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ORIGINAL ARTICLE

Effect of Irbesartan treatment on plasma and urinary markers of protein damage in patients with type 2 diabetes and microalbuminuria

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Abstract The aim of this study was to assess the effect of the angiotensin II receptor blocker Irbesartan on protein damage by glycation, oxidation and nitration in patients with type 2 diabetes and microalbuminuria. In a double-masked randomised crossover trial of 52 hypertensive type 2 diabetic patients, antihypertensive treatment was replaced with bendroflumethiazide. After 2-months wash-out, patients were treated randomly with Irbesartan 300, 600, and 900 mg o.d., each dose for 2 months in a three-way crossover study. Glycation, oxidation and nitration adduct residues in plasma protein and related urinary free adducts were determined by stable isotopic dilution analysis liquid chromatography–tandem mass spectrometry. Treatment with Irbesartan decreased urinary excretion of advanced glycation endproducts (AGEs)—methylglyoxal- and glyoxal-derived hydroimidazolones, MG-H1 and G-H1. Urinary AGEs were decreased by 30–32%. In plasma protein, treatment with Irbesartan increased content of glycation adducts N_{ε}-fructosyl-lysine, AGEs N_{ε}-carboxymethyl-lysine, N_{ε}-carboxyethyl-lysine and pentosidine, and also increased content of oxidation markers *N*-formylkynurenine and dityrosine. This was attributed to decreased clearance of plasma protein modified by N_{ε}-fructosyl-lysine and oxidative markers through the glomerular filter tightened by Irbesartan treatment. Treatment of patients with type 2 diabetes with Irbesartan decreased urinary excretion of MG-H1, G-H1 and 3-NT, which may result from decreased exposure to these AGEs. This is likely achieved by blocking angiotensin II signalling and related down-regulation of glyoxalase 1 and may contribute to health benefits of Irbesartan therapy.

Keywords Diabetic nephropathy · Microalbuminuria · Angiotensin receptor blocker · Glycation · Oxidative stress · Nitrotyrosine · Methylglyoxal · Glycosylation gap

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Introduction

Proteins in physiological systems are susceptible to spontaneous modifications by glycation, oxidation and nitration. Glycation of proteins is a complex series of parallel and sequential reactions collectively called the Maillard reaction. Early stage reactions are directed to lysine and N-terminal amino acid residues leading to the formation of the early glycation adduct, fructosyl-lysine (FL), and other fructosamine derivatives. Later stage reactions produce advanced glycation endproducts (AGEs). The most important AGEs quantitatively are hydroimidazolones derived from arginine residues modified by glyoxal, methylglyoxal and 3-deoxyglucosone - G-H1, MG-H1 and 3DG-H (mixture of 3 isomers), respectively (Ahmed et al. 2002). Other important and widely studied AGEs are N_{ε}-carboxymethyl-lysine (CML), N_{ε}-carboxyethyl-lysine (CEL) and crosslinks glyoxal- and methylglyoxal-derived lysine dimers (GOLD and MOLD, respectively) and pentosidine (PENT). Markers of irreversible oxidative damage to proteins are dityrosine (DT) and *N*-formylkynurenine (NFK)—products of

oxidation of tyrosine and tryptophan residues, respectively. A widely studied marker of nitration damage to proteins is 3-NT (Thornalley 2006) (Fig. 1a–c). Glycated, oxidised and nitrated proteins undergo cellular proteolysis leading to the formation of glycated, oxidised and nitrated amino acids called glycation, oxidation and nitration free adducts. These are released into plasma, readily filtered in renal glomeruli (molecular mass < 500 Da) and, unless repaired or further metabolised, are excreted in the urine (Fig. 1d).

Protein glycation, oxidation and nitration adduct residues in plasma protein are increased in experimental and clinical diabetes (Ahmed et al. 2005; Karachalias et al. 2010). Some therapeutic options to reverse early stage nephropathy in diabetes decrease the exposure to protein AGE, oxidation and nitration adducts, as judged by decreasing in urinary excretion of AGE, oxidation and nitration free adducts (Karachalias et al. 2010). Inhibition of the renin-angiotensin-aldosterone system (RAAS) by the use of angiotensin II receptor blockers (ARBs) and angiotensin converting enzyme inhibitors, usually combined with diuretics, is considered a first-line treatment modality for diabetic patients with microalbuminuria to delay onset of renal insufficiency (American Diabetes Association 2009). Irbesartan is a potent and selective angiotensin II subtype 1 receptor ARB used in the treatment of diabetic nephropathy (DN). In two large clinical

