

# Targeted reduction of advanced glycation improves renal function in obesity

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Obesity is highly prevalent in Western populations and is considered a risk factor for the development of renal impairment. Interventions that reduce the tissue burden of advanced glycation end-products (AGEs) have shown promise in stemming the progression of chronic disease. Here we tested if treatments that lower tissue AGE burden in patients and mice would improve obesity-related renal dysfunction. Overweight and obese individuals (body mass index (BMI) 26–39 kg/m<sup>2</sup>) were recruited to a randomized, crossover clinical trial involving 2 weeks each on a low- and a high-AGE-containing diet. Renal function and an inflammatory profile (monocyte chemoattractant protein-1 (MCP-1) and macrophage migration inhibitory factor (MIF)) were improved following the low-AGE diet. Mechanisms of advanced glycation-related renal damage were investigated in a mouse model of obesity using the AGE-lowering pharmaceutical, alagebrium, and mice in which the receptor for AGE (RAGE) was deleted. Obesity, resulting from a diet high in both fat and AGE, caused renal impairment; however, treatment of the RAGE knockout mice with alagebrium improved urinary albumin excretion, creatinine clearance, the inflammatory profile, and renal oxidative stress. Alagebrium treatment, however, resulted in decreased weight gain and improved glycemic control compared with wild-type mice on a high-fat Western diet. Thus, targeted reduction of the advanced glycation pathway improved renal function in obesity.

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KEYWORDS: alagebrium chloride; nephropathy; obesity; RAGE

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Obesity is an important risk factor for type 2 diabetes and its subsequent complications including renal and cardiovascular diseases. Between 2010 and 2030, it is estimated that worldwide numbers of diabetes cases will increase by 54%.<sup>1</sup> As such, the International Diabetes Federation has proposed lifestyle changes as a cost-effective method of preventing or delaying the onset of type 2 diabetes,<sup>2</sup> which would likely extend to manifestations of obesity such as an increased risk of chronic kidney disease.<sup>3</sup> Current figures show that 30–50% of individuals with diabetes will develop nephropathy.<sup>4</sup>

It is well known that certain lifestyle choices such as diets high in saturated fat and processed foods contribute to obesity and the development of type 2 diabetes, although the exact mechanisms involved have not been fully defined. Dietary fat and processed foods are extremely high in a group of sugar modifications known as advanced glycation end-products (AGEs). These molecules improve taste, reduce food spoilage, and promote longer shelf life. Excessive dietary intake of AGEs has recently been shown to contribute to renal<sup>5</sup> and cardiovascular<sup>6</sup> diseases and the development of type 2 diabetes, especially in the context of a high-fat diet in animal models.<sup>7</sup> Once in circulation,<sup>8–10</sup> dietary AGEs may cause inflammation and free oxygen radical production by modulation of specific receptors, including the receptor for AGE (RAGE). Interestingly, the kidney is the main organ responsible for the removal of AGEs from the bloodstream.<sup>11</sup> This high exposure of the kidney to AGEs is likely to make the organ particularly susceptible to AGE-mediated damage. The potential for reduction in dietary AGEs to improve renal function in nonobese, renal failure patients has been demonstrated after a 4-week low-AGE diet that reduced serum creatinine concentrations by 30–40%.<sup>9</sup>

This study investigated the effects of lowering the accumulation of AGEs or interrupting RAGE downstream signaling pathways using a model of obesity-related renal disease in mice. The efficacy of a reducing dietary AGE intake

to improve renal function in obese humans was also examined.

## RESULTS

### Clinical study

The baseline characteristics of the 11 participants are shown in Table 1. Although diets were isocaloric and matched for macronutrient content, on a 9 MJ/day diet, individuals were calculated to consume 14,090 kJ *N*-carboxymethyllysine (CML) on the high-AGE diet and 3302 kJ CML on the low-AGE diet. There was no effect of the dietary interventions on body weight, body mass index (BMI), or adiposity, which remained elevated (Table 2).

**Renal function and inflammatory makers.** Urinary albumin/creatinine ratios were significantly better following the low AGE dietary period in obese individuals (low- vs high-AGE diet:  $P=0.02$ , Figure 1a). Plasma cystatin C levels were elevated following consumption of a high-AGE diet for 2 weeks (low vs high:  $P=0.02$ , Figure 1b). Plasma CML concentrations following high AGE consumption declined (low vs high:  $P=0.01$ , Figure 1c), whereas urinary CML concentrations increased following consumption of the high-AGE diet (low vs high:  $P=0.03$ , Figure 1d). The high-AGE diet increased urinary 8-isoprostanes (low vs high:  $P=0.02$ , Figure 1e). Plasma monocyte chemoattractant protein-1 (MCP-1), also known as chemokine (C-C motif) ligand 2 (CCL2), was increased as a result of high AGE dietary consumption (low vs high:  $P=0.04$ , Figure 1f). Conversely, however, plasma macrophage migration inhibitory factor (MIF) significantly declined after consumption of the high-AGE diet (low vs high:  $P=0.04$ , Figure 1g). There were no significant effects of the order in which the diets were consumed on any of the parameters, when these data were analyzed via repeated measures analysis of variance with order as a between-subject factor. There were no differences in other circulating cytokines and transcription factors including endogenous secreted form of RAGE, soluble RAGE (sRAGE), nuclear factor- $\kappa$ B, interleukin-6, and high-sensitivity C-reactive protein between diets (data not shown).

**Table 1 | Baseline anthropometric and biochemical data in obese individuals recruited for the dietary intervention study ( $n=11$  patients)**

	Mean ( $\pm$ s.d.)	Range
<i>N</i>	11	
Age (years)	30 ( $\pm$ 9)	21–50
BMI ( $\text{kg}/\text{m}^2$ )	31.8 ( $\pm$ 4.8)	27–36
Waist circumference (cm)	96.9 ( $\pm$ 18.4)	78.5–115.3
Waist/hip ratio	0.91 ( $\pm$ 0.12)	0.78–1.30
24 h Creatinine clearance (ml/s)	2.4 ( $\pm$ 1.1)	1.3–4.2
Urinary CML (nmol/mol lysine)	11.5 ( $\pm$ 14.4)	0.35–44.4
Serum CML ( $\mu\text{mol}/\text{mol}$ lysine)	224.5 ( $\pm$ 166.9)	122.9–859.8
Fasting plasma glucose (mmol/l)	4.7 ( $\pm$ 0.4)	4.1–5.5
Fasting plasma insulin (mU/ml)	10.2 ( $\pm$ 4.1)	6.3–19.1
Insulin sensitivity (mg glucose/kg/min)	7.8 ( $\pm$ 3.4)	2.5–17.1

Abbreviations: BMI, body mass index; CML, *N*-carboxymethyllysine.

