germany) was measured by an in-house assay essentially as previously described (21).

A continuous infusion of ^3H -palmitate was used to determine the rate of appearance for palmitate ($\text{Ra}_{\text{palmitate}}$) at time=240 min. An abdominal subcutaneous adipose tissue (SAT) biopsy was obtained at time=135 min. Fat biopsies were successfully obtained and blotted for $n=7$. Western blot analysis was used to measure relative content and phosphorylation of relevant protein targets using the Bio-Rad Criterion system (Bio-Rad). Primary antibodies used were raised against phosphorylated (ser⁶⁶⁰) and HSL (4107 and 4126 Cell Signaling).

Indirect calorimetry

An Oxycon Pro calorimeter (Intramedic, Gentofte, Denmark) with a canopy was applied at time=180 min. A 15-min period collection of respiratory gases was used to estimate resting energy expenditure and the respiratory quotient. Fat oxidation rates were calculated as previously described (22). Due to technical problems, calorimetry measurements were only performed in seven subjects.

Statistics

All statistical analyses and graphs were made using Stata 13 (College Station, Texas, USA) and SigmaPlot 11 (San

Jose, California, USA). Unequal distributed data were logarithmically converted. P -Values <0.05 were considered significant. Two-way repeated measure ANOVA was used to test for interactions in sCD163 concentrations during time in the study period and between groups. In the event of significant interaction, multiple pairwise comparisons were performed using the Student–Newman–Keuls method. Correlations between ΔGIR and ΔsCD163 were evaluated using parametric linear regression. Correlations between sCD163 and other outcomes were evaluated using a mixed model linear regression adjusting for the crossover design and reuse of test subjects.

Results

Subjects

The test subjects had a median age of 26 years (range: 25–32), a median BMI of 23 kg/m^2 (range: 22–26) and a median body weight of 79 kg (range 68–85 kg) as reported previously (16).

Concentrations of sCD163

The mean concentration of sCD163 was comparable at baseline (placebo and LPS at time=0 min, $P=0.78$) but increased rapidly 2–3-fold and remained elevated throughout the study period during LPS conditions compared with placebo (Fig. 1, two-way repeated measure

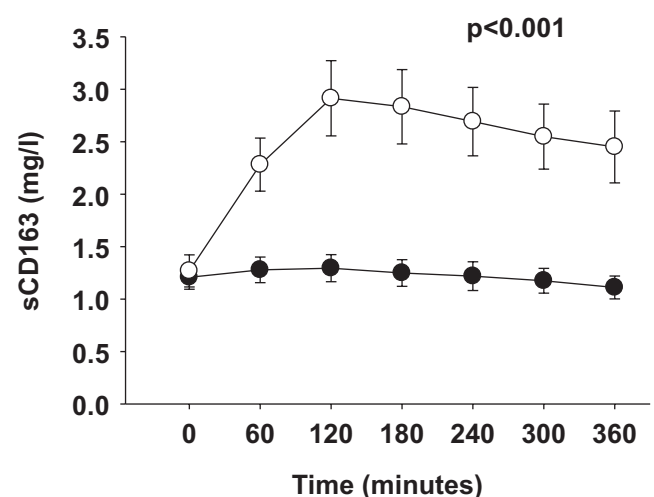


Figure 1

Plasma sCD163 concentrations. The mean plasma concentration ($\pm\text{s.e.m.}$) of sCD163 during the study period is shown for placebo (●) and LPS (○) conditions. Repeated measurement two-way ANOVA analysis was used to test for differences between groups. $N=8$.