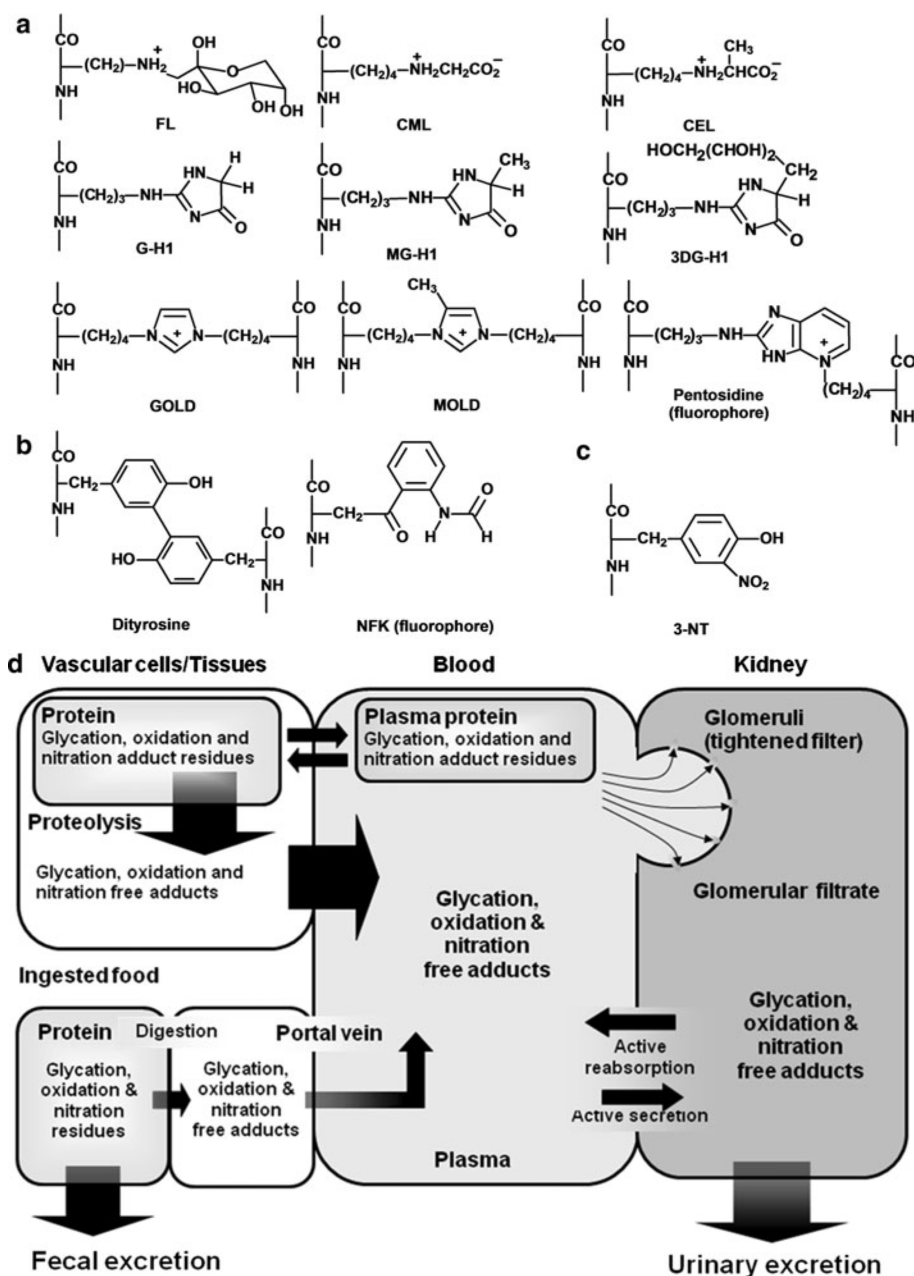


Fig. 1 Protein damage by glycation, oxidation and nitration. **a** Glycation adducts: early glycation adduct FL and advanced glycation endproducts. **b** Oxidation adducts, and **c** nitration adduct 3-NT. Adduct residues of damaged proteins are shown. The structure of one isomer of 3DG-H, 3DG-H1 (Ahmed et al. 2002). For the corresponding free adducts at physiological pH, the N-terminal amino group is protonated $-NH_3^+$ and the C-terminal carbonyl is a carboxylate $-CO_2^-$ moiety. **d** Multi-compartment model of formation, proteolytic processing and excretion of proteins damaged by glycation, oxidation and nitration in diabetic nephropathy

studies (Lewis et al. 2001; Parving et al. 2001), Irbesartan exerted a renoprotective effect in hypertensive patients with type 2 diabetes at both the early and later stages of DN. These long-term studies using Irbesartan 300 mg o.d. have demonstrated reduced progression to DN in microalbuminuric patients (Parving et al. 2001) and prevention or delay of the combined endpoint of death, end stage renal disease or doubling of creatinine in patients with DN (Lewis et al. 2001). At least part of the beneficial effect of ARBs in DN appears to be independent of hypotensive action. Decrease of markers of low-grade inflammation (C-reactive protein) and blood coagulation (fibrinogen) have been found (Persson et al. 2006).

In this report, we describe the effect of Irbesartan treatment of patients with type 2 diabetes and microalbuminuria on protein glycation, oxidation and nitration. Plasma and urine samples were analysed retrospectively of a trial to assess the dose-dependence of Irbesartan on urinary albumin excretion (UAE), glomerular filtration rate (GFR), blood pressure and other conventional biochemical and clinical variables (Rossing et al. 2005). Plasma protein content of glycation, oxidation and nitration adducts and urinary excretion of protein glycation, oxidation and nitration adducts were quantified by stable isotopic dilution analysis liquid chromatography with tandem mass spectrometric detection (LC–MS/MS).

Materials and methods

Patients and trial design

Patient recruitment, study design and clinical procedures were described previously (Rossing et al. 2005). Briefly, all patients were recruited from the Steno Diabetes Center. The study was a double-masked randomised three-way crossover trial including 52 (41 males) hypertensive type 2 diabetic patients with microalbuminuria on ongoing antihypertensive medication randomly assigned to crossover subgroups 1, 2 and 3 of 17, 17 and 18 patients, respectively. At inclusion, antihypertensive treatment was discontinued and replaced with bendroflumethiazide, 5 mg once daily, for the entire study. Following 2-months washout (baseline), patients were treated randomly with Irbesartan 300, 600, and 900 mg once daily, each dose for 2 months. Endpoints evaluated at the end of each study period included UAE (mean of three 24-h collections), 24-h ambulatory blood pressure and GFR (chromium 51 ethylenediaminetetraacetic acid method). Blood pressure, glycated haemoglobin HbA_{1c}, total cholesterol, LDL, VLDL, HDL and triglycerides were reported previously (Rossing et al. 2005). All doses of Irbesartan significantly decreased UAE, ambulatory blood pressure, and GFR from baseline; UAE was decreased more by Irbesartan 900 mg compared to other doses (Rossing et al. 2005). The local ethical committee approved the study, and all patients gave their written informed consent to participate in the study after the nature of the study had been explained. The study was performed in accordance with the Helsinki Declaration.

