INTRODUCTION

Although obesity is the leading risk factor for metabolic syndrome, insulin resistance and type 2 diabetes, not all obese individuals go on to develop disease. Type 2 diabetes is a heterogeneous condition with aetiologies reflecting a complex interaction between genes and environment, including diet, physical activity and factors operating in early life. Among these exposures, low-grade inflammation (assessed by a range of markers including C-reactive protein, sialic acid, tumour necrosis factor (TNF) and interleukin-6 (IL-6)) has emerged as a consistent correlate of metabolic syndrome and a strong predictor of the risk of obesity and diabetes. Germ-free mice are protected of the gut microflora may be an important mediator of the risk of obesity and diabetes. Germ-free mice are protected from obesity and diabetes. Confirmation of these findings and elucidation of the role of the microbiota, gut damage and the pathways for translocation of bacterial debris, could open new avenues for prevention and treatment of type 2 diabetes.

OBJECTIVE: Emerging evidence from animal models suggests that translocation of bacterial debris across a leaky gut may trigger low-grade inflammation, which in turn drives insulin resistance. The current study set out to investigate this phenomenon, termed ‘metabolic endotoxia’, in Gambian women.

METHODS: In a cross-sectional study, we recruited 93 age-matched middle-aged urban Gambian women into three groups: lean (body mass index (BMI): 18.5–22.9 kg m⁻²), obese non-diabetic (BMI: ≥ 30.0 kg m⁻²) and obese diabetic (BMI: ≥ 30.0 kg m⁻² and attending a diabetic clinic). We measured serum bacterial lipopolysaccharide (LPS) and endotoxin-core IgM and IgG antibodies (EndoCAb) as measures of endotoxin exposure and interleukin-6 (IL-6) as a marker of inflammation.

RESULTS: Inflammation (IL-6) was independently and positively associated with both obesity and diabetes (F = 12.7, P < 0.001). LPS levels were highest in the obese-diabetic group compared with the other two groups (F = 4.4, P < 0.02). IgM EndoCAb (but not total IgM) was highly significantly reduced in the obese (55% of lean value) and obese diabetic women (30% of lean; F = 21.7, P < 0.0001 for trend) compared with lean women.

CONCLUSION: These data support the hypothesis that gut-derived inflammatory products are associated with obesity and diabetes. Confirmation of these findings and elucidation of the role of the microbiota, gut damage and the pathways for translocation of bacterial debris, could open new avenues for prevention and treatment of type 2 diabetes.

Keywords: type 2 diabetes; bacteria; metabolic endotoxia; The Gambia
and diabetes. Here we present data on the cross-sectional association between diabetes and endotoxemia in urban-dwelling lean, obese and obese diabetic Gambian women.

SUBJECTS AND METHODS
Three groups of women were recruited into the study from the urban Bakau district of the Gambia, West Africa. We selected a lean (body mass index [BMI]; 18.5–22.9 kg m$^{-2}$) and obese (BMI $>30$ kg m$^{-2}$) group from the general population using convenience sampling by screening households in the area and matching recruited women for age. A third group of obese women with diagnosed type 2 diabetes mellitus were recruited from the diabetic clinic at the Royal Victoria Teaching Hospital in the Gambian capital of Banjul. The study protocol was approved by the Scientific Coordinating Committee of Medical Research Council (MRC) Unit, the Gambia, and ethics permission was granted by the joint Gambian Government/MRC Ethics Committee. Full informed consent was obtained from each participant before enrolment into the study.

Participants were invited to attend a study morning at MRC Fajara in a fasted state. Weight was measured to the nearest 0.1 kg using daily calibrated stadiometer (Leicester height measure, Seca 214, Birmingham, UK) to the nearest 0.1 cm and BMI was defined as weight(kg)/height(m)$^2$. Body composition was further assessed by bioelectrical impedance analysis using the Tanita BC-418MA (Tanita Corporation) segmental analyser. Blood pressure was measured in triplicate using the automated Omron 705IT device (Omron, Kyoto, Japan). Plasma samples were collected in EDTA-coated collection tubes and frozen at $-80\,^\circ$C for analysis at a later date. Women were also interviewed during the study visit and data were recorded on their level of education and various assets owned by their household.