### Murine study

**Biochemical and metabolic parameters.** Both wild-type (WT) and RAGE $^{-/-}$  mice consuming the Western-style diet, high in AGEs and fat content, were obese by week 16 ( $\Delta$ BW; Table 3), with significant increases in epididymal and omental adipose depots (Table 3). Increases in body weight and fat deposition after the Western diet were prevented using the AGE-lowering therapy, alagebrium (ALA, Table 3). Kidney size was unaffected by dietary consumption of a western-style diet (Table 3). Fasting plasma glucose and insulin concentrations were increased in obese mice following the consumption of the western diet in both WT and RAGE $^{-/-}$  mouse strains (Table 3), and the parameters were significantly improved in the mice treated with ALA.

**Renal functional parameters.** Renal function was assessed by albumin excretion rate and creatinine clearance. Obese WT mice consuming the Western-style diet had albuminuria (Figure 2a), which was reduced in obese RAGE $^{-/-}$  mice fed a Western diet but not with ALA. Creatinine clearance was elevated in obese WT mice and significantly improved by ALA (Figure 2b). Furthermore, a western diet did not induce hyperfiltration in RAGE $^{-/-}$  mice (Figure 2b). All obese mice had lower plasma CML concentrations (Figure 2c) despite consuming more dietary AGEs than lean low-AGE-fed mice (Figure 2d). Urinary CML excretion was below detectable limits (5.6 nmol/mol lysine) in all mice. Also of interest was that obese WT ( $16.8 \pm 11.2$  kJ/day) and RAGE $^{-/-}$  mice ( $29.7 \pm 6.9$  kJ/day) consumed less kilojoules per day than both lean low-AGE-fed mice ( $50.3 \pm 3.4$  kJ/day;  $P<0.05$  vs obese WT) or obese mice treated with ALA ( $34.9 \pm 9.4$  kJ/day;  $P<0.05$  vs obese WT).

Concentrations of the AGE CML in renal cortices were significantly increased in obese and obese ALA-treated animals but not in obese RAGE knockout mice when measured via enzyme-linked immunosorbent assay (ELISA; Figure 2e). Immunohistochemistry confirmed that there were increases in CML in renal cortices taken from obese mice that were not seen in lean low-AGE-fed mice (Figure 2f).

**RAGE protein expression and inflammation.** Membranous RAGE protein concentrations in renal cortices taken from obese WT mice were significantly higher than those in lean mice consuming a low-AGE diet (Figure 3a). This parameter was not affected by treatment with ALA (Figure 3a). Circulating levels of sRAGE, measured via ELISA, tended to be higher in obese mice, although they were significantly lower after ALA therapy (lean low AGE ( $216.6 \pm 65.98$  pg/ml) vs obese ( $352.2 \pm 172.4$  pg/ml) RAGE,  $P<0.05$ ; obese vs obese ALA ( $152.2 \pm 55.67$  pg/ml) RAGE,  $P<0.05$ ). As expected, there was no expression of membranous or soluble RAGE protein detected in RAGE $^{-/-}$  mice (data not shown). Renal MCP-1 levels were significantly lower in obese ALA-treated animals and obese RAGE $^{-/-}$  mice (Figure 3b) when compared with untreated obese WT mice. Plasma MIF concentrations in mice were decreased with obesity and significantly increased by ALA treatment or in obese

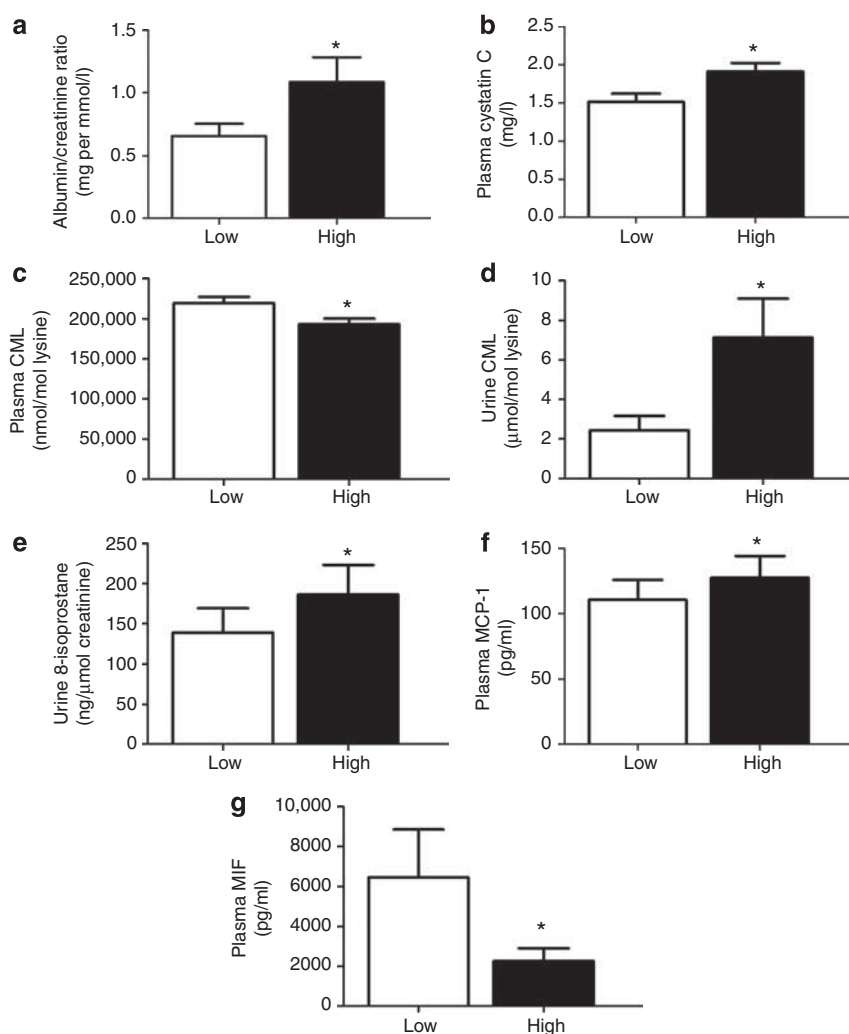
**Table 2 | Anthropometric and biochemical data at the completion of 2 weeks of dietary consumption of either a low- or high-AGE diet (n=11 patients)**