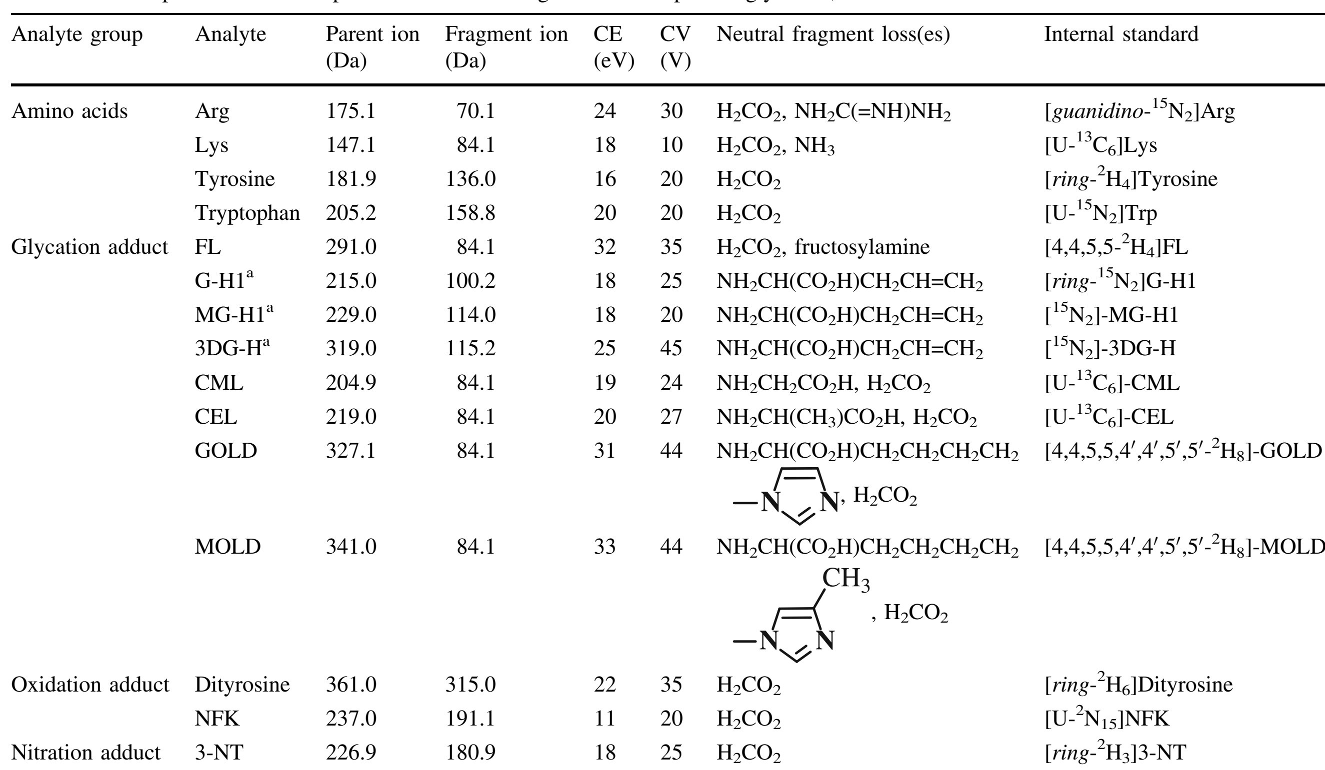
Protein damage marker analysis

Protein glycation, oxidation and nitration adducts were determined by LC–MS/MS with quantitation by stable isotopic dilution analysis (Thornalley et al. 2003). Optimised conditions for analyte detection are given in Table 1. Twelve analytes were determined: FL, CML, CEL, G-H1, MG-H1, 3DG-H, GOLD, MOLD, NFK, DT and 3-NT by stable isotopic dilution analysis LC–MS/MS, and PENT by concurrent fluorescence detection (excitation wavelength 320 nm, emission wavelength 365 nm). They were quantified by reference to calibration curve response of authentic standard. LC–MS/MS and fluorescence detection was performed with a Waters Acquity UPLC system with Acquity fluorescence detector and Quattro Premier XE tandem mass spectrometer. Urinary glycation, oxidation and nitration free adducts were determined by direct analysis of urine ultrafiltrates (3 kDa cut-off). Glycation, oxidation and nitration adduct residues of plasma protein were determined after exhaustive enzymatic hydrolysis by consecutive incubation with pepsin, pronase E, and finally aminopeptidase and prolidase, under nitrogen (Thornalley et al. 2003). Plasma was prepared immediately after blood collection. Twenty-four-hour urine samples were collected at ambient temperature without additives. Both were then stored at −80°C until analysis. Previous validation studies have shown that protein damage marker free adduct contents do not change significantly under these collection and storage conditions (Thornalley et al. 2003). Sample identity was blinded from the investigator.

Statistical analysis

Data are mean ± SD for parametric data and median (upper–lower quartile) for non-parametric data. Significance of difference between mean changes was assessed by Student's paired *t* test and two-way ANOVA. Significance of difference between median changes was assessed by Wilcoxon's signed ranks test and Friedman's two-way analysis of variance. Crossover subgroup data were compared by one-way ANOVA and Kruskal–Wallis test. Correlation analysis was by the Spearman non-parametric method. Bonferroni correction was applied for a 12 variable composite analysis of protein damage markers.