Insulin was measured using the AxSYM Microparticle Enzyme Immunoassay (Abbott Laboratories, North Chicago, IL, USA) with a detection limit of 1.0 $\mu$L insulin per ml. Glucose was measured on the VITROS 5600 Integrated System (Ortho-Clinical Diagnostics, Rochester, NY, USA) using the slide method with a detection range of 1.11–34.69 mmol l$^{-1}$. Plasma IL-6 was measured using the eBioscience IL-6 high-sensitivity ELISA with a detection limit of 0.03 pg ml$^{-1}$ [Bender MedSystems, Vienna, Austria: intra-assay CV: 4.9%]. Faecal calprotectin was measured using the CALPRO Calprotectin ELISA Test (ALP) with a detection limit of 6.25 pg ml$^{-1}$ [Firefly Scientific, Manchester, UK: intra-assay CV: 1.9%]. Calprotectin was assayed because faecal levels have been shown to correlate with the number of polymorphonuclear granulocytes entering the gut lumen. Serum endotoxin concentrations were measured using the Endpoint Chromogenic Limulus Amoebocyte Lysate Test for Gram-negative endotoxin (LAL; QCL-1000, Basel, Switzerland) with a detection limit of 0.1 EU ml$^{-1}$. Samples were carefully processed for this assay using endotoxin-free consumables. Total IgM was measured using an ELISA test kit (Immunology Consultants Laboratory Inc., Portland, OR, USA).

Finally, endotoxin-core IgM and IgG antibodies (EndoCAb) were measured by ELISA as described elsewhere.22 In brief, polystyrene microplates were pre-coated with an equimolar mixture of incomplete-core rough mutant endotoxins from each of four species of Gram-negative bacteria, complexed with polymyxin B. An eight-point standard curve was constructed using doubling dilutions of a pooled serum calibrated in EndoCAb median units and was used on every plate. Test samples were diluted 1:200 with dilution buffer and 100 $\mu$L of each sample added in duplicate to the plate and incubated for 60 min at 37 $^\circ$C. After washing four times with wash buffer, 100 $\mu$L of a diluted alkaline phosphatase-conjugated goat anti-human IgG or IgM (lg heavy-chain specific) antibody was added to each well and the plate was incubated for a further 60 min at 37 $^\circ$C. The washing step was then repeated four times and 100 $\mu$L of the substrate solution (1 mg ml$^{-1}$ disodium p-nitrophenylphosphate dissolved in 1 $\mu$L diethanolamine buffer) was added to the plate wells. The plate was incubated in the dark for 25 min and the reaction was stopped with 500 $\mu$L per well of 2 $\mu$L sodium hydroxide and read at 405 nm. Repeat blood samples were collected on participants in the lean and obese non-diabetic categories approximately 2 weeks after the initial study visit in order to assess the reliability of the endotoxin and endotoxin core antibody measurements.

Statistical analysis
Data were analysed using STATA 11 (Stata Corporation, College Station, TX, USA). All biochemistry variables had right-skewed distributions and residuals that were not normally distributed and were therefore log-transformed for the analysis. Geometric means (95% confidence intervals) have been presented for the descriptive statistics of these variables. Impaired fasting glucose was defined as fasting glucose $\geq 6.1–6.9$ mmol l$^{-1}$ whereas diabetes was defined as fasting glucose $>7.0$ mmol l$^{-1}$ in accordance with WHO recommendations.21 Insulin sensitivity was assessed by the QUICKI index (calculated as $1/($insulin(log) + glucose(oglog))) as several insulin values were below 20 pm, the lower limit for which the Homeostasis Model Assessment was validated.24 The EndoCAb values for IgG and IgM in the obese and obese diabetic groups were calculated as a percentage of the mean value for lean subjects. Thus, the lean group acted as a healthy control group and the values for the other two groups were normalised with respect to these controls; all EndoCAb values are expressed as percentage median units (PMUs) where the mean for lean individuals is 100 PMU or 100%.

