CMKLR1 activation ex vivo does not increase proportionally to serum total chemerin in obese humans

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Abstract

Prochemerin is the inactive precursor of the adipokine chemerin. Proteolytic processing is obligatory for the conversion of prochemerin into active chemerin and subsequent regulation of cellular processes via the chemokine-like receptor 1 (CMKLR1). Elevated plasma or serum chemerin concentrations and differential processing of prochemerin have been reported in obese humans. The impact of these changes on CMKLR1 signalling in humans is unknown. The objective of this pilot study was to develop a cellular bioassay to measure CMKLR1 activation by chemerin present in human serum and to characterise how obesity modifies serum activation of CMKLR1 under fasted and fed conditions. Blood samples were collected from control (N=4, BMI 20–25) and obese (N=4, BMI >30) female subjects after an overnight fast (n=2) and at regular intervals (n=7) following consumption of breakfast over a period of 6 h. A cellular CMKLR1-luminescent reporter assay and a pan-chemerin ELISA were used to determine CMKLR1 activation and total chemerin concentrations, respectively. Serum total chemerin concentration (averaged across all samples) was higher in obese vs control subjects (17.9±1.8 vs 10.9±0.5 nM, P<0.05), but serum activation of CMKLR1 was similar in both groups. The CMKLR1 activation/total chemerin ratio was lower in obese vs control subjects (0.33±0.04 vs 0.58±0.05, P<0.05). After breakfast, serum total chemerin or CMKLR1 activation did not differ from baseline values. In conclusion, the unexpected observation that obese serum activation of CMKLR1 did not match increased total chemerin concentrations suggests impaired processing to and/or enhanced degradation of active chemerin in serum of obese humans.

Introduction

After characterisation as an adipokine by our group in 2007, research concerning chemerin and its relationship to obesity has risen steadily. Chemerin is secreted as an inactive precursor prochemerin, which undergoes processing at distinct sites on the C-terminus by proteases of the inflammatory, coagulation and fibrinolytic cascades to become biologically active chemerin (herein active chemerin) (1, 2, 3, 4). Several isoforms of active chemerin can be produced that vary in their C-terminal amino acid, their ability to bind and activate the chemokine-like receptor 1 (CMKLR1) as well as their capacity to facilitate their distinctive inflammatory, immune and metabolic processes (1, 2, 3, 4, 5, 6, 7, 8, 9, 10).
The established biological functions of chemerin including the recruitment of immune cells to sites of inflammation (3, 9, 11) and the regulation of adipogenesis, lipolysis and glucose metabolism (12, 13, 14, 15, 16, 17) have implicated chemerin as a target of interest in the pathophysiology of obesity and the metabolic syndrome. In line with a reputed role for chemerin in obesity, total chemerin concentrations are elevated in murine and human obesity, are correlated to markers of adiposity including body mass index (BMI), waist-to-hip ratio (WHR) and fat mass and decline after surgical, diet and activity-based weight loss (13, 14, 18, 19, 20, 21, 22). Independent observations in humans, animals and cell models further support adipose tissue/adipocytes as a significant and modifiable source of circulating total chemerin (pro and active chemerin) (14, 18, 19, 23, 24, 25, 26, 27). The exact role, beneficial or detrimental, that chemerin plays in obesity is complicated by the fact that both pro- (28, 29, 30) and anti-inflammatory effects (31, 32, 33) have been described for chemerin/CMKLR1 signalling. Furthermore, the biological effects of chemerin are not exclusively dependent upon total circulating concentrations. Rather, the mechanisms by which prochemerin is activated, the relative abundance of chemerin isoforms and their activity at CMKLR1 are important determinants of biological function (1, 2, 4, 6, 8, 19). Accordingly, a significant limitation of the majority of previous studies has been a reliance on quantifying total but not active chemerin. As such, much of the clinical chemerin research to date has inferred a role for elevated chemerin in obesity and metabolic disorders based on the assumption that parity exists between total and active chemerin concentrations. However, this assumption is not supported by in vitro or in vivo studies that demonstrated a large discrepancy between total and active chemerin under a TNF-mediated acute inflammatory response and in synovial fluid of arthritic patients (18, 19, 34). A disparity between total and active chemerin is further supported by a recent human study that discovered elevated concentrations of shorter and previously uncharacterised chemerin isoforms in plasma of obese humans compared to lean controls (23). How these novel degraded chemerin isoforms influence CMKLR1 activation and the biological processes that ensue in obesity remains unknown. The objective of our study was to utilise a cellular bioassay to measure CMKLR1 activation by chemerin present within human serum samples to address whether CMKLR1 activation parallels total chemerin concentrations in obese and normal weight humans under fed and fasted conditions.