Table 1 Mass spectrometric multiple reaction monitoring detection of protein glycation, oxidation and nitration adducts



Analyte group	Analyte	Parent ion (Da)	Fragment ion (Da)	CE (eV)	CV (V)	Neutral fragment loss(es)	Internal standard
Amino acids	Arg	175.1	70.1	24	30	H_2CO_2, $NH_2C(=NH)NH_2$	[*guanidino*-$^{15}N_2$]Arg
	Lys	147.1	84.1	18	10	H_2CO_2, NH_3	[U-$^{13}C_6$]Lys
	Tyrosine	181.9	136.0	16	20	H_2CO_2	[*ring*-$^2H_4$]Tyrosine
	Tryptophan	205.2	158.8	20	20	H_2CO_2	[U-$^{15}N_2$]Trp
Glycation adduct	FL	291.0	84.1	32	35	H_2CO_2, fructosylamine	[4,4,5,5-$^2H_4$]FL
	G-H1[a]	215.0	100.2	18	25	$NH_2CH(CO_2H)CH_2CH{=}CH_2$	[*ring*-$^{15}N_2$]G-H1
	MG-H1[a]	229.0	114.0	18	20	$NH_2CH(CO_2H)CH_2CH{=}CH_2$	[$^{15}N_2$]-MG-H1
	3DG-H[a]	319.0	115.2	25	45	$NH_2CH(CO_2H)CH_2CH{=}CH_2$	[$^{15}N_2$]-3DG-H
	CML	204.9	84.1	19	24	$NH_2CH_2CO_2H$, H_2CO_2	[U-$^{13}C_6$]-CML
	CEL	219.0	84.1	20	27	$NH_2CH(CH_3)CO_2H$, H_2CO_2	[U-$^{13}C_6$]-CEL
	GOLD	327.1	84.1	31	44	$NH_2CH(CO_2H)CH_2CH_2CH_2CH_2$ —N(imidazole)N, H_2CO_2	[4,4,5,5,4′,4′,5′,5′-$^2H_8$]-GOLD
	MOLD	341.0	84.1	33	44	$NH_2CH(CO_2H)CH_2CH_2CH_2CH_2$ —N(imidazole, CH_3)N, H_2CO_2	[4,4,5,5,4′,4′,5′,5′-$^2H_8$]-MOLD
Oxidation adduct	Dityrosine	361.0	315.0	22	35	H_2CO_2	[*ring*-$^2H_6$]Dityrosine
	NFK	237.0	191.1	11	20	H_2CO_2	[U-$^2N_{15}$]NFK
Nitration adduct	3-NT	226.9	180.9	18	25	H_2CO_2	[*ring*-$^2H_3$]3-NT

[a] For hydroimidazolones, structural isomer-1 is denoted, although for 3DG-H detection, structural isomers 3DG-H1, 3DG-H2 and 3DG-H3 are all detected (Thornalley et al. 2003). Molecular and fragment ion detection was optimised to ±0.1 Da and collision energy and cone voltage to ±1 eV and 1 V, respectively. Other instrument settings were: capillary voltage 1.00 kV, source temperature 120°C, desolvation gas temperature 350°C, desolvation gas flow rate 898 l/h, cone gas flow rate 87 l/h, collision gas (argon) pressure 5.0×10^{-3} mbar, mobile phase flow rate 0.2 ml/min. Anlaytes were detection with a Quattro Premier XE tandem mass spectrometer (Waters, Manchester, UK)

Results

Clinical characteristics of type 2 diabetic patients with microalbuminuria during treatment with Irbesartan

Clinical characteristics at baseline and during study have previously been published (Rossing et al. 2005) but are summarised in Table 2. Mean UAE was decreased by 41–51% and GFR by 5–8% by Irbesartan treatment. Mean systolic blood pressure (BP) was decreased from 140 to 130–132 mmHg and mean diastolic BP was decreased from 77 to 70–71 mmHg by Irbesartan treatment. There were minor increases of plasma creatinine and HDL and minor decreases in fasting plasma glucose, total cholesterol and LDL cholesterol during Irbesartan treatment.

Protein damage markers

Patients with type 2 diabetes and microalbuminuria treated with Irbesartan had no significant change in plasma protein contents of AGEs MG-H1, G-H1, GOLD and MOLD, and also of 3-NT. Irbesartan treatment produced a small decrease in plasma protein content of 3DG-H; median plasma protein 3DG-H-Irbesartan treatment 0.21–0.22 mmol/mol arg, baseline 0.25 mmol/mol arg ($P < 0.001$). In contrast, plasma protein FL content was increased by 66–72% from baseline, independent of Irbesartan dose and plasma protein contents of CML, CEL and PENT were increased by 51–62, 149–180 and 34–43%, respectively, with Irbesartan treatment independent of dose. For protein oxidation markers, the plasma protein contents of NFK and DT were increased 40–60 and 60–81%, respectively, by Irbesartan therapy (Table 3; Fig. 2a–f).

For urinary excretion of protein damage markers, there was no significant change in urinary excretions of FL, CML, CEL, 3DG-H, GOLD, MOLD, PENT and NFK free adducts with Irbesartan treatment (Table 3). Urinary excretion of the major AGE, hydroimidazolone MG-H1 free adduct, was decreased 31–32% by Irbesartan, independent of dose (Fig. 3a). Excretion of G-H1 was decreased 35–45% by Irbesartan treatment independent of dose (Fig. 3b). Together, excretion of MG-H1 and G-H1 was decreased by 30–32% by Irbesartan treatment independent of dose. For oxidative markers, urinary excretion of DT was increased by 25% and 3-NT was decreased 44%

Table 2 Clinical variables of patients with type 2 diabetes and microalbuminuria at baseline and after Irbesartan treatment