Descriptive statistics have been presented for the study participants divided into the three recruitment groups: lean, obese, obese diabetic. Analysis of variance was used to assess the difference in markers of endotoxin and inflammation across the exposure groups. The association between obesity and diabetes status and the markers of endotoxin and inflammation was further assessed by using linear regression models first fitting obesity (yes/no) as the explanatory variable and then fitting both obesity and diabetes as binary variables to assess any independent effect.

RESULTS
In total, 93 women were recruited; mean BMI for the lean group was 20.8 kg m$^{-2}$, for the obese group it was 34.3 kg m$^{-2}$, and for the obese diabetic group was 33.3 kg m$^{-2}$ (Table 1). Total and trunkal fat masses were over 2.5-fold higher in the obese and obese diabetic groups (total fat: 14.2, 39.5 and 37.8 kg; trunkal fat: 6.3, 20.0 and 19.1 kg). The majority of women (55%) had been born in the urban region and the median amount of time spent living there for individuals who had been born elsewhere was 25 years. All women in the obese diabetic group were receiving medication to treat their condition. There was a marginal difference in age between the three groups (analysis of variance, F: 2.89; P-value: 0.06, df: 2,88), with women in the lean group on average 3.6 years younger than women in the diabetic group. As would be expected, diabetes prevalence (defined as fasting glucose above 7.0 mmol l$^{-1}$) was very different between the three groups with no women in the lean group, one woman in the obese group and 70% of the obese diabetic group (calculated as a percentage of the obese diabetic group). For the nine individuals in the diabetic group who did not have frank diabetes, their mean fasting glucose was still elevated at 5.9 mmol l$^{-1}$ (s.d. 0.7). Some of the markers of socioeconomic status were different between the groups with a much higher proportion of women in both obese groups having completed at least primary school education compared with lean participants. Women in both obese categories also reported owning a larger number of assets and having greater access to a flush toilet compared with women in the lean category.

Mean endotoxin detected in plasma for the entire sample was 4.25 EU ml$^{-1}$ (95% confidence interval (CI): 3.65, 4.96). Repeat samples were conducted on 48 individuals and the correlation between the first and second measurements was poor at $r=0.2$. Mean anti-endotoxin IgG antibody for the entire sample was 109.6 PMU (95% CI: 94.53, 127.0) and the correlation between repeat samples was 0.54. Mean anti-endotoxin IgM antibody for the entire sample was 55.45 PMU (95% CI: 46.54, 66.07) and the correlation between repeat samples was 0.82. The analysis presented focuses on samples obtained from the first study visit. Stool samples were more difficult to obtain than blood samples due to reluctance by some participants and a total of 72 samples were collected for the assessment of faecal calprotectin, representing 77% of participants. Mean calprotectin was 45.73 mg kg$^{-1}$ (95% CI: 34.42, 60.76). Mean IL-6 for the entire sample was 1.11 pg ml$^{-1}$ (95% CI: 0.92, 1.33).