Materials and methods

Subjects

The study was open to men and women, ages 18–55 years, with a BMI of 20–25 (control weight, n = 4) or BMI >30 (obese, n = 4). Subjects were recruited through the Izaak Walton Killam (IWK) Health Centre. Exclusion criteria included subjects taking medications associated with enhanced chemerin production (insulin and metformin) (26) or inhibition of degradation (ACE inhibitors) (6, 35). Additionally, subjects with renal impairment (creatinine clearance <60 mL/min), type 2 diabetes mellitus or who have previously undergone gastric bypass surgery were excluded as these conditions may influence chemerin concentrations independent of obesity (36). The IWK Research Ethics Board approved the experimental protocol (Project # 1013395), and all participants gave their written informed consent before their inclusion in the study.

Clinical procedures

Subjects fasted overnight (10–12 h) prior to their arrival to the Clinical Research Unit of the IWK Health Centre at approximately 06:00 h on the study day. All subjects remained in the unit for the duration of the study. Upon admission, subjects were assessed for anthropometric measures of body fat including BMI, WHR and waist circumference in addition to their systolic (SBP) and diastolic (DBP) blood pressure and heart rate. A saline lock was subsequently placed, and fasted blood samples were collected at 07:00 h (baseline 1) and 08:00 h (baseline 2). Immediately after collection of the second sample at 08:00 h, subjects were offered a selection of breakfast foods (fruit cups, fresh fruit, assorted bagels, muffins and cereals, yogurt, cheese and hard-boiled eggs), beverages (fruit juices, milk, tea and coffee) and condiments (cream cheese, peanut butter and sugar). Subjects chose from these breakfast items without any restrictions and were allowed to consume breakfast over a 30-min period. Blood samples were subsequently taken at 08:30, 09:00, 09:30, 10:00, 11:00, 12:00 and 13:00 h. Two samples (4 mL each) were collected at each time point and placed into a 3.2% sodium citrate tube for plasma preparation and an uncoated tube for serum. Based on reported time for maximal activation of chemerin in serum (9), blood samples taken for serum were left to clot at room temperature for 2 h after collection. All samples
were centrifuged at 2500 \( g \) for 5 min at 4°C, aliquoted and stored at \(-70°C\) until the appropriate analysis was performed.

**Quantifying CMKLR1 activation using the CMKLR1 bioassay**

The CMKLR1 bioassay methods were based on our previously reported protocol for measuring CMKLR1 activation by chemerin in serum and adipocyte media samples \((18, 19)\). Briefly, a genetically modified HEK293 cell line (HTLA) that constitutively expresses a fusion protein comprised a tobacco etch virus (TEV) protease linked to human \( \beta \)-arrestin2, and a transcriptional-transactivator (tTA)-dependent luciferase reporter gene was transiently transfected with a plasmid-expressing human \( CMKLR1 \) fused to a tTA via a TEV protease recognition sequence \((37)\). A batch transfection