Variable	Baseline	Irbesartan treatment		
		300 mg o.d.	600 mg o.d.	900 mg o.d.
24 h UAE (μg/min)	93 (72–118)	45 (40–50)***	47 (43–53)***	38 (34–43)***
GFR (ml/min/1.73 m^2)	103 ± 3	98 ± 3*	95 ± 3***	95 ± 3***
Plasma creatinine (μM)	94.5 ± 2.0	97.2 ± 2.1*	98.2 ± 2.1**	96.9 ± 2.3**
Fasting glucose (mM)	10.4 ± 0.4	9.6 ± 0.3**	9.7 ± 0.4	9.7 ± 0.3*
HbA1c (%)	8.4 ± 0.2	8.2 ± 0.2	8.3 ± 0.2	8.2 ± 0.2
Total cholesterol (mM)	4.32 ± 0.14	4.04 ± 0.14*	4.18 ± 0.10*	4.00 ± 0.09*
LDL (mM)	2.61 ± 0.11	1.88 ± 0.09*	2.03 ± 0.09*	1.94 ± 0.09*
HDL (mM)	1.21 ± 0.05	1.25 ± 0.05*	1.25 ± 0.05*	1.24 ± 0.05*
Triglyceride (mM)	2.05 ± 0.20	1.87 ± 0.18	1.94 ± 1.17	1.81 ± 0.17
Systolic BP (mmHg)	140 ± 2	132 ± 2***	130 ± 2***	130 ± 2***
Diastolic BP (mmHg)	77 ± 1	71 ± 1***	70 ± 1***	70 ± 1***

Data are mean ± SEM except for UAE which are geometric mean (95% CI)

Significance: *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ with respect to baseline

Table 3 Protein damage markers in plasma protein and urine of patients with type 2 diabetes and microalbuminuria at baseline

Plasma protein damage marker	Type 2 diabetic patients with microalbuminuria at baseline (n = 52)	Urinary protein damage marker free adduct (μmol/24 h)	Type 2 diabetic patients with microalbuminuria at baseline (n = 52)
FL (mmol/mol lys)	6.91 ± 1.29	FL	145 (92–206)
CML (mmol/mol lys)	0.052 ± 0.014	CML	10.3 (7.3–14.9)
CEL (mmol/mol lys)	0.008 (0.005–0.021)	CEL	3.8 (2.8–4.6)
G-H1 (mmol/mol arg)	0.075 (0.053–0.145)	G-H1	6.36 (4.22–11.63)
MG-H1 (mmol/mol arg)	0.40 (0.34–0.44)	MG-H1	118 (77–157)
3DG-H (mmol/mol arg)	0.25 (0.23–0.28)	3DG-H	5.43 (4.03–7.38)
GOLD (mmol/mol lys)	0.00034 (0.00019–0.0004)	GOLD	0.0061 (0.0037–0.0104)
MOLD (mmol/mol lys)	0.0034 (0.0026–0.0107)	MOLD	0.126 (0.084–0.202)
PENT (mmol/mol lys)	0.0012 (0.0010–0.0015)	PENT	0.066 (0.033–0.214)
NFK (mmol/mol trp)	0.38 (0.027–0.057)	NFK	0.52 (0.40–0.80)
DT (mmol/mol tyr)	0.0022 (0.0019–0.0030)	DT	0.0022 (0.0019–0.0030)
3-NT (mmol/mol tyr)	0.0071 (0.0034–0.0095)	3-NT	0.058 (0.023–0.162)

Data are mean ± SD or median (lower–upper quartile)

but only at the 300 mg o.d. Irbesartan dose with borderline significance ($P < 0.05$, Friedman) (Fig. 3c, d).

Correlation of protein damage and clinical variables was investigated for patients at baseline and after 300 mg o.d. Irbesartan treatment; correlations were similar for other Irbesartan doses. There were relatively few correlations of plasma protein damage markers at baseline and with Irbesartan treatment. Only plasma protein FL correlated with HbA_{1c} (Table 4). There were a greater number of significant correlations of urinary protein damage marker free adducts at baseline and an increase in positive correlations with Irbesartan treatment. Urinary CML, CEL, MG-H1 and PENT correlated positively with urinary FL at baseline with correlations with CML and MG-H1 remaining with Irbesartan treatment (Table 5).

Surprisingly, there were no correlations of plasma protein damage markers or urinary free adducts with GFR and UAE at baseline and only correlation of urinary NFK ($r = 0.48$, $P < 0.01$) and 3-NT ($r = 0.41$, $P < 0.05$) with GFR after Irbesartan treatment. Systolic blood pressure correlated positively with plasma protein MOLD ($r = 0.46$, $P < 0.05$) and negatively with plasma protein 3-NT ($r = -0.39$, $P < 0.05$). After Irbesartan therapy, these correlations were lost.