Endotoxin levels were higher in the obese diabetic group compared with both the lean and obese non-diabetic groups.
of the lean comparison group (F-ratio for trend showed a very strong reverse association; both the obese non-diabetic and diabetic groups. Applying a Bonferroni correction (data not shown). There was also no association between anti-endotoxin IgG antibodies (but not between log fasting insulin and log endotoxin (P: 0.03)) and between log fasting insulin and anti-endotoxin IgM antibodies (P: 0.001) to account for multiple comparisons between groups still reveals a difference in anti-endotoxin IgM and IL-6 levels between the three groups. Simple linear regression analysis for all groups combined showed a positive association between log fasting glucose and log endotoxin levels (P: 0.20) but not between log fasting insulin and log endotoxin (P: 0.02). There was a negative association between log fasting glucose and log anti-endotoxin IgM antibodies (P: 0.02) and between log fasting insulin and anti-endotoxin IgM antibodies (P: 0.20) but not between anti-endotoxin IgG antibodies and either glucose or insulin (data not shown). There was a negative association between whole-body fat mass and log anti-endotoxin IgM antibodies (P: 0.03). There was also no association between anti-endotoxin IgG antibodies and either glucose or insulin (data not shown). There was a positive association between QUICKI index and log anti-endotoxin IgM antibodies (P: 0.03) and between log fasting insulin and anti-endotoxin IgM antibodies (P: 0.20) but not between either endotoxin or anti-endotoxin IgG antibodies (data not shown). There was also no association between anti-endotoxin IgG antibodies and either glucose or insulin (data not shown). There was a negative association between whole-body fat mass and log anti-endotoxin IgM antibodies (P: 0.03). There was also no association between anti-endotoxin IgG antibodies and either glucose or insulin (data not shown). There was a positive association between QUICKI index and log anti-endotoxin IgM antibodies (P: 0.03) and between log fasting insulin and anti-endotoxin IgM antibodies (P: 0.20) but not between either endotoxin or anti-endotoxin IgG antibodies (data not shown). There was also no association between anti-endotoxin IgG antibodies and either glucose or insulin (data not shown). There was a positive association between QUICKI index and log anti-endotoxin IgM antibodies (P: 0.03) and between log fasting insulin and anti-endotoxin IgM antibodies (P: 0.20) but not between either endotoxin or anti-endotoxin IgG antibodies (data not shown). There was also no association between anti-endotoxin IgG antibodies and either glucose or insulin (data not shown). There was a positive association between QUICKI index and log anti-endotoxin IgM antibodies (P: 0.03) and between log fasting insulin and anti-endotoxin IgM antibodies (P: 0.20) but not between either endotoxin or anti-endotoxin IgG antibodies (data not shown). There was also no association between anti-endotoxin IgG antibodies and either glucose or insulin (data not shown). There was a positive association between QUICKI index and log anti-endotoxin IgM antibodies (P: 0.03) and between log fasting insulin and anti-endotoxin IgM antibodies (P: 0.20) but not between either endotoxin or anti-endotoxin IgG antibodies (data not shown). There was also no association between anti-endotoxin IgG antibodies and either glucose or insulin (data not shown). There was a positive association between QUICKI index and log anti-endotoxin IgM antibodies (P: 0.03) and between log fasting insulin and anti-endotoxin IgM antibodies (P: 0.20) but not between either endotoxin or anti-endotoxin IgG antibodies (data not shown). There was also no association between anti-endotoxin IgG antibodies and either glucose or insulin (data not shown). There was a positive association between QUICKI index and log anti-endotoxin IgM antibodies (P: 0.03) and between log fasting insulin and anti-endotoxin IgM antibodies (P: 0.20) but not between either endotoxin or anti-endotoxin IgG antibodies (data not shown). There was also no association between anti-endotoxin IgG antibodies and either glucose or insulin (data not shown).
fat mass and endotoxin or anti-endotoxin IgG antibodies (data not shown).

An adjusted regression analysis was conducted to investigate the independent effect of obesity and diabetes on endotoxin and inflammatory markers. The initial model included obesity only with the subsequent models fitting both obesity and diabetes variables to assess any independent association with the outcomes of interest (Table 3). For this analysis, only individuals with fasting plasma glucose > 7.0 mmol l\(^{-1}\) were defined as having type 2 diabetes rather than including all individuals recruited from the diabetic clinic. Endotoxin was associated only with diabetes, whereas anti-endotoxin IgM antibodies and IL-6 were associated independently with both obesity and diabetes. Fitting potential confounding variables to the models (age, education, assets owned and water or waste management) did not affect the results (data not shown).

**DISCUSSION**

This ‘proof-of-principal’ study reveals some of the strongest human evidence to date in support of the theory that gut microbiota-derived bacterial products may drive a chronic low-grade inflammation that predisposes to insulin resistance and diabetes.

The potential role of the gut microbiota in the development of obesity and associated metabolic complications is receiving increasing attention. Animal studies have allowed for experiments to assess independent association. Mean difference refers to difference in independent variable for obese compared with lean individuals or for diabetic (defined as fasting plasma glucose > 7.0 mmol l\(^{-1}\) ) compared with non-diabetic individuals. Endotoxin were determined in ‘median units’ relative to a standard of healthy UK blood donors and normalised to the lean population where the mean is shown as 100 PMU.