	Following low-AGE diet Mean ( $\pm$ s.d.)	Following high-AGE diet Mean ( $\pm$ s.d.)	P-value for change
Weight (kg)	93.2 ( $\pm$ 15.9)	93.9 ( $\pm$ 15.8)	NS
BMI (kg/m <sup>2</sup> )	31.5 ( $\pm$ 4.2)	31.4 ( $\pm$ 4.2)	NS
Body fat (%)	29.3 ( $\pm$ 6.4)	29.2 ( $\pm$ 6.8)	NS
Urine albumin (mg/day)*	20.27 ( $\pm$ 34.8)	16.05 ( $\pm$ 20.9)	NS
Serum creatinine ( $\mu$ mol/l)	72.3 ( $\pm$ 18.3)	70.2 ( $\pm$ 13.5)	NS
Total cholesterol (mmol/l)	4.2 ( $\pm$ 0.9)	4.2 ( $\pm$ 1.0)	NS
Fasting plasma glucose (mmol/l)	5.1 ( $\pm$ 0.3)	4.8 ( $\pm$ 0.3)	NS

Abbreviations: AGE, advanced glycation end-product; BMI, body mass index; NS, not significant ( $P > 0.05$ ).

This clinical study in obese individuals (BMI 31.8 ( $\pm$  4.8)) was performed as a single-blinded, randomized, crossover dietary intervention study.

\*A nonparametric analysis was performed.



**Figure 1 | Renal and inflammatory parameters in obese humans following dietary interventions.** Assays were performed in samples collected from the same obese individuals following consumption of a diet either low or high in advanced glycation end-product (AGE) content for 2 weeks. (a) Urinary albumin/creatinine ratios, (b) plasma cystatin C concentration, (c) plasma concentrations of the AGE, N-carboxymethyllysine (CML), (d) urinary CML concentrations, (e) urinary 8-isoprostanes, (f) plasma monocyte chemotactic protein-1 (MCP-1) concentrations, and (g) plasma macrophage migration inhibitory factor (MIF) concentrations. \* $P < 0.05$  low- versus high-AGE diet, Student's paired *t*-test.

RAGE<sup>-/-</sup> mice (Figure 3c). Kidney MIF levels were increased in obese mice, which were not affected by ALA treatment; however, deletion of RAGE significantly decreased renal MIF

concentrations (Figure 3d). Collagen IV deposition in glomerular cortices was not significantly different among treatment groups (Figure 3e and f).

**Table 3 | Murine physiological and metabolic parameters at study completion (week 16)**

	$\Delta$ Body weight (g)	Left kidney weight (g)	Omental adipose tissue (g)	Epididymal adipose (g)	KW/BW ratio ( $\times 10^3$ )	Plasma glucose (mmol/l)	Plasma insulin (ng/ml)
<i>C57BL/6J</i>							
Lean	2.9 ( $\pm 2.4$ )	0.19 ( $\pm 0.02$ )	33.5 ( $\pm 1.8$ )	0.89 ( $\pm 0.3$ )	11.35 ( $\pm 1.2$ )	5.3 ( $\pm 1.8$ )	0.24 ( $\pm 0.21$ )
Obese	11.0 ( $\pm 1.6$ )*	0.19 ( $\pm 0.01$ )	40.8 ( $\pm 2.6$ )*	1.59 ( $\pm 0.2$ )*	9.35 ( $\pm 1.2$ )*	8.5 ( $\pm 1.5$ )*	1.69 ( $\pm 0.68$ )*
Obese ALA	8.1 ( $\pm 2.5$ )* <sup>†</sup>	0.17 ( $\pm 0.02$ )	37.8 ( $\pm 3.8$ )*	1.31 ( $\pm 0.3$ )* <sup>†</sup>	9.74 ( $\pm 0.8$ )	6.6 ( $\pm 1.5$ )	0.66 ( $\pm 0.5$ )* <sup>†</sup>
<i>RAGE<sup>-/-</sup></i>							
Obese	15.4 ( $\pm 2.2$ )* <sup>†</sup>	0.19 ( $\pm 0.01$ )	39.7 ( $\pm 2.6$ ) <sup>†</sup>	1.94 ( $\pm 0.3$ ) <sup>†</sup>	9.8 ( $\pm 0.6$ )	10.3 ( $\pm 3.1$ )	3.36 ( $\pm 0.97$ )* <sup>†</sup>

Abbreviations: AGE, advanced glycation end-product; ALA, alagebrium chloride; BW, body weight; KW, kidney weight; RAGE, receptor for AGE.

Data are presented as mean ( $\pm$  s.d.).

Obese (high AGE/high-fat diet), ALA (AGE-lowering therapy, alagebrium chloride 1 mg/kg/day), and RAGE<sup>-/-</sup> (RAGE deletion).

\* $P < 0.05$  vs lean low AGE, <sup>†</sup> $P < 0.05$  vs obese.

Obesity induced excess cortical superoxide production in the mitochondrial (Figure 4a) and cytosolic compartments (Figure 4b). Treatment with ALA and the deletion of the RAGE gene significantly decreased renal superoxide levels (Figure 4a and b). Urinary 8-isoprostane concentrations were increased in obese mice; however, this was attenuated with ALA therapy (Figure 4b).

## DISCUSSION

This study has provided evidence that intervention using diets low in AGE content may attenuate renal changes seen with obesity. Although our current human study did not encourage weight loss in obese participants because of matching of caloric intake and the short duration of dietary intervention (2 weeks), we were able to demonstrate that altering dietary AGE content alone is sufficient to improve inflammatory profiles and early renal disease. These findings are consistent with a previous study of patients with advanced end-stage renal disease.<sup>12</sup> To complement these findings, we performed studies in mice to further define potential mechanisms linking the AGE/RAGE axis to renal functional changes in the context of obesity. Indeed, our studies in obese mice highlighted that interfering with the AGE/RAGE axis by either preventing AGE tissue accumulation with the AGE-lowering therapy, ALA, or via RAGE deletion in RAGE<sup>-/-</sup> mice is protective against obesity-related renal dysfunction. These findings are consistent with previous evidence that AGE formation is important in the pathogenesis of other chronic kidney diseases.<sup>13–17</sup>