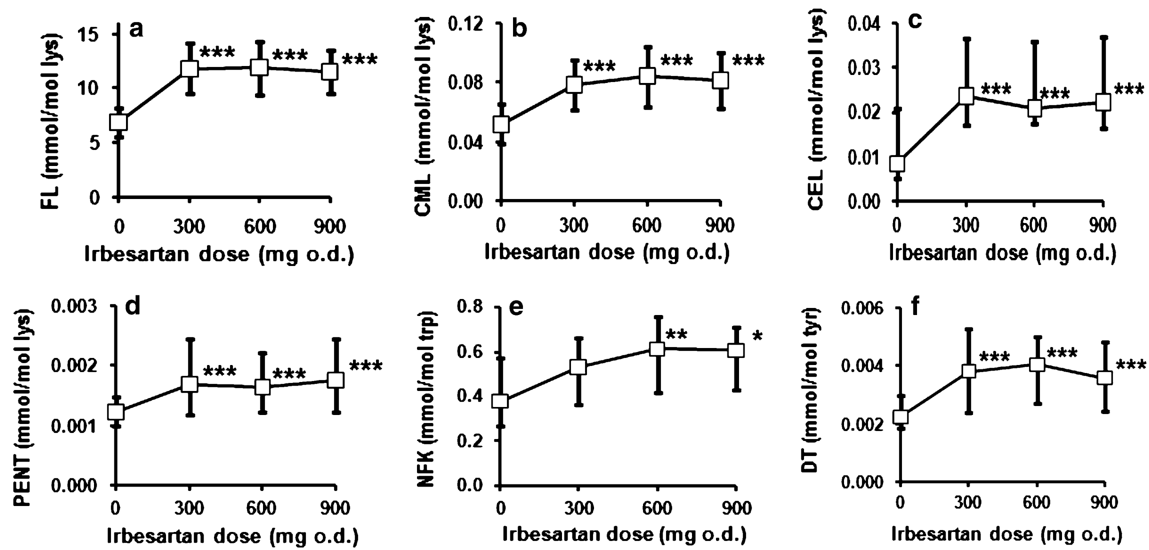


Fig. 2 Effect of Irbesartan dose on plasma protein glycation and oxidation adducts. **a** FL, **b** CML, **c** CEL, **d** PENT, **e** NFK and **f** DT. Data are normalised to the amino acid residue modified and are median (upper–lower quartile) except mean ± SD for **a** and **b**. Significance: *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ with respect to baseline (Wilcoxon's signed ranks test or paired *t* test (**a** and **b**) with Bonferroni correction)

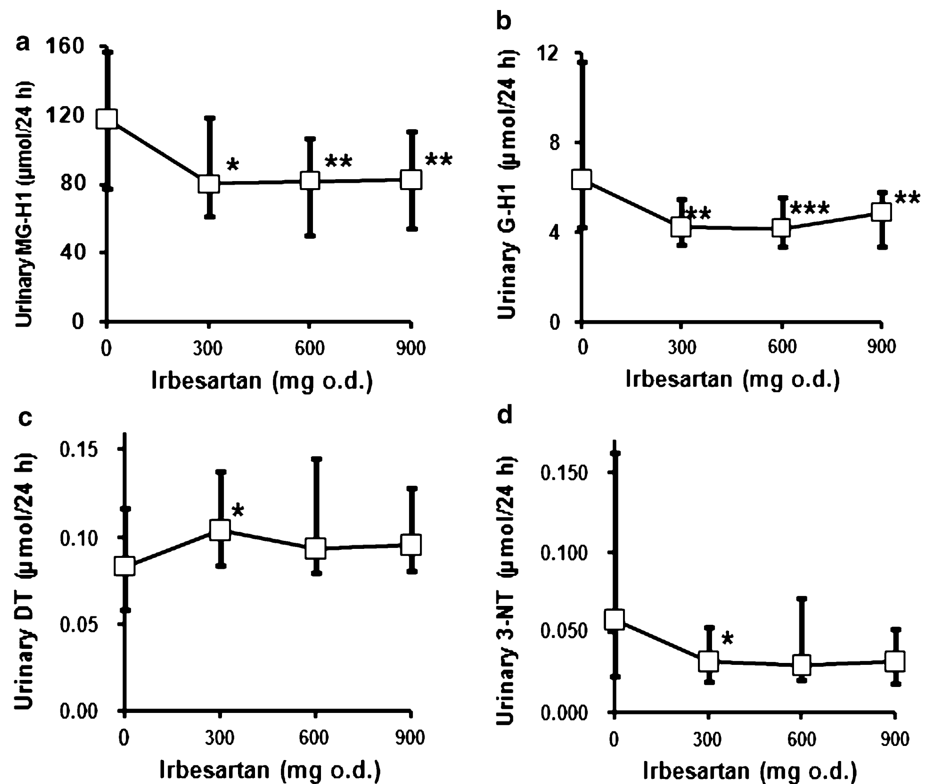
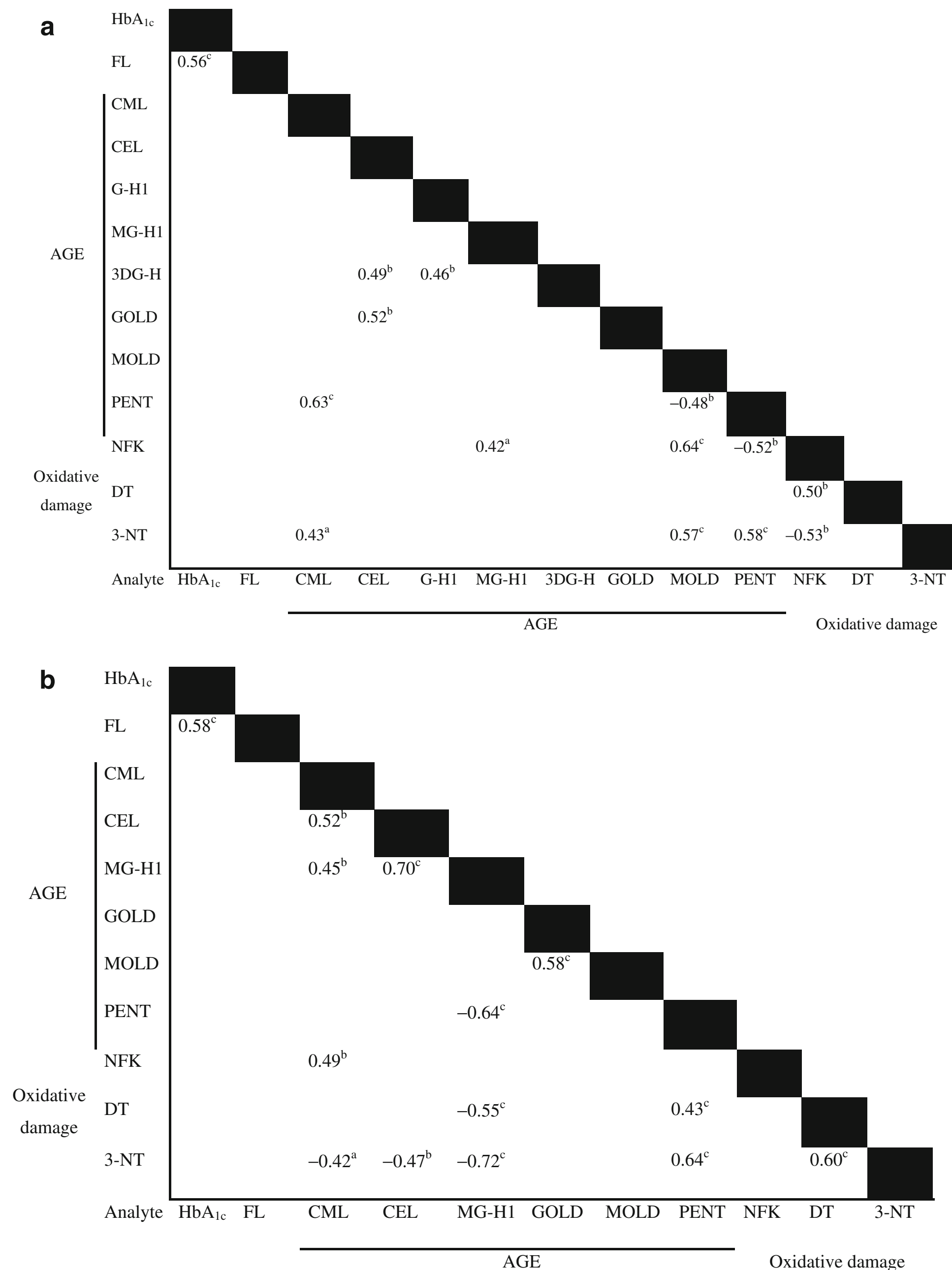