**Table 3.** Association between endotoxin and inflammatory markers with obesity and diabetes separately

| Variable                      | Condition                      | Mean difference (95% CI)\(^{a}\) | P-value |
|-------------------------------|-------------------------------|---------------------------------|---------|
| Log endotoxin (EU ml\(^{-1}\)) | Obesity                       | 0.14 (–0.08, 0.37)              | 0.21    |
|                               | Obesity adj for diabetes      | 0.001 (–0.24, 0.24)             | 0.99    |
|                               | Diabetes adj for obesity      | 0.43 (0.16, 0.70)               | 0.002   |
| Log EndoCAb-IgG (PMU)^b       | Obesity                       | 0.14 (–0.18, 0.45)             | 0.39    |
|                               | Obesity adj for diabetes      | 0.19 (–0.16, 0.54)             | 0.29    |
|                               | Diabetes adj for obesity      | –0.07 (–0.46, 0.32)            | 0.73    |
| Log EndoCAb-IgM (PMU)^b       | Obesity                       | –0.88 (–1.21, –0.55)           | <0.001  |
|                               | Obesity adj for diabetes      | –0.69 (–1.04, –0.34)           | <0.001  |
|                               | Diabetes adj for obesity      | –0.53 (–0.92, –0.14)           | 0.01    |
| Log total IgM (mg dl\(^{-1}\))| Obesity                       | 0.06 (–0.44, 0.56)             | 0.81    |
|                               | Obesity adj for diabetes      | 0.17 (–0.39, 0.74)             | 0.55    |
|                               | Diabetes adj for obesity      | –0.27 (–0.89, 0.35)            | 0.38    |
| Log faecal calprotectin (mg kg\(^{-1}\)) | Obesity               | 0.22 (–0.38, 0.83)         | 0.46    |
|                               | Obesity adj for diabetes      | 0.20 (–0.47, 0.86)             | 0.56    |
|                               | Diabetes adj for obesity      | 0.29 (–0.46, 1.03)             | 0.45    |
| Log IL-6 (pg ml\(^{-1}\))     | Obesity                       | 0.38 (0.21, 0.56)              | <0.001  |
|                               | Obesity adj for diabetes      | 0.31 (0.12, 0.51)              | 0.002   |
|                               | Diabetes adj for obesity      | 0.21 (–0.01, 0.43)             | 0.06    |

Abbreviations: adj, adjusted; CI, confidence interval; EndoCAb, endotoxin core antibodies; PMU, percentage median unit. *Effect estimates from linear regression analysis. Obesity model includes obesity (coded 1,0) only. †Obesity adj for diabetes and †Diabetes adj for obesity includes both obesity and diabetes variables (coded 1,0) fitted to the same model to assess independent association. Mean difference refers to difference in independent variable for obese compared with lean individuals or for diabetic (defined as fasting plasma glucose > 7.0 mmol l\(^{-1}\) ) compared with non-diabetic individuals. Endotoxin were determined in ‘median units’ relative to a standard of healthy UK blood donors and normalised to the lean population where the mean is shown as 100 PMU.

The mechanisms of high-fat-induced low-grade inflammation are unclear and may also suggest a link with the gut microbiota. For example, single-meal intervention studies have indicated that certain food types (fat and glucose) lead to raised endotoxin as part of post-prandial inflammation. In mouse models, antibiotic-induced changes to the gut microbiota have been shown to result in reduced endotoxemia and improvements in metabolic disorders including adipose tissue inflammation and macrophage infiltration. In human studies, ex-vivo treatment of adipose tissue with LPS causes an increase in the secretion of inflammatory markers TNF-α and IL-6.