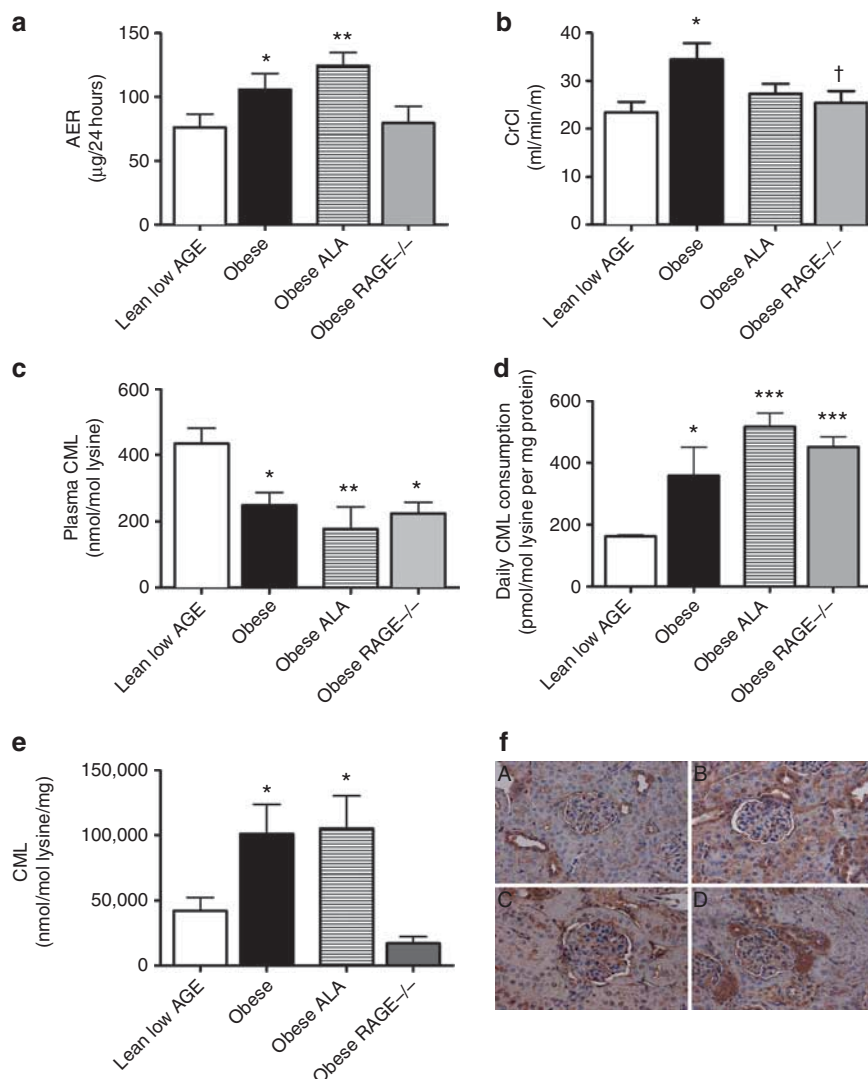
The increases in the expression of the proinflammatory protein RAGE in kidney cortices taken from obese mice, and its contributory role to obesity-related renal dysfunction in this model, was further suggested in obese RAGE<sup>-/-</sup> mice who had better renal function and less inflammation. Elevations in the circulating concentrations of sRAGE were also seen in obese mice, consistent with findings in type 2 diabetic individuals with nephropathy who are generally obese.<sup>18,19</sup> Although sRAGE was not changed after a low-AGE diet in our human study, this was most likely because of the short duration of the dietary intervention. It is possible that a longer dietary duration would have ultimately led to lower

circulating sRAGE concentrations. This hypothesis is supported by the improved inflammatory profile seen with consumption of a low-AGE diet as reflected by decreased MCP-1 and MIF concentrations which in the context of previous studies which have associated increases in sRAGE with systemic inflammation.<sup>19–21</sup>

We have also demonstrated for the first time that total AGE burden is likely a combination of circulating, tissue and excreted AGE concentrations in obesity. Furthermore this can be modulated via alteration of diet or treatment with therapies, as was the case in our murine study. Previous other studies have reported that circulating AGE concentrations may be an indication of renal disease progression as they showed increases.<sup>22</sup> As a result of our findings, we therefore heed caution at this becoming a gold standard marker.

Given the findings of this study and the previously reported roles of RAGE, it is possible that inflammation plays a role in modulating the changes seen in this study. In both obese humans and mice, there was evidence of low-grade inflammation, which was enhanced by consumption of a high-AGE diet. This increased plasma MCP-1 and lowered MIF concentrations, attenuated by interrupting the AGE/RAGE axis, either by lowering the tissue AGE burden using dietary means, the AGE-lowering therapy ALA, or by deletion of RAGE. Activation of RAGE has already been reported to be crucial for macrophage recruitment, as highlighted by its role in host–pathogen defense.<sup>23</sup> Therefore, it is likely that RAGE activation as a result of AGE stimulation is a modulator of MCP-1 and MIF secretion in this study. However, obesity-related changes in circulating insulin concentrations seen in both humans and mice may also be indirectly modulating the expression of MIF (localized in the pancreatic islets<sup>24</sup>) and MCP-1 (from white adipose tissue<sup>21,25</sup>) that are known to affect insulin secretion and action, respectively.

AGEs and RAGE are also known to contribute to renal dysfunction via excess generation of reactive oxygen species.<sup>26–28</sup> High-AGE diets in both obese humans and mice appear to influence oxidative stress as reflected by increases in urinary isoprostanes and renal superoxide production. This pro-oxidant effect of AGEs is further suggested by the findings in obese mice that received ALA that appeared to



**Figure 2 | Murine renal and biochemical parameters at study completion.** Groups of mice were followed for 16 weeks. **(a)** Urinary albumin excretion rate (AER) over 24 h measured by enzyme-linked immunosorbent assay (ELISA). **(b)** Creatinine clearance (CrCl) as determined by high-performance liquid chromatography (HPLC) following correction for body surface area. **(c–e)** *N*-Carboxymethyllysine (CML) analyzed by ELISA in plasma **(c)**, dietary CML consumption over 24 h **(d)**, and kidney cortex protein **(e)**. **(f)** CML immunohistochemistry staining on paraffin-fixed kidney sections from (A) lean low advanced glycation end-product (AGE), (B) obese, (C) obese alagebrium (ALA), and (D) obese RAGE<sup>-/-</sup>. Obese (high AGE/high-fat diet), ALA (AGE-lowering therapy, alagebrium chloride 1 mg/kg/day), and RAGE<sup>-/-</sup> (RAGE deletion). Data for AER were logarithmically transformed as these were not normally distributed. Other data are presented as mean  $\pm$  s.d. \* $P < 0.05$  vs lean low AGE, \*\* $P < 0.01$  vs lean low AGE, \*\*\* $P < 0.001$  vs lean low AGE, † $P < 0.05$  vs obese.