| Parameter | Normal weight (BMI 20–25) | Obese (BMI >30) | Ref. range |
|-----------|---------------------------|-----------------|------------|
| Physical characteristics | | | |
| Age (years) | 30.8±11.6 | 39±8.0 | NA |
| Weight (kg) | 66.8±12.1 | 106.0±18.6* | NA |
| Height (cm) | 170.2±9.8 | 163.5±6.0 | NA |
| Waist-to-hip ratio (WHR) | 0.81±0.03 | 0.87±0.05 | ≥0.85* |
| Body mass index (BMI) (kg/m\(^2\)) | 23.0±1.3 | 38.8±7.5* | ≥30\(^b\) |
| Cardiovascular and renal | | | |
| Heart rate (beats/min) | 74±3 | 72±20 | NA |
| Systolic BP (mm/Hg) | 116.8±9.8 | 125.3±14.6 | NA |
| Diastolic BP (mm/Hg) | 72.0±8.5 | 78.5±14.3 | NA |
| Creatinine (µmol/L) | 59.0±8.5 | 58.8±7.5 | <98 |
| eGFR (mL/min) | 117.1±21.1 | 99.4±19.8 | ≥90 |
| Metabolism/inflammation | | | |
| Insulin (pmol/L) | 69±22 | 118±118 | ≤209 |
| Glucose (mmol/L) | 4.65±0.48 | 5.53±0.96 | 3.3–5.6 |
| Triglycerides (mmol/L) | 0.78±0.17 | 1.85±1.08 | 0.5–1.6 |
| Free fatty acids (µmol/L) | 573±168 | 490±239 | 100–900 |
| Cholesterol (mmol/L) | 3.93±0.43 | 4.55±0.87 | 2.9–5.7 |
| Hs-CRP (mg/L) | 8.25±11.6 | 8.23±6.17 | 0–5.0 |
| PAI (ng/mL) | 4.48±0.44 | 9.49±2.26* | NA |
| Immunological | | | |
| White blood cells (x10^9/L) | 6.67±1.77 | 5.65±1.05 | 4.0–11 |
| Abs neutrophils (x10^9/L) | 3.90±1.11 | 3.33±1.24 | 1.5–8.0 |
| Abs lymphocytes (x10^9/L) | 1.80±0.71 | 1.80±0.29 | 1.5–5.0 |
| Abs monocytes (x10^9/L) | 0.63±0.14 | 0.38±0.10* | 0.0–1.0 |
| Abs eosinophils (x10^9/L) | 0.30±0.13 | 0.15±0.06 | 0.0–0.5 |
| % Neutrophils | 59.2±4.0 | 57.2±10.9 | 44–79 |
| % Lymphocytes | 26.5±5.3 | 33.3±10.5 | 28–48 |
| % Monocytes | 9.4±1.2 | 6.7±1.2* | 1.0–9.0 |
| % Eosinophils | 4.5±2.3 | 2.3±0.83 | 0.0–5.0 |
| % Basophils | 0.37±0.06 | 0.43±0.29 | 0.0–1.0 |
| Haematological | | | |
| Red blood cells (x10^12/L) | 4.17±0.39 | 4.27±0.31 | 4.1–5.1 |
| Haemoglobin (g/L) | 128.3±9.1 | 112.3±10.6* | 120–160 |
| Haematocrit (L/L) | 0.39±0.03 | 0.35±0.03 | 0.36–0.46 |
| Mean corpuscular volume (fl) | 92.4±3.6 | 82.3±1.9* | 77–102 |
| Mean corpuscular haemoglobin (pg) | 30.8±1.0 | 26.3±0.9* | 26.0–35.0 |
| Mean corpuscular haemoglobin concentration (g/L) | 334±10 | 320±15 | 320–370 |
| Red cell distribution width (%) | 12.6±0.3 | 15.3±0.2* | 12.2–14.3 |
| Platelet count (x10^10) | 225±61 | 258±45 | 186–353 |
| Mean platelet volume (fl) | 10.9±0.4 | 10.6±1.2 | 6.8–11.2 |

All of the above parameters were measured in the 1st morning baseline plasma or serum samples after an overnight fast. All values are listed as the mean ± s.d. mean (n = 4 per group). The typical reference values for various parameters are shown in the right hand column.

* A WHR ≥0.85 was used as the cutoff to define central obesity. ** A BMI of ≥30 kg/m² to define obesity. The estimated glomerular filtration rate (eGFR) was determined from the measured serum creatinine concentration using the Cockcroft–Gault equation with correction for ideal body weight. *The values were significantly different (P < 0.05) compared to the control group by both unpaired t-test.

NA, not applicable; PAI-1, plasminogen activator inhibitor 1.
mixture was prepared containing (per well) 40 µL Opti-MEM, 200 ng human CMKLR1-tTA plasmid, 200 ng β-galactosidase reference plasmid and 2 × 10^4 HTLA cells (at passage 5–20) in 160 µL DMEM supplemented with 0.4 µL of 1 mg/mL polyethylenimine (PEI) and added to a 96-well plate. After 24h, the transfection mix was aspirated and replaced with 50 µL of diluted (1:10 with optimum) human serum. To match the sample compositions, recombinant human chemerin (R&D Systems) 50 µL standards were prepared in heat-inactivated bovine serum diluted 1:10 in Opti-MEM (0.1, 0.3, 0.6, 1, 3, 6, 10 and 30 nM final concentrations). In this assay, activation of CMKLR1 leads to the recruitment of the β-arrestin2-TEV protease to the C-terminus of the CMKLR1-tTA fusion protein, resulting in tTA cleavage and migration of free tTA to the nucleus where it transcribes the luciferase reporter gene. After 16h, the serum or standards were aspirated and the cells were incubated for 5 min with shaking (1000 rev min⁻¹) in 100 µL reporter lysis buffer (RLT; Promega) followed by a single rapid freeze/thaw cycle. To quantify the luciferase activity, 10 µL of lysate or RLT blank was transferred to a 96-well white luminometer plate. Eighty microlitres of luciferase assay reagent (Promega) was injected into each well, and the luminescence was monitored for 10 ms using a Biotek synergy HT plate reader (Biotek). For the β-galactosidase assay, 30 µL of lysate or blank was transferred to a clear 96-well plate and incubated with 30 µL of 2× β-galactosidase assay buffer (200 mM NaPO₄ pH 7.3, 2 mM MgCl₂, 100 mM 2-mercaptoethanol and 1.33 mg/mL ortho-nitrophenyl-β-galactoside (ONPG)) for 15 min at 37°C. The reaction was stopped by the addition of 100 µL of 1 M Na₂CO₃, and the absorbance at 420 nm was measured. The luciferase and β-galactosidase measurements were corrected for the respective blanks.