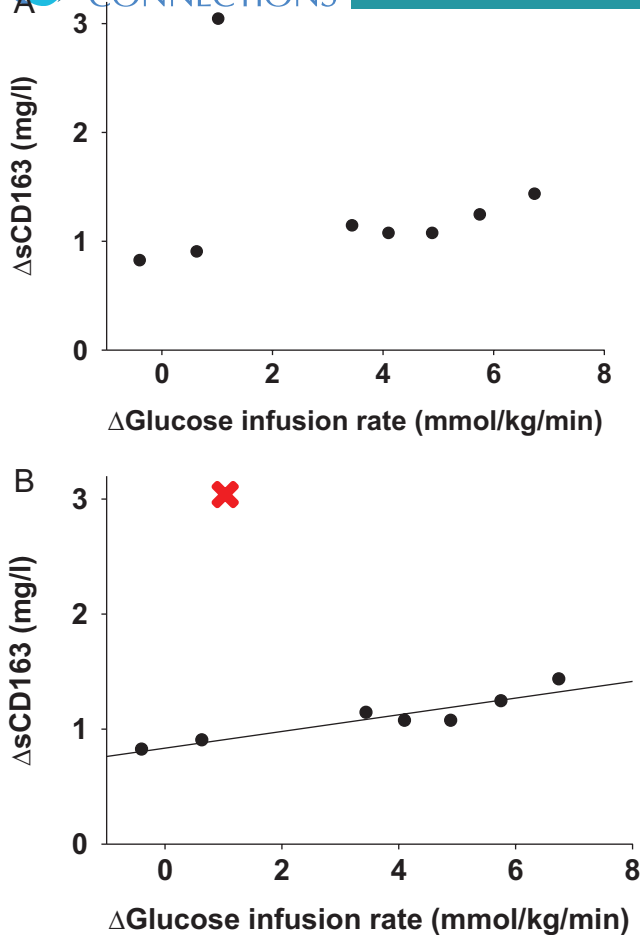


Figure 2 Insulin resistance and sCD163. The change in plasma concentrations of sCD163 ($\Delta sCD163 = sCD163_{LPS} - sCD163_{placebo}$) and the change in glucose infusion rates during the hyperinsulinemic euglycemic clamp period (GIR, $\Delta GIR = GIR_{placebo} - GIR_{LPS}$) during LPS and placebo exposures are shown for the eight test subjects (A). A parametric linear regression analysis showed no significant correlation between $\Delta sCD163$ and ΔGIR ($P = 0.8$). $N = 8$. When excluding the outlier (marked with a red cross), parametric linear regression showed a significant ($P < 0.001$, $r^2 = 0.87$) positive correlation between ΔGIR (insulin resistance) and $\Delta sCD163$ (B). $N = 7$.

ANOVA interaction $P < 0.001$). One of the test subjects developed markedly higher concentrations of sCD163 (≈ 4 -fold increase) during time = 60–360 min compared with the other seven test subjects (≈ 2 -fold increase) under LPS conditions. All test subjects had roughly the same plasma concentration of sCD163 during placebo conditions (≈ 1.2 mg/L).

Insulin resistance

We used the hyperinsulinaemic euglycaemic clamp technique to obtain GIR values during both placebo and LPS conditions. There was no significant correlation between the increase in sCD163 concentrations ($\Delta sCD163 = sCD163_{LPS} - sCD163_{placebo}$) and the increase in insulin resistance ($\Delta GIR = GIR_{placebo} - GIR_{LPS}$) during LPS conditions compared with placebo (Fig. 2A). However, when excluding the abovementioned outlier, there was a significant (simple linear regression $P < 0.001$) positive correlation between $\Delta sCD163$ and ΔGIR (Fig. 2B).

Lipid metabolism

There was a significant positive correlation between plasma concentrations of FFA ($P < 0.005$), lipid oxidation rates ($P = 0.007$), $Ra_{palmitate}$ ($P < 0.005$) and plasma concentrations of BHB ($P = 0.006$) with the plasma concentrations of sCD163 at time ≈ 240 min (Fig. 3 and Table 1). Additionally, sCD163 concentrations also had a significant ($P < 0.005$) positive correlation with the phosphorylated amount of HSL (involved in lipolysis) in SAT biopsies (Fig. 3).

Inflammation

The peak increments in plasma cortisol, glucagon, IL-6, IL-10 and TNF- α all had a significant (all, $P < 0.005$) positive

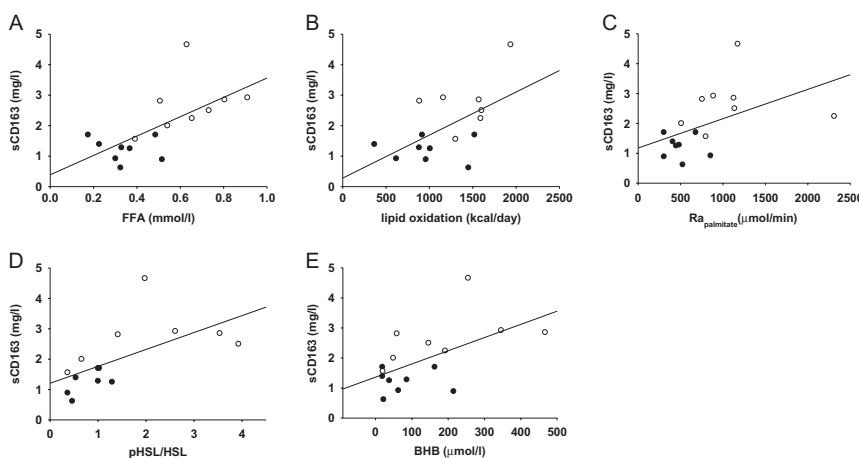


Figure 3 Lipid metabolism and sCD163. Data are presented as dot plots showing (A) plasma concentrations of free fatty acids (FFAs), (B) lipid oxidation rates, (C) rate of appearance for palmitat ($Ra_{palmitate}$), (D) ratio of phosphorylated hormone-sensitive lipase at ser⁶⁶⁰ (HSL) to content of HSL (pHSL/HSL) and (E) plasma concentrations of β -hydroxybutyrate (BHB) on the horizontal axis and concentrations of sCD163 on the vertical axis following exposure to placebo (●) and LPS (○). All measurements were performed around time = 240 min (at the end of the non-insulin-stimulated period of the trial day). A mixed model linear regression analysis was used to test for correlations. $N = 8$ in graphs (A), (C) and (E). $N = 7$ in graphs (B) and (D).