have less oxidative stress. RAGE deficiency did not improve obesity-related increases in urinary isoprostane excretion, which was interesting given that this group also had a lack of effect on adiposity and obesity-related abnormalities in glycemic control. This suggests that the benefits afforded by low-AGE diets and ALA on oxidative stress may be partly independent of RAGE. This is not totally surprising as AGEs can interact with other receptors in addition to RAGE, and ALA is likely to have additive actions that may be relevant including a modest effect as an antioxidant.<sup>15,29</sup>

In conclusion, this study suggests that a low-AGE diet has an impact on modulating renal function in healthy obese individuals. Studies in murine models suggest that the

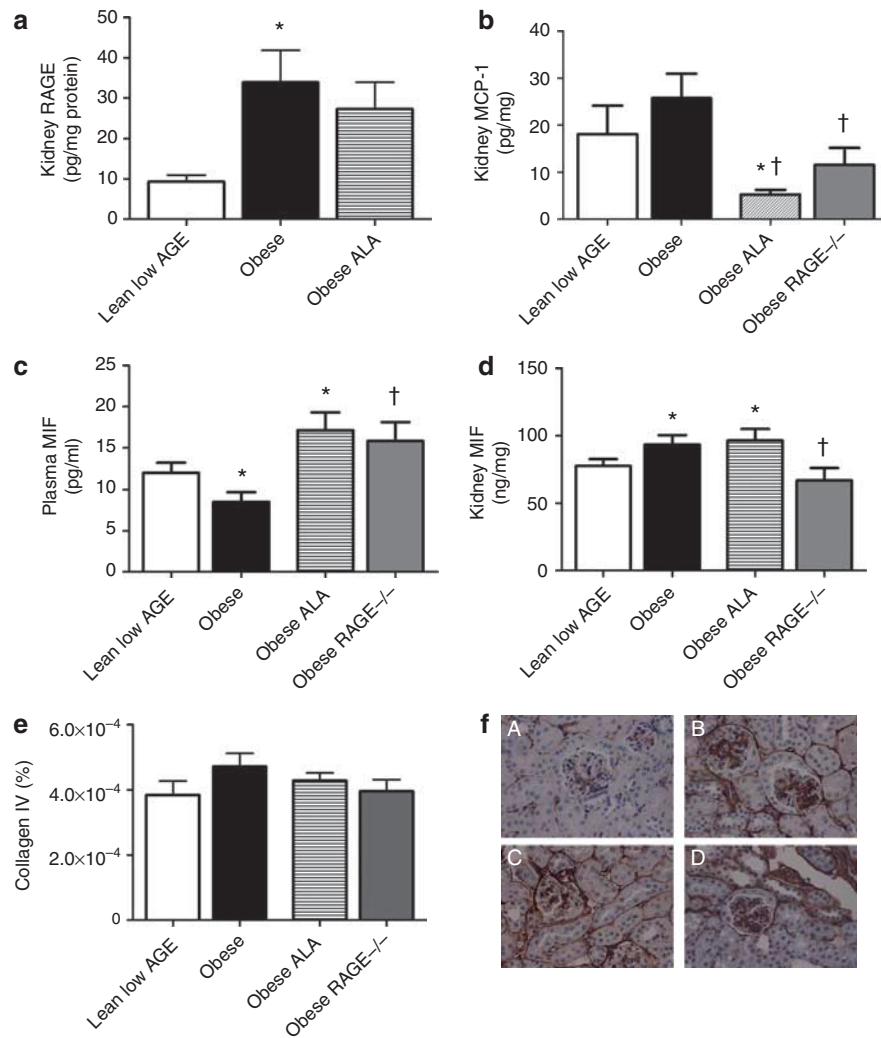
mechanism responsible for AGE effects on renal function is likely to involve its receptor RAGE and include improvements in inflammation, oxidative stress, and glycemic control.

## MATERIALS AND METHODS

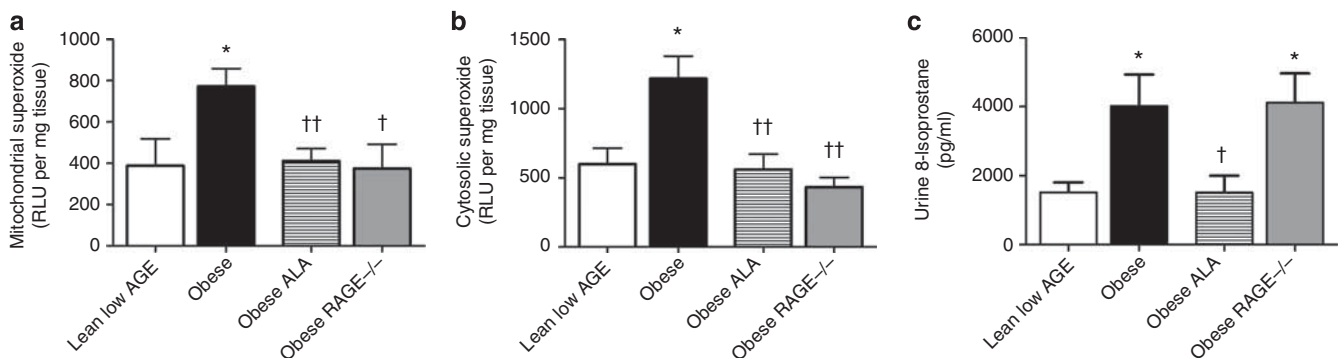
### Clinical study

**Participant selection.** This study was approved by the Alfred Hospital Ethics Committee and conducted according to the Declaration of Helsinki Principles. All individuals gave written informed consent before commencement of the study. Participants were males, aged between 18 and 50 years with stable body weight (weight change  $< 5$  kg in last year), BMI  $\geq 25$  kg/m<sup>2</sup>, normal glucose





**Figure 3 | Murine inflammatory parameters following 16 weeks of high advanced glycation end-product (AGE) dietary feeding.** (a) The receptor for AGE (RAGE) protein content in kidney cortices measured by mouse-specific enzyme-linked immunosorbent assay (ELISA). Kidney cortices from obese RAGE<sup>-/-</sup> did not have measurable membrane RAGE. (b) Renal cytosolic monocyte chemoattractant protein-1 (MCP-1) assayed by ELISA. (c) Plasma macrophage migration inhibitory factor (MIF) concentration assayed by ELISA. (d) Renal cytosolic MIF content assayed by ELISA. (e) Semiquantification of collagen IV in glomeruli. (f) Representative collagen IV immunohistochemistry staining used for semiquantification of (A) lean low AGE, (B) obese, (C) obese ALA, and (D) obese RAGE<sup>-/-</sup>. Obese (high AGE/high-fat diet), ALA (AGE-lowering therapy, alagebrium chloride 1 mg/kg/day), and RAGE<sup>-/-</sup> (RAGE deletion). \**P* < 0.05 vs lean low AGE, †*P* < 0.05 vs obese.