Certain constituents of the diet also are known to stimulate the release of pro-inflammatory cytokines; meals with a high-fat content have been implicated as a trigger of post-prandial inflammation through the activation of NF-κB. However, the mechanisms of high-fat-induced low-grade inflammation are unclear and may also suggest a link with the gut microbiota. For example, single-meal intervention studies have indicated that certain food types (fat and glucose) lead to raised endotoxin as part of post-prandial inflammation. In mouse models, antibiotic-induced changes to the gut microbiota have been shown to result in reduced endotoxemia and improvements in metabolic disorders including adipose tissue inflammation and macrophage infiltration. In human studies, ex-vivo treatment of adipose tissue with LPS causes an increase in the secretion of inflammatory markers TNF-α and IL-6. ENDOTOXIN LEVELS IN OBESITY AND DIABETES

Endotoxin levels observed in the current study were similar to those in a cross-sectional study of patients attending hospital in Saudi Arabia, where mean levels for non-diabetics were 4.2 EU ml\(^{-1}\) and for diabetics treated with insulin were...
A separate study of diabetic subjects and BMI-matched controls reported a 76% higher endotoxin concentration among the diabetics. In the present study, both EndoCaB-IgG and IgM antibodies were measured in addition to circulating endotoxin. EndoCaB-IgG levels were similar for the three groups. In contrast, EndoCaB-IgM levels were highly significantly lower in the obese and obese diabetic groups compared with their lean counterparts (F = 21.7, P < 0.0001). There was virtually no overlap in EndoCaB-IgM levels between the three groups. Total IgM antibodies remained similar across the groups demonstrating an EndoCaB-specific effect. Note that the strong correlation between repeated measures of EndoCaB IgM validates our choice of this as a more robust marker of chronic exposure than LPS levels, which may vary with day-to-day changes in diet and showed a very poor intra-subject reproducibility. The paradoxical reduction in EndoCaB IgM levels in the obese and obese diabetics were unexpected and are challenging to explain. We hypothesise that it could be caused by degradation of IgM–LPS complex that is constantly neutralising a persistent endotoxin leakage from the gut. This might also help to explain why there is no such trend in EndoCaB-IgG levels. In settings with high background antibody levels, repeated exposure to endotoxin may not necessarily stimulate a new IgG-mediated immune response if there is sufficient antibody to neutralise incoming LPS. Depletion of circulating EndoCaB levels as the LPS–antibody complex is cleared may erode IgM antibodies much faster than IgG antibodies, as only 20% of IgG is in circulation and will be supplemented from the 80% extravascular pool of IgG. The relative kinetics of IgM and IgG EndoCaB in this context is currently unknown and should be explored in future studies. Alternatively, chronic exposure among the obese and diabetic obese patients may have induced antibody class switching or even immune tolerance. Although there was no evidence for increased EndoCaB-IgG, we did not measure IgG isotypes in this study. It remains possible that the chronically exposed individuals produce an IgG of higher affinity rather than simply larger quantities.

Faecal calprotectin has been validated as a marker of gut inflammation against endoscopic and histologic grading of disease, and excretion of indium-111-labelled neutrophils, in inflammation against endoscopic and histologic grading of quantities. Producing an IgG of higher affinity rather than simply larger antibodies, as only 20% of IgG is in circulation and will be supplemented from the 80% extravascular pool of IgG. The relative kinetics of IgM and IgG EndoCaB in this context is currently unknown and should be explored in future studies. Alternatively, chronic exposure among the obese and diabetic obese patients may have induced antibody class switching or even immune tolerance. Although there was no evidence for increased EndoCaB-IgG, we did not measure IgG isotypes in this study. It remains possible that the chronically exposed individuals produce an IgG of higher affinity rather than simply larger quantities.

In conclusion, we have shown that in urban Gambian women type 2 diabetes is associated with convincing evidence of ME, presumably due to translocation of bacterial products originating in the gut. The paradoxical reduction in anti-endotoxin IgM antibodies tentatively suggests that constant immunosurveillance may be depleting the available neutralising antibody. The markedly lower levels in diabetics could indicate either a greater exposure (driven by an altered microbiota and/or a more leaky gut and/or altered diet) or an impaired ability to sustain neutralising defences. Whether this is a particular attribute of diabetes in a low-income setting, where a persistent ‘environmental enteropathy’ has long been recognised as a contributor to growth faltering in children, remains to be tested. If these findings are replicated in other studies, and a causal pathway emerges between diet composition, the composition and behaviour of the microbiota, damage to gut integrity and translocation of inflammatory agonists that ultimately impair insulin signalling, then this would open new avenues for intervention.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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