Table 1 Correlations between sCD163 and metabolic and inflammatory parameters.

sCD163	β -Coefficient	95% CI	P-Value
Metabolism			
FFA (mmol/L)	3.2	1.5; 4.9	<0.005
Lipid oxidation (kcal/day)	0.001	0.0004; 0.002	0.007
Ra _{palmitate} (μ mol/min)	3.3	1.8; 4.8	<0.005
pHSL/HSL	0.6	0.2; 0.9	<0.005
BHB (mmol/L)	0.004	0.001; 0.008	0.006
Inflammation			
IL-6 (ng/mL)	0.0008	0.0006; 0.001	<0.005
IL-10 (ng/mL)	0.01	0.005; 0.02	<0.005
TNF- α (ng/mL)	0.003	0.003; 0.003	<0.005
Glucagon (μ g/L)	0.02	0.01; 0.03	<0.005
Cortisol (μ g/mL)	0.008	0.006; 0.010	<0.005
Heart rate (beats/min)	0.05	0.03; 0.06	<0.005
Temperature ($^{\circ}$ C)	0.6	0.4; 0.8	<0.005

Correlations were evaluated using a mixed model linear regression. The β -coefficient, 95% confidence interval (95% CI) and P-value of the tests are shown.

BHB, β -hydroxybutyrate; FFA, free fatty acids; HSL, hormone-sensitive lipase; IL, interleukine; Ra, rate of appearance; TNF, tumour necrotic factor.

correlation with the peak increment of sCD163 (Fig. 4 and Table 1). Additionally, the peak in body temperature and heart rate also correlated significantly (both, $P < 0.005$) with the peak concentration of sCD163 (Table 1).

Discussion

Here, we demonstrate how sCD163 concentrations increased during the hours following LPS exposure. As novel findings, we show significant positive correlations between sCD163 and insulin resistance, different key factors in lipid metabolism, pro-inflammatory cytokines and inflammatory hormones. These findings bring

new insight and understanding to the field of research concerning obesity, insulin resistance, DM2 and NAFLD and suggest macrophages to play an important role.

Exposure to LPS rapidly increased sCD163 concentrations, which remained elevated for the six-hour trial period, as reported in previous studies investigating sCD163 following LPS administration (13, 17). These studies reported slightly higher concentrations of both sCD163 (≈ 5 mg/L vs ≈ 2 mg/L in our study) and TNF- α (≈ 700 pg/mL vs ≈ 400 pg/mL in our study). Most likely, this difference is due to the higher doses of LPS given in these studies (4 ng/kg) compared with the dose given in our study (1 ng/kg).

The sCD163 has already been associated with insulin resistance, obesity and DM2 (3, 23, 24). Studies investigating sCD163 and insulin resistance have primarily used the HOMA index to quantify insulin resistance, while only few have used the hyperinsulinemic euglycemic clamp technique (25), which is considered the gold standard method to measure insulin resistance. We used the hyperinsulinemic euglycemic clamp and found a significant positive correlation between the change in sCD163 and the change in insulin resistance (change in GIR = Δ GIR) when excluding the outlier from our data. These findings are in accordance with the abovementioned studies, but notably these studies were cross-sectional observational studies investigating obese, prediabetic and DM2 patients. In contrast, we applied a randomised crossover design and explored the acute changes in sCD163 and insulin resistance, thereby excluding possible inter-individual confounding.

We extensively investigated lipid metabolism using palmitate tracer technique, indirect calorimetry, SAT biopsies and blood sample collection during both placebo and LPS exposures demonstrating how concentrations