**Table 2** Serum CMKLR1 bioassay performance.

| Chemerin standards (nM) | Predicted chemerin concentrations | Accuracy | Inter-assay % CV | Intra-assay % CV |
|-------------------------|----------------------------------|----------|------------------|-----------------|
| 0.10                    | 142.8%                           | 51.4%    | 26.8%            |
| 0.30                    | 102.8%                           | 13.6%    | 12.6%            |
| 0.60                    | 97.7%                            | 13.8%    | 5.6%             |
| 1.00                    | 97.8%                            | 4.5%     | 12.8%            |
| 3.00                    | 102.2%                           | 35.9%    | 14.5%            |
| 6.00                    | 79.2%                            | 47.2%    | 21.2%            |
| 10.00                   | 64.6%                            | 55.2%    | 46.8%            |
| 30.00                   | 56.6%                            | 98.5%    | 61%              |

The assay performance values were based on 4 independent experimental replicates with each standard measured in duplicate.
equivalents) in each sample was interpolated based on its luciferase/β-galactosidase activity.

**Total chemerin measurements**

Total chemerin was quantified using a human pan-chemerin ELISA (LOT # 1316723, R&D Systems) according to the manufacturer’s instructions and our previously reported methods (38). A dose-response curve using a best fit of a 4-parameter logistic equation was used to calculate the total chemerin concentration.

**Quantification of other factors in plasma and serum**

The 1st baseline sample was used for the baseline complete blood cell count, metabolic/inflammatory and serum creatinine analysis. These assays were performed in the IWK clinical chemistry facility using standard methods. In addition, we used commercially available analytical kits to quantify glucose, free fatty acids (FFA), high-sensitivity C-reactive protein (hs-CRP), cholesterol, triglycerides (Cayman Chemical) and insulin (Millipore) in all samples in order to compare changes in these parameters in the post-prandial vs baseline period. A commercially available ELISA assay was used for measures of plasminogen activator inhibitor-1 (PAI-1) in the baseline samples (Abcam). The manufacturer’s instructions were followed for all assays.

**Data and statistical analysis**

The primary outcome was to compare the average CMKLR1 activation, total chemerin and CMKLR1 equivalents). The effect of obesity on serum total chemerin and serum activation of CMKLR1. Serum total chemerin (A), serum activation of CMKLR1 expressed as chemerin157 equivalents (C) and CMKLR1 activation/total chemerin (E) were measured in 4 obese (BMI >30) and 4 normal weight females (BMI 20–25) after an overnight fast (baseline, BL), which was followed by breakfast (BF) consumed over 30 min and then intermittent sample measurements over a 5.5-h duration in the post-breakfast period. The between-group statistical analysis was performed on the average data for all time points combined (B, D, F). **P < 0.01 and ***P < 0.001 compared to the BMI >30 group, unpaired t-test.
activation/total chemerin ratios between the obese and control groups. For these variables, the average of 9 samples over time in plasma and serum in each patient was calculated. The average values were compared using an unpaired 2-tailed *t*-test. The secondary outcome was to evaluate for differences in average CMKLR1 activation, total chemerin and CMKLR1 activation/total chemerin ratios in the baseline vs post-prandial period within each group. For this analysis, the average of the two baseline measures were compared to the maximum and minimum values for CMKLR1 activation, total chemerin and CMKLR1 activation/total chemerin that were observed in the post-prandial period. A one-way analysis of variance with Bonferroni post hoc test was used. Differences between groups were considered significant when *P* < 0.05. SPSS, version 23 was used for statistical analysis.