**Figure 4 | Murine oxidative parameters following 16 weeks of dietary intervention.** (a) Mitochondrial NADH-dependent superoxide production in fresh kidney cortices, measured via lucigenin-enhanced chemiluminescence. (b) Cytosolic NADPH-dependent superoxide production in fresh kidney tissue. (c) 8-Isoprostane measured via enzyme-linked immunosorbent assay (ELISA) in urine. Obese (high advanced glycation end-product (AGE)/high-fat diet), ALA (AGE-lowering therapy, alagebrium chloride 1 mg/kg/day), RAGE<sup>-/-</sup> (RAGE deletion). NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate. \**P* < 0.05 vs lean low AGE, †*P* < 0.05 vs obese, ††*P* < 0.01 vs obese.

**Table 4 | Representative example of isocaloric meal selections for high-AGE versus low-AGE diets consumed by obese individuals**

High-AGE diet	AGE (kU) <sup>a</sup>	Low-AGE diet	AGE (kU)
<i>Breakfast</i>		<i>Breakfast</i>	
2 scrambled eggs	2749	2 lightly poached eggs	628
1.5 slices toasted white bread (with crusts)	310	2 slices of fresh white bread (without crusts)	12
Commercial orange juice	9	Juice from an orange	0
<i>Lunch</i>		<i>Lunch</i>	
One apple	19	One apple	19
One toasted bacon sandwich (with crusts)	4026	One avocado and ham sandwich (without crusts)	1217
One glass cola <sup>b</sup>	16	One glass diet lemonade	2
<i>Dinner</i>		<i>Dinner</i>	
Pan-fried chicken breast	5387	Steamed chicken breast	989
Vegetables	391	Steamed vegetables	36
(fried in olive oil)	300	Olive oil dressing	300
Fried white rice	66	Boiled white rice	18
One apple	19	One apple	19
One glass cola	16	One glass diet lemonade	2
<i>Evening snack</i>		<i>Evening snack</i>	
One glass of heated skim milk	138	One glass cold full-cream milk	48
Shortbread biscuits	644	Angel food cake	11
<i>Total AGE content (kU)</i>	<i>14,090</i>	<i>Total AGE content (kU)</i>	<i>3302</i>
Total energy (MJ)	9.0	Total energy (MJ)	9.0
Protein (%E)	16	Protein (%E)	16
Total fat (%E)	30	Total fat (%E)	30
Carbohydrate (%E)	54	Carbohydrate (%E)	55
Saturated fat (g)	10	Saturated fat (g)	10

Abbreviations: AGE, advanced glycation end-product; %E, percent of total energy.

<sup>a</sup>Approximate values only as calculated from available American data for the N-carboxymethyllysine (CML) content of foods.<sup>12</sup> While Australian foods may differ in AGE content, all foods prepared for the high-AGE diet were subjected to a high level of browning.

<sup>b</sup>Cola is a rich source of methylglyoxal in addition to CML.

tolerance (by oral glucose tolerance test), and healthy according to medical history, examination, and basic blood screening. Exclusion criteria included morbid obesity (BMI  $\geq 40$  kg/m<sup>2</sup>), current smoking habit, high alcohol use or a positive urine drug screening test, any medication taken within 1 month before commencing the study, presence of acute inflammation (by history, physical, or laboratory examination), or highly unusual dietary habits or vegan diet.

**Clinical study design and anthropometric and metabolic measurements.** In all, 11 healthy overweight males participated in a clinical dietary intervention study involving 2 weeks each of low- and high-AGE diet separated by a 4-week wash-out period. Participants kept a 3-day diet record (two weekdays and one weekend day) based on household measures. Nutrient content was analyzed with SERVE (SERVE Nutrition Systems, St Ives, NSW, Australia), based on Australian Food Composition tables plus US data for food AGE content.<sup>30</sup> Results guided food selection and indicated the approximate habitual AGE intake. A menu of carefully matched alternative food choices (Table 4), each similar in macronutrients and total kilojoules but differing in total AGE content, were prepared for each meal of the day, including snacks and beverages, according to previously described guidelines.<sup>30</sup> All foodstuffs for the low- and high-AGE diets were provided to the individuals, in addition to instructions for storage and preparation of meals (method, temperature, and duration of cooking). Participants were instructed to eat to appetite, and maintain normal physical activity as measured by IPAQ (International Physical Activity Questionnaire)<sup>31</sup> and by accelerometer (Respirotrics Mini-mitter, Bend, OR). Participants had a 6-week run-in period of the

high-AGE diet as this was generally similar to their normal dietary habits, and were then randomized to either the low-AGE or high-AGE diet for 2 weeks. At the commencement and conclusion of each 2-week dietary test period, body weight, waist-hip ratio, and adiposity by four-point bioimpedance analysis (Body Composition Analyser, Model BC-418MA; Tanita, Middlesex, UK) were measured and BMI calculated. A 24-h urine collection and fasting plasma sample were taken at the commencement of the study and further spot urine and fasting plasma samples were taken before and after dietary interventions. Fasting plasma samples were analyzed for glucose (Radiometer, Copenhagen, Denmark) and insulin via ELISA.

**Renal function.** Before and after each dietary period, spot urines and plasma samples were taken to assess serum creatinine and urinary albumin/creatinine ratios. Creatinine clearance was estimated via the Cockcroft–Gault formula,<sup>32</sup> and albumin excretion rates assayed in 24 h urine collections at baseline.

**CML indirect ELISA.** CML was measured in human serum (1:8000) and urine samples (1:4) before and after each diet at their respective dilutions, using an in-house indirect CML ELISA that has been previously described.<sup>33</sup> CML was also measured in mouse chow, murine plasma, urine, and renal cortices using the previously described methods.<sup>15,29</sup>

**Immunohistochemistry.** Immunohistochemistry analysis for CML and collagen IV was performed on paraffin-embedded neutral buffered formalin-fixed murine kidneys as previously described.<sup>34</sup>

**Cystatin C, MIF, and MCP-1 ELISAs.** Cystatin C was measured in human plasma samples according to the manufacturer's instructions in a 1:1000 dilution (Human Cystatin C; BioVendor,

Mordice, Czech Republic). MIF was measured in human plasma (R&D Systems, Minneapolis, MN) and murine plasma and renal cytosolic fractions (USCN Life, Wuhan, China) according to the manufacturer's guidelines. MCP-1 was assayed in human plasma (R&D Systems) and murine renal cortex cytosolic protein fractions (Raybiotech, Norcross, GA).