Fig. 3 Effect of Irbesartan dose on urinary free adduct excretion of glycation, oxidation and nitration adducts. **a** MG-H1, **b** G-H1, **c** DT and **d** 3-NT. Plasma protein adduct data are normalised to the amino acid residue modified. Data are median (upper–lower quartile). Significance: *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ with respect to baseline (Wilcoxon's signed ranks test with Bonferroni correction)

Carryover effects of Irbesartan treatment on protein damage marker analysis

Carryover effects, the effect of prior Irbesartan treatment in the second and third crossover periods, were examined by comparing plasma protein and urinary protein damage analyte levels in patient crossover subgroups 1–3 receiving 300 mg o.d. Irbesartan in successive 10-week treatment periods. Analytes showing significant difference over the three treatment periods with the 300 mg o.d. dose were:

Table 4 Correlation triangle of glycemic control and plasma protein damage-related variables at baseline (a) and after Irbesartan treatment (b)



a

Group	Analyte	HbA_{1c}	FL	CML	CEL	G-H1	MG-H1	3DG-H	GOLD	MOLD	PENT	NFK	DT	3-NT
	HbA_{1c}													
	FL	0.56[c]												
AGE	CML													
AGE	CEL													
AGE	G-H1													
AGE	MG-H1													
AGE	3DG-H				0.49[b]	0.46[b]								
AGE	GOLD				0.52[b]									
AGE	MOLD													
AGE	PENT			0.63[c]						−0.48[b]				
Oxidative damage	NFK						0.42[a]			0.64[c]	−0.52[b]			
Oxidative damage	DT											0.50[b]		
Oxidative damage	3-NT			0.43[a]						0.57[c]	0.58[c]	−0.53[b]		