**Results**

**Subject characteristics**

The 1st eight subjects that applied for entry into the study met the inclusion criteria and were enrolled after their initial screening visit. No subjects were excluded based on the predetermined exclusion criteria. Individual subject characteristics are summarised in Table 1. All subjects were female and were categorised as either normal weight (*n* = 4) with BMIs between 20 and 25 or obese (*n* = 4) with BMIs >30. As per the study inclusion criteria, weight and BMI were significantly higher in the obese vs control group. Age, height, WHR, cardiovascular and renal parameters were similar between groups. High-sensitivity C-reactive protein was more than two-fold higher than the upper normal values for one normal weight and 2 obese subjects, but did not differ between groups. Plasminogen activator inhibitor-1 (PAI-1) was significantly higher in the BMI >30 group. Immune cell counts fell with the normal reference range for both groups, although absolute and percent monocyte counts were significantly lower in the BMI >30 group. Haemoglobin, mean corpuscular volume and mean corpuscular haemoglobin were lower and red cell distribution width was higher in the obese vs normal weight groups and was suggestive of mild iron-deficiency anaemia. After breakfast, both groups displayed a significant rise in insulin concentration (after 1.5–2h) and a later reduction in free fatty acids (2.5–5h) (Supplementary Fig. 1 and Supplementary Table 1, see section on supplementary data given at the end of this article). In the post-prandial period, significant increases in glucose, triglycerides and cholesterol were observed in the normal weight but not obese group.

**CMKLR1 bioassay performance**

The CMKLR1 bioassay accuracy and inter- and intra-assay coefficients of variation for each standard curve chemerin concentration are shown in Table 2. The lower limit for the quantification of CMKLR1 activation occurred at approximately 100pM chemerin, but the standard curve typically overestimated the actual chemerin concentrations in this range, and the inter-assay and intra-assay CVs were unacceptably high. Between the

**Table 3** Post-prandial changes in serum total chemerin and CMKLR1 activation.

| Parameter                      | Baseline       | Maximum        | Minimum        | *t*<sub>max</sub> (h) | *t*<sub>min</sub> (h) |
|--------------------------------|----------------|----------------|----------------|-----------------------|-----------------------|
| Serum total chemerin (nmol/L)  |                |                |                |                       |                       |
| Normal weight                  | 10.4 ± 1.1     | 13.5 ± 1.4     | 8.69 ± 0.61    | 1.75 (1.5–3.0)        | 1.75 (1.5–5.0)        |
| Obese                          | 17.0 ± 3.1     | 22.9 ± 3.4     | 13.2 ± 2.2     | 4.5 (3.0–6.0)         | 4.0 (1.5–6.0)         |
| Serum CMKLR1 activation (expressed as nmol/L chemerin<sub>57</sub> equivalents) |                |                |                |                       |                       |
| Normal weight                  | 6.03 ± 1.28    | 7.45 ± 0.97    | 5.24 ± 0.98    | 4.0 (2.5–6.0)         | 2.0 (1.5–5.0)         |
| Obese                          | 5.70 ± 1.74    | 6.51 ± 1.99    | 5.34 ± 1.82    | 3.0 (2.0–4.0)         | 2.5 (2.0–6.0)         |
| Serum CMKLR1 activation/total chemerin ratio |                |                |                |                       |                       |
| Normal weight                  | 0.583 ± 0.136  | 0.710 ± 0.154  | 0.436 ± 0.091* | 4.0 (3.0–6.0)         | 1.75 (1.5–2.0)        |
| Obese                          | 0.333 ± 0.087  | 0.423 ± 0.145  | 0.259 ± 0.056  | 4.0 (2.0–6.0)         | 2.25 (1.5–2.5)        |
| Plasma total chemerin (nmol/L) |                |                |                |                       |                       |
| Normal weight                  | 10.3 ± 1.2     | 11.5 ± 1.2     | 7.56 ± 0.42    | 4.25 (1.5–6)          | 4.0 (2.5–5.0)         |
| Obese                          | 14.0 ± 3.6     | 17.5 ± 2.0     | 12.2 ± 3.0     | 3.0 (2.5–6.0)         | 3.0 (1.5–6.0)         |

*t*<sub>max</sub> and *t*<sub>min</sub> are the time at which the maximum and minimum values occurred, respectively. Baseline, maximum and minimum values are expressed as mean ± 1.0. and *t*<sub>max</sub> and *t*<sub>min</sub> as median with lower and upper values in brackets. For each parameter, the average of the two baseline samples was compared to the maximum (or minimum) value obtained in the post-feeding period using a one-way repeated-measures ANOVA, followed by Bonferroni post hoc comparison.

*P* < 0.05 compared to baseline.
concentrations of 0.3–1.0 nM, the assay was highly accurate and the inter- and intra-assay coefficients were in the acceptable range (<15%). The assay performance progressively deteriorated with increasing chemerin concentrations at above 3.0 nM. Two representative standard curves along with samples from two normal weight and two obese subjects demonstrated that a 1:10 dilution of serum samples provided predicted chemerin values within the most accurate and reproducible range (0.3–1.0 nM) of the standard curve (Fig. 1).