### Murine study

**Study design.** Male WT, C57BL/6J (WT), and RAGE-deficient mice (RAGE<sup>-/-</sup>)<sup>35</sup> on a C57BL/6J background were housed in a temperature-controlled environment with a 12 h light-dark cycle (Alfred Medical Research and Education (AMREP) Precinct Animal Centre, Melbourne, Australia). At 8 weeks of age, groups of C57BL/6J mice ( $n = 10$ /group) were randomized to either (1) a high-AGE, high-fat, Western diet (obese; SF05-031, Specialty Feeds, Perth, Australia, baked for 1 h at 160 °C, 101.9 nmol/mol lysine of CML per 100 mg) (2) a high-AGE, high-fat Western diet plus the AGE-lowering therapy ALA (obese ALA; 1 mg/kg/day oral gavage; Synvista Therapeutics, Montvale, NJ), or (3) a low-AGE standard fat diet (lean; AIN-93G, Specialty Feeds, unbaked; 20.9 nmol/mol lysine of CML per 100 mg). Food intake and water access was *ad libitum* with diets matched for vitamin and amino-acid content. However, 40% of total energy in the Western diet was derived from animal fat (Ghee; 210 g/kg) versus 16% of total energy in the low-AGE diet. One further group of RAGE<sup>-/-</sup> mice consuming the Western diet were also studied ( $n = 10$ ; obese RAGE<sup>-/-</sup>). All animal studies were performed in accordance with the guidelines from the AMREP Animal Ethics Committee and the National Health and Medical Research Council of Australia.

**Murine physiological and biochemical parameters.** Body weight, fasting plasma glucose, and fasting plasma insulin were measured at 16 weeks as previously described.<sup>36</sup> The 24 h metabolic caging to collect urine and measure food and water intake was performed at weeks 8 and 16 of the study. Albumin excretion rate was assessed using a mouse albumin ELISA kit according to the manufacturer's instructions (Bethyl Laboratories, Montgomery, TX). Creatinine clearance was determined following HPLC (Agilent HP1100 system, Hewlett Packard, Nuremberg, Germany) measurement of creatinine content in timed plasma and urine samples as previously described and in accordance with AMDCC (Animal Models of Diabetic Complications Consortium) guidelines.<sup>37</sup> Frozen renal cortex was processed via ultracentrifugation as previously described<sup>15</sup> in order to generate membrane, cytosol, and nuclear protein fractions.

**Urinary isoprostane concentrations.** As a noninvasive measure of oxidative stress, 8-isoprostane F<sub>2</sub> was measured in 24 h human urine samples collected before and after each diet by competitive ELISA (Oxford Biomedical Research, Oxford, MI). Human urine samples were assayed neat and the assay was conducted as per the manufacturer's instructions. Murine urine samples were also analyzed neat for 8-isoprostane, according to the manufacturer's instructions (8-isoprostane enzyme immunoassay; Cayman Chemical, Ann Arbor, MI).

**Superoxide production.** Renal superoxide was measured in fresh murine renal cortical tissue as previously described via chemiluminescence of lucigenin.<sup>38,39</sup>

**Renal RAGE expression.** Murine renal cytosolic protein fractions were assayed for RAGE protein using an ELISA specific for mouse (R&D Systems). Unknown values were calculated relative to a four-parameter logistic standard curve generated using the

GraphPad Prism program (GraphPad Prism, San Diego, CA). All assays were run according to the manufacturer's instructions.

**Statistical analyses.** Human data were expressed as mean  $\pm$  s.e.m. unless otherwise stated and were analyzed using paired Student's *t*-test analysis. Urinary albumin/creatinine values were nonparametric and were therefore logarithmically transformed before analysis. Order effect of the diets was analyzed via repeated measures analysis of variance with order as a between-subject factor. Human statistical analyses were performed using SPSS (SPSS Statistics 17.0, IBM, Somers, NY).

Murine study analyses were performed by one-way analysis of variance followed by Tukey's *post hoc* analysis (GraphPad Prism, 5.2). Mouse data are presented as mean  $\pm$  s.d. Mouse albuminuria data were not normally distributed and were therefore logarithmically transformed before analysis. A  $P < 0.05$  was considered to be statistically significant.

### DISCLOSURE

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### DISCLAIMER

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### REFERENCES

- Shaw JE, Sicree RA, Zimmet PZ. Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Res Clin Pract* 2010; **87**: 4–14.
- Alberti KG, Zimmet P, Shaw J. International Diabetes Federation: a consensus on type 2 diabetes prevention. *Diabet Med* 2007; **24**: 451–463.
- Sharma K, Ramachandrarao S, Qiu G *et al.* Adiponectin regulates albuminuria and podocyte function in mice. *J Clin Invest* 2008; **118**: 1645–1656.
- Hossain P, Kawan B, El Nahas M. Obesity and diabetes in the developing world—a growing challenge. *N Engl J Med* 2007; **356**: 213–215.
- Sebekova K, Faist V, Hofmann T *et al.* Effects of a diet rich in advanced glycation end products in the rat remnant kidney model. *Am J Kidney Dis* 2003; **41**: S48–S51.
- Tikellis C, Thomas MC, Harcourt BE *et al.* Cardiac inflammation associated with a Western diet is mediated via activation of RAGE by AGEs. *Am J Physiol Endocrinol Metab* 2008; **295**: E323–E330.
- Sandu O, Song K, Cai W *et al.* Insulin resistance and type 2 diabetes in high-fat-fed mice are linked to high glycotxin intake. *Diabetes* 2005; **54**: 2314–2319.
- Tuohy KM, Hinton DJ, Davies SJ *et al.* Metabolism of Maillard reaction products by the human gut microbiota—implications for health. *Mol Nutr Food Res* 2006; **50**: 847–857.
- Uribarri J, Peppas M, Cai W *et al.* Dietary glycotoxins correlate with circulating advanced glycation end product levels in renal failure patients. *Am J Kidney Dis* 2003; **42**: 532–538.
- Vlassara H, Cai W, Crandall J *et al.* Inflammatory mediators are induced by dietary glycotoxins, a major risk factor for diabetic angiopathy. *Proc Natl Acad Sci USA* 2002; **99**: 15596–15601.