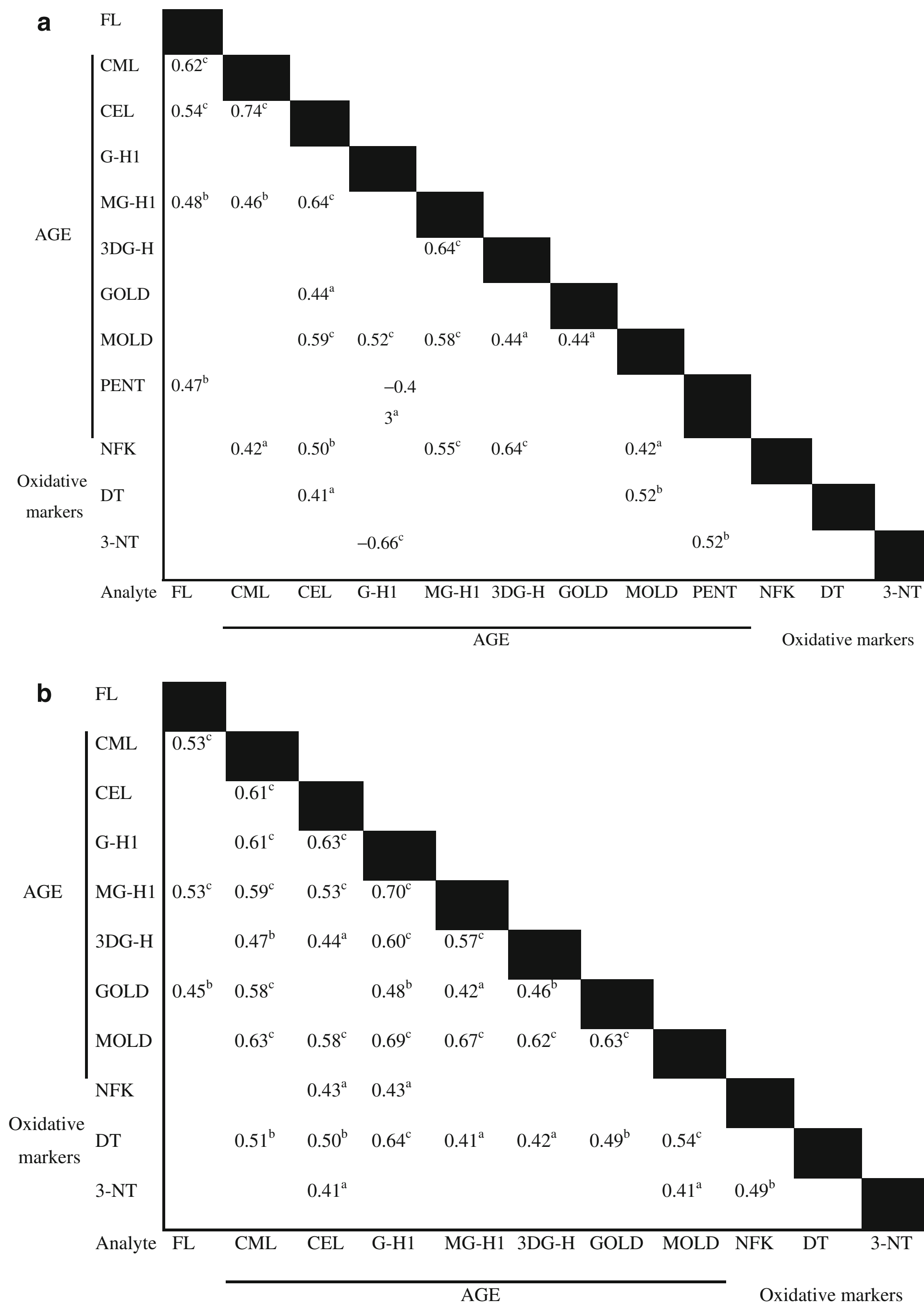
b

Group	Analyte	HbA_{1c}	FL	CML	CEL	MG-H1	GOLD	MOLD	PENT	NFK	DT	3-NT
	HbA_{1c}											
	FL	0.58[c]										
AGE	CML											
AGE	CEL			0.52[b]								
AGE	MG-H1			0.45[b]	0.70[c]							
AGE	GOLD											
AGE	MOLD						0.58[c]					
AGE	PENT					−0.64[c]						
Oxidative damage	NFK			0.49[b]								
Oxidative damage	DT					−0.55[c]			0.43[c]			
Oxidative damage	3-NT			−0.42[a]	−0.47[b]	−0.72[c]			0.64[c]		0.60[c]	

Data are correlation coefficients (Spearman) with significance: [a]$P < 0.05$, [b]$P < 0.01$ and [c]$P < 0.001$

plasma protein markers—CML, CEL, MG-H1, pentosidine and 3-NT, and urinary CEL ($P < 0.05$; Kruskal–Wallis). The variation of these analytes over the three treatment periods compared to the related baseline level was examined (Fig. 4). This revealed that plasma protein CML was increased modestly and CEL unchanged in the first treatment period and increased further in the second and third treatment periods, where patients had received higher dose Irbesartan in the previous treatment periods (Fig. 4a, b). Plasma protein content of MG-H1 was decreased in the initial treatment period but not thereafter (Fig. 4c). Plasma protein contents of pentosidine and 3-NT were increased in the initial treatment period but not thereafter, with plasma protein 3-NT content decreasing below baseline in the third treatment period (Fig. 4d, e). To eliminate carryover effects and to examine the dependence of change on

Table 5 Correlation triangle of glycemic control and urinary protein damage-related variables at baseline (a) and after Irbesartan treatment (b)



a

Group	Analyte	FL	CML	CEL	G-H1	MG-H1	3DG-H	GOLD	MOLD	PENT	NFK	DT	3-NT
	FL												
AGE	CML	0.62[c]											
AGE	CEL	0.54[c]	0.74[c]										
AGE	G-H1												
AGE	MG-H1	0.48[b]	0.46[b]	0.64[c]									
AGE	3DG-H					0.64[c]							
AGE	GOLD			0.44[a]									
AGE	MOLD			0.59[c]	0.52[c]	0.58[c]	0.44[a]	0.44[a]					
AGE	PENT	0.47[b]			−0.43[a]								
Oxidative markers	NFK		0.42[a]	0.50[b]		0.55[c]	0.64[c]		0.42[a]				
Oxidative markers	DT			0.41[a]					0.52[b]				
Oxidative markers	3-NT				−0.66[c]					0.52[b]			

Columns CML–PENT: AGE; NFK, DT, 3-NT: Oxidative markers

b

Group	Analyte	FL	CML	CEL	G-H1	MG-H1	3DG-H	GOLD	MOLD	NFK	DT	3-NT
	FL											
AGE	CML	0.53[c]										
AGE	CEL		0.61[c]									
AGE	G-H1		0.61[c]	0.63[c]								
AGE	MG-H1	0.53[c]	0.59[c]	0.53[c]	0.70[c]							
AGE	3DG-H		0.47[b]	0.44[a]	0.60[c]	0.57[c]						
AGE	GOLD	0.45[b]	0.58[c]		0.48[b]	0.42[a]	0.46[b]					
AGE	MOLD		0.63[c]	0.58[c]	0.69[c]	0.67[c]	0.62[c]	0.63[c]				
Oxidative markers	NFK			0.43[a]	0.43[a]							
Oxidative markers	DT		0.51[b]	0.50[b]	0.64[c]	0.41[a]	0.42[a]	0.49[b]	0.54[c]			
Oxidative markers	3-NT			0.41[a]					0.41[a]	0.49[b]		

Columns CML–MOLD: AGE; NFK, DT, 3-NT: Oxidative markers

Data are correlation coefficients (Spearman) with significance: [a]$P < 0.05$, [b]$P < 0.01$ and [c]$P < 0.001$

Irbesartan dose on protein damage markers, the levels of all analytes for all three doses of Irbesartan were compared to baseline levels at the end of the first dosing period. Treatment with Irbesartan increased plasma protein contents of FL, CML, pentosidine and dityrosine and decreased plasma protein content of MG-H1 at the 300 mg o.d. dose (Fig. 5). Overall, the effect was independent of Irbesartan dose. For urinary protein damage markers, there was no significant effect of Irbesartan treatment after the first treatment period for patient crossover subgroups. Since the effects of Irbesartan were independent of dose, the patient subgroups were combined and plasma protein