**Total chemerin was elevated in the serum of obese females but did not lead to greater CMKLR1 activation**

Serum total chemerin concentrations (measured by a pan-chemerin ELISA) were consistently higher in the obese vs normal weight group, and this was reflected by a significant 1.6-fold higher average total chemerin over the entire sampling duration (Fig. 2A and B). In comparison, CMKLR1 activation (expressed as chemerin_{157} equivalents) in the bioassay was similar between groups (Fig. 2C and D). In matched samples, the average apparent chemerin_{157} concentration determined by the CMKLR1 bioassay ranged between 43% and 66% (normal weight group) and 25% and 45% (obese group) of serum total chemerin concentrations. Consistent with this, the average ratio of CMKLR1 activation to total chemerin concentrations was significantly higher in the normal weight vs obese groups (Fig. 2E and F). Reflecting a small degree of fluctuation in serum total chemerin and CMKLR1 activation over time, the maximum and minimum values for total chemerin and CMKLR1 activation after feeding did not differ from baseline in the normal weight and obese groups (Table 3). The times at which the maximum (t\text{max}) and minimum (t\text{min}) values were observed were also highly variable (Table 3). With respect to the CMKLR1 activation/total chemerin ratio, a significant nadir was observed in the normal weight but not obese group at between 1.5 and 2 h after breakfast.

**Assessment of plasma total chemerin and plasma activation of CMKRL1**

Similar to serum, plasma total chemerin was significantly increased in obese vs normal weight subjects (Fig. 3A and B) and did not differ in the post-prandial period vs baseline in either group (Table 3). When data from all eight subjects were combined, the mean serum total chemerin concentration (14.4 ± 3.9) was significantly higher than the mean plasma total chemerin concentration (11.9 ± 3.3), P = 0.01 paired t-test. We attempted to measure CMKLR1 activation by plasma samples using the CMKLR1 bioassay. During assay development, it was determined that a 1:20 dilution of bovine plasma with 3.2% Na citrate provided optimal signal to background and generated chemerin standard curves with similar assay performance characteristics as observed for 1:10 diluted serum standards. However, when the subject plasma samples were diluted to 1:20, the level of CMKLR1 activation was at or below the lower limit of quantification.
in the assay. Therefore, these data are not presented, and firm conclusions regarding the activation of CMKLR1 by plasma chemerin were not possible.

Discussion

Previous studies examining total chemerin concentrations in humans and animals have resulted in the hypothesis that chemerin is relevant to obesity and obesity-associated comorbidities (12, 39, 40, 41, 42). However, by focusing solely on total chemerin, the majority of these studies have not accounted for chemerin processing to active forms and subsequent activation of CMKLR1. Thus, the biological significance of elevated chemerin in the context of human obesity remains unclear. Our proof-of-concept study has advanced understanding in this area by adopting a CMKLR1 cellular-reporter gene assay (18, 19, 37) to determine if CMKLR1 activation by human serum paralleled total chemerin concentrations and if this relationship differed in lean and obese subjects.

As expected, obese subjects had significantly increased serum total chemerin. Unexpectedly, this did not result in proportionally greater activation of CMKLR1 in the bioassay. The importance of this result is that functional alterations in chemerin/CMKLR1 signalling in obesity, whether pro- or anti-inflammatory may be less than expected based on measures of total chemerin alone. A possible explanation for the observed disparity between total chemerin concentrations and CMKLR1 activation by serum is that prochemerin is degraded to shorter chemerin products that were detected by the pan-chemerin ELISA but did not activate CMKLR1. Supporting the latter argument, Chang and coworkers demonstrated increased plasma total chemerin in obesity using the pan-chemerin ELISA assay, but using isof orm-specific ELISAs did not demonstrate significant changes in high-activity chemerin\textsubscript{155}, low-activity chemerin\textsubscript{155} and inactive prochemerin (23). Furthermore, the combined concentrations of chemerin\textsubscript{155}, chemerin\textsubscript{157} and prochemerin only accounted for 57% of total chemerin detected by the pan-chemerin ELISA. The remaining 43% was attributed to chemerin isoforms that underwent further C-terminal processing, resulting in shorter proteins. As the ability of chemerin to activate CMKLR1 depends primarily on the 9 C-terminal (149–157) residues (43), these shorter chemerin proteins would be predicted to be inactive. Additionally, it is plausible that these peptides may act as competitive antagonists for the active chemerin forms. However, both of these possibilities remain to be tested experimentally.