11. Miyata T, Ueda Y, Yoshida A *et al.* Clearance of pentosidine, an advanced glycation end product, by different modalities of renal replacement therapy. *Kidney Int* 1997; **51**: 880–887.
12. Uribarri J, Peppas M, Cai W *et al.* Restriction of dietary glycotoxins reduces excessive advanced glycation end products in renal failure patients. *J Am Soc Nephrol* 2003; **14**: 728–731.
13. Guo J, Ananthakrishnan R, Qu W *et al.* RAGE mediates podocyte injury in adriamycin-induced glomerulosclerosis. *J Am Soc Nephrol* 2008; **19**: 961–972.
14. Linden E, Cai W, He JC *et al.* Endothelial dysfunction in patients with chronic kidney disease results from advanced glycation end products (AGE)-mediated inhibition of endothelial nitric oxide synthase through RAGE activation. *Clin J Am Soc Nephrol* 2008; **3**: 691–698.
15. Tan AL, Sourris KC, Harcourt BE *et al.* Disparate effects on renal and oxidative parameters following RAGE deletion, AGE accumulation inhibition, or dietary AGE control in experimental diabetic nephropathy. *Am J Physiol Renal Physiol* 2010; **298**: F763–F770.
16. Yamamoto Y, Doi T, Kato I *et al.* Receptor for advanced glycation end products is a promising target of diabetic nephropathy. *Ann NY Acad Sci* 2005; **1043**: 562–566.
17. Yamamoto Y, Kato I, Doi T *et al.* Development and prevention of advanced diabetic nephropathy in RAGE-overexpressing mice. *J Clin Invest* 2001; **108**: 261–268.
18. Humpert PM, Djuric Z, Kopf S *et al.* Soluble RAGE but not endogenous secretory RAGE is associated with albuminuria in patients with type 2 diabetes. *Cardiovasc Diabetol* 2007; **6**: 9.
19. Nakamura K, Yamagishi S, Adachi H *et al.* Serum levels of sRAGE, the soluble form of receptor for advanced glycation end products, are associated with inflammatory markers in patients with type 2 diabetes. *Mol Med* 2007; **13**: 185–189.
20. Bopp C, Hofer S, Weitz J *et al.* sRAGE is elevated in septic patients and associated with patients outcome. *J Surg Res* 2008; **147**: 79–83.
21. Chao PC, Huang CN, Hsu CC *et al.* Association of dietary AGEs with circulating AGEs, glycated LDL, IL-1 $\alpha$  and MCP-1 levels in type 2 diabetic patients. *Eur J Nutr* 2010; **49**: 429–434.
22. Uribarri J, Cai W, Sandu O *et al.* Diet-derived advanced glycation end products are major contributors to the body's AGE pool and induce inflammation in healthy subjects. *Ann NY Acad Sci* 2005; **1043**: 461–466.
23. Yan SD, Chen X, Fu J *et al.* RAGE and amyloid-beta peptide neurotoxicity in Alzheimer's disease. *Nature* 1996; **382**: 685–691.
24. Waeber G, Calandra T, Roduit R *et al.* Insulin secretion is regulated by the glucose-dependent production of islet beta cell macrophage migration inhibitory factor. *Proc Natl Acad Sci USA* 1997; **94**: 4782–4787.
25. Sartipy P, Loskutoff DJ. Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proc Natl Acad Sci USA* 2003; **100**: 7265–7270.
26. Coughlan MT, Thorburn DR, Penfold SA *et al.* RAGE-induced cytosolic ROS promote mitochondrial superoxide generation in diabetes. *J Am Soc Nephrol* 2009; **20**: 742–752.
27. Rosca MG, Monnier VM, Szewda LI *et al.* Alterations in renal mitochondrial respiration in response to the reactive oxoaldehyde methylglyoxal. *Am J Physiol Renal Physiol* 2002; **283**: F52–F59.
28. Wautier MP, Chappey O, Corda S *et al.* Activation of NADPH oxidase by AGE links oxidant stress to altered gene expression via RAGE. *Am J Physiol Endocrinol Metab* 2001; **280**: E685–E694.
29. Coughlan MT, Thallas-Bonke V, Pete J *et al.* Combination therapy with the advanced glycation end product cross-link breaker, alagebrium, and angiotensin converting enzyme inhibitors in diabetes: synergy or redundancy? *Endocrinology* 2007; **148**: 886–895.
30. Goldberg T, Cai W, Peppas M *et al.* Advanced glycoxidation end products in commonly consumed foods. *J Am Diet Assoc* 2004; **104**: 1287–1291.
31. Maddison R, Ni Mhurchu C, Jiang Y *et al.* International Physical Activity Questionnaire (IPAQ) and New Zealand Physical Activity Questionnaire (NZPAQ): a doubly labelled water validation. *Int J Behav Nutr Phys Act* 2007; **4**: 62.
32. Cockcroft DW, Gault MH. Prediction of creatinine clearance from serum creatinine. *Nephron* 1976; **16**: 31–41.
33. Norman PE, Davis WA, Coughlan MT *et al.* Serum carboxymethyllysine concentrations are reduced in diabetic men with abdominal aortic aneurysms: Health in Men study. *J Vasc Surg* 2009; **50**: 626–631.
34. Forbes JM, Thallas V, Thomas MC *et al.* The breakdown of preexisting advanced glycation end products is associated with reduced renal fibrosis in experimental diabetes. *FASEB J* 2003; **17**: 1762–1764.
35. Bierhaus A, Haslbeck KM, Humpert PM *et al.* Loss of pain perception in diabetes is dependent on a receptor of the immunoglobulin superfamily. *J Clin Invest* 2004; **114**: 1741–1751.
36. Forbes JM, Yee LT, Thallas V *et al.* Advanced glycation end product interventions reduce diabetes-accelerated atherosclerosis. *Diabetes* 2004; **53**: 1813–1823.
37. Dunn SR, Qi Z, Bottinger EP *et al.* Utility of endogenous creatinine clearance as a measure of renal function in mice. *Kidney Int* 2004; **65**: 1959–1967.
38. Coughlan MT, Forbes JM, Cooper ME. Role of the AGE crosslink breaker, alagebrium, as a renoprotective agent in diabetes. *Kidney Int Suppl* 2007; **72**: S54–S60.
39. Thallas-Bonke V, Thorpe SR, Coughlan MT *et al.* Inhibition of NADPH oxidase prevents advanced glycation end product-mediated damage in diabetic nephropathy through a protein kinase C- $\alpha$ -dependent pathway. *Diabetes* 2008; **57**: 460–469.