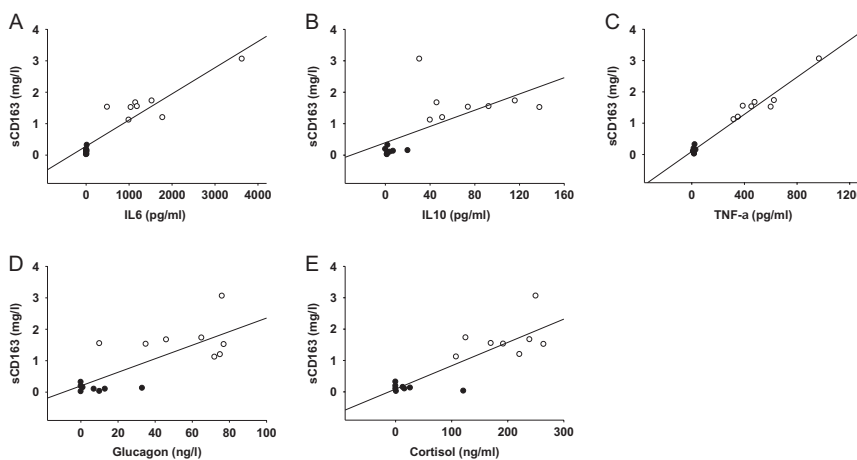


Figure 4 Inflammatory cytokines/hormones and sCD163. Data are presented as dot plots showing peak plasma concentrations of (A) interleukin (IL)-6, (B) IL-10, (C) tumour necrotic factor (TNF)- α , (D) cortisol and (E) glucagon on the horizontal axis and peak plasma concentrations of sCD163 on the vertical axis following exposure to placebo (●) and LPS (○). $N = 8$ in all graphs. A mixed model linear regression analysis was used to test for correlations.

of sCD163 were positively correlated with accelerated lipolysis, increased plasma FFA, lipid oxidation, $Ra_{\text{palmitate}}$ and phosphorylation of HSL in SAT biopsies. The increased circulating FFA may result in adipose tissue insulin resistance with ectopic lipid deposition, which has been proposed to precede insulin resistance in muscle and liver (26, 27, 28). Ultimately, elevated circulating FFAs are beta-oxidised in the liver resulting in increased concentrations of ketone bodies, e.g. BHB. Here, we demonstrated how plasma concentration of sCD163 correlated positively with plasma concentrations of BHB ($P < 0.005$) in agreement with the elevated circulating levels of FFA providing increased substrate supply to the liver.

We used a human model simulating the initial stage of sepsis in healthy lean subjects. Albeit these subjects were not suffering from obesity, DM2 and NAFLD, the model inducted metabolic adaptations characterising these conditions: insulin resistance and increased lipolysis. Thus, conditions such as obesity, DM2 and NAFLD are all characterised by chronic low-grade inflammation; on the other hand, we investigated an acute and more pronounced inflammatory condition. It is well known that the release of cytokines following LPS exposure is dynamic and changes during time (10). Furthermore, intracellular signals involved in the regulation of lipolysis also change during time (18, 19). Extrapolations should therefore be done with caution. Despite these facts, our results indicate that sCD163 and thereby macrophage activation may play an important role in the adaptations occurring in lipid and glucose metabolism during the acute phase of inflammation.

The increment in sCD163 levels following LPS exposure was highly correlated with TNF- α , which is in line with preexisting literature indicating that TNF- α and sCD163 are released by ectodomain shedding following LPS attachment to the TLR-4 and subsequently activation of the TNF- α converting enzyme (TACE/ADAM17) in macrophages (13, 17, 29). Even though several studies have already shown that the shedding of sCD163 can be induced by LPS (17), PMA (phorbol 12-myristate 13-acetate) (30), oxidative stress (31) and thrombin (32), most of these are *in vitro* studies using monocyte/macrophage cell cultures with only sparse human *in vivo* studies investigating sCD163 preceding LPS (17). Decisively, sCD163 increases rapidly and in close alignment with TNF- α , but the exact function of sCD163 remains elusive. In addition to TNF- α , we also found other inflammatory parameters such

as IL-6, IL-10, cortisol, glucagon, body temperature and heart rate that correlated positively with sCD163 concentrations ($P < 0.05$). All these abovementioned inflammatory parameters peaked in alignment with sCD163 concentrations within the first 3 h preceding LPS exposure (data not shown), emphasising sCD163 as an inflammatory biomarker.

Strengths of the study include the randomised controlled crossover design and use of human test subjects especially considering the fact that the majority of studies investigating sCD163 apply an observational cross-sectional or *in vitro* design. Additionally, we thoroughly investigated metabolic adaptations using ^3H -palmitate tracer technique, hyperinsulinemic euglycemic clamp, indirect calorimetry and western blotting of SAT biopsies. Our study is limited by the use of only eight healthy, lean, male test subjects and the use of a disease model. Therefore, extrapolation of results to clinical conditions should be done with caution.

In conclusion, LPS exposure elevates plasma sCD163 concentrations, which was positively correlated with insulin resistance, key elements of lipid metabolism and inflammatory cytokines (e.g. TNF- α). These novel findings support preexisting theory that inflammation and macrophage activation play a vital role in lipid metabolic adaptations during conditions such as obesity, sepsis, DM2 and NAFLD.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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