In addition to increased degradation, there could be reduced conversion of prochemerin to active chemerin isoforms. In one pathway, prochemerin is converted to chemerin\textsubscript{157} via a two-step process involving cleavage of amino acids 159–163 by plasmin or tryptase to form chemerin\textsubscript{158}, an isoform with low activity following removal of the C-terminal lysine by plasma carboxypeptidase B or N to form chemerin\textsubscript{157} (1, 4). In obese humans, increased circulating concentrations of PAI-1, a serum protease inhibitor that blocks the enzymatic conversion of plasminogen to plasmin, have been well documented (44). Consistent with this, baseline PAI-1 was elevated in our obese subjects, suggesting that the plasmin/carboxypeptidase pathway of chemerin\textsubscript{157} formation could be less active.

We report the novel observation that within subjects, total chemerin was elevated (21%) in serum compared to plasma. There are at least two possible explanations for this. Platelets store prochemerin and secrete it upon activation (1). Therefore, it is possible that platelet activation during blood clotting increases prochemerin release and total chemerin concentrations in serum vs plasma. The chemerin ELISA demonstrates lower reactivity towards prochemerin, chemerin\textsubscript{158} and chemerin\textsubscript{153} compared to chemerin\textsubscript{157} (23). Thus, it is also possible that the ELISA assay underestimates total chemerin concentrations in plasma, where prochemerin predominates while providing more accurate determinations in serum where chemerin\textsubscript{157} concentrations are higher.

We previously observed that serum chemerin concentrations in mice oscillated with time of day, and the pattern of oscillation was altered with obesity (18). Other studies have demonstrated that short-term or long-term changes in nutritional status (fasting and re-feeding) affected chemerin mRNA expression and chemerin protein secretion from adipocytes and/or serum chemerin concentrations (22, 45). These latter effects may have been related to insulin stimulation of prochemerin secretion by adipocytes (26, 45). Despite these observations, the majority of clinical experimental studies have assessed plasma or serum chemerin concentrations in a single sample after an overnight fast and have thus not accounted for potential effects of time of day or nutritional effects on chemerin. To begin to address this gap in understanding, the clinical sampling procedures of our pilot study were designed to assess if serum total chemerin and CMKLR1 activation change acutely in association with insulin secretion.

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after breakfast. The observation of similar values for serum total chemerin and CMKLR1 activation in the post-prandial vs baseline periods suggested that the post-prandial insulin response did not appreciably affect serum chemerin concentrations and CMKLR1 activation. Furthermore, if insulin stimulation of adipose tissue chemerin production was linked to serum total chemerin, the rise and fall of chemerin should have been delayed relative to the insulin response and occurred over a narrow time range such as that observed for free fatty acid concentrations. This was not observed. Our results agreed with the findings of Bauer and coworkers in which a similar acute insulin increase produced by a fasting oral glucose tolerance test did not increase serum chemerin over a period of 1–2 h (46). Our results also expanded on these earlier findings by evaluating a typical breakfast, evaluating more time points over a longer duration and showing that immunodetectable chemerin and CMKLR1 activation are similarly unaffected. Our findings differed from those of Tan and coworkers, which demonstrated an approximate 2-fold increase in plasma chemerin 4 h after initiating a hyperinsulinaemic normoglycemic clamp (26). As the peak insulin concentrations observed in our study were similar to those in the study by Tan and coworkers, our data reaffirm that insulin regulation of chemerin is most likely to occur in conditions when there are sustained rather than acutely elevated insulin concentrations.

There are a number of limitations to our study. The bioassay provided a measure of CMKLR1 activation expressed as chemerin$_{157}$ equivalents but did not measure the ‘true’ chemerin$_{157}$ concentration and we were unable to reference the CMKLR1 bioassay results to purified prochemerin or other chemerin isoforms. Based on the generation of multiple chemerin isoforms by adipocytes and the presence of multiple chemerin isoforms in plasma (5, 23), there are also likely to be multiple chemerin forms in serum, which may be active or inactive to varying degrees and/or activate the CMKLR1 bioassay with a differing stoichiometry than chemerin$_{157}$. Furthermore, it is unknown if there could be interactions between multiple chemerin isoforms in human serum that could influence the activation of the receptor compared to chemerin$_{157}$ alone.

A second limitation is that the CMKLR1 bioassay only assesses one aspect of CMKLR1 activation and signalling, that being the β-arrestin2-mediated pathway. Emerging research supports that chemerin/CMKLR1 signalling is complex and not completely defined (47). The pleiotropic functions of chemerin involves classical CMKLR1 signalling via Ga$_{i/o}$ proteins as well as β-arrestin2 with the involvement of several intracellular effector pathways including intracellular calcium, p38 mitogen-activated protein kinases (MAPKs), extracellular signal-related kinases (ERK1/2) and phosphoinositide-3-kinase/protein kinase B (PI3K/AKT) and the RhoA/Rock pathway (3, 47, 48, 49). Although we can conclude that the CMKLR1 β-arrestin2 pathway is activated by human serum, we cannot currently make inferences regarding biased ligand signalling, activation of specific downstream effector molecules and the resulting impacts on cellular functions and/or CMKLR1 internalisation and desensitisation.

A third limitation was that we assessed the activation of CMKLR1 and not other chemerin receptors. In addition to CMKLR1, chemerin binds G-protein receptor 1 (GPR1) and the non-signalling chemokine C–C motif receptor-like 2 (CCRL2) (30, 37). The majority of chemerin functions have been attributed to CMKLR1 signalling (50). However, recent studies have confirmed functional human GPR1 signalling, which mediates chemotaxis of human gastric adenocarcinoma cells (47). Future studies that compare β-arrestin2 and Ga$_{i/o}$ protein-based CMKLR1 and GPR1 bioassays along with signalling reporter assays for the downstream pathways (as described by Rourke and coworkers (47)) will be important to further delineate the mechanisms of chemerin signalling and determine whether β-arrestin2 vs Ga$_{i/o}$ biased signalling can be influenced by the variation in chemerin isoforms produced in normal weight vs obese subjects. Our results also cannot account for potential effects of CCRL2, which binds and increases local levels of active chemerin facilitating chemerin interactions with CMKLR1 on neighbouring cells (30).

Although the small number of subjects was a limitation, this was offset by the repeated sampling procedures used for the primary analysis. Furthermore, the mean differences in plasma total chemerin between obese and normal weight subjects were similar to historical data, providing confidence in our primary analyses (38, 46, 51). Given the small sample size, we conducted a conservative comparison of post-feeding minimum and maximum values to baseline for our secondary analysis. The relatively stable serum total chemerin concentrations and CMKLR1 activation during early daytime hours observed in our study is consistent with the results of Tan and coworkers in which plasma chemerin concentrations measured at 30 min to 4-h intervals over 24 h in a small group of control subjects demonstrated little fluctuation (26). However, it is possible that we missed significant changes, given the 30- to 60-min frequency of sampling and that we were
only able to sample for part of the day. Nonetheless, the pilot data provide the basis for sample size determinations for future studies aimed at conducting a more robust two-way mixed analysis of variance to assess the effects of time and obesity on chemerin concentrations and CMKLR1 and GPR1 activation.

The inability to measure CMKLR1 activation by plasma was a limitation. Plasma should better represent circulating active chemerin isoforms, whereas serum activation of CMKLR1 accounts for active chemerin that is formed during the coagulation process. It is possible that the relationships between time and obesity on CMKLR1 activation by plasma could differ from those observed for serum. The ability to detect CMKLR1 activation by serum but not plasma was likely the result of lower concentrations of active chemerin in plasma (10, 23) and is consistent with previous studies demonstrating that serum- but not plasma-mediated chemotaxis of CMKLR1-expressing β-lymphoma cells (9).

The literature, although mixed, suggests that gender has small or insignificant effects on plasma or serum total chemerin (52, 53, 54, 55, 56, 57). Furthermore, Chang and coworkers, did not observe an effect of gender on the plasma concentration of specific chemerin isoforms (23). However, as only women enrolled in the study, we cannot identify or rule out gender differences in serum activation of CMKLR1.

Our study included healthy obese subjects only. The assessment of whether the relationship between total chemerin and CMKLR1 activation changes with more severe obesity coupled with comorbidities remains to be determined.

In summary, our study is the first to identify a disparity between serum total chemerin and CMKLR1 activation in obesity, in that obese subjects displayed increased immunodetectable chemerin that did not lead to a comparable increase in ex vivo CMKLR1 activation. These results affirm our position that inferences regarding chemerin signalling or function based solely on measures of serum total chemerin concentrations should be made with extreme caution. Ideally, future clinical studies should include measures of total chemerin or specific chemerin isoforms (using isoform-specific antibodies or mass spectrometry) combined with bioassay measures of CMKLR1 and/or GPR1 activation.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/EC-16-0065.

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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Author contribution statement
J T performed the research, generated experimental data, analysed the data and co-wrote the manuscript. S D P conceived and designed the research and co-wrote the manuscript. K S served as the clinical study Co-ordinator/Pharmacist and recruited and assessed study subjects and edited the manuscript. C J S conceived and designed the research and edited the manuscript. S M supervised the clinical procedures as the study physician and edited the manuscript. K B G was the study's Principal Investigator, conceived and designed the research, analysed data and co-wrote the manuscript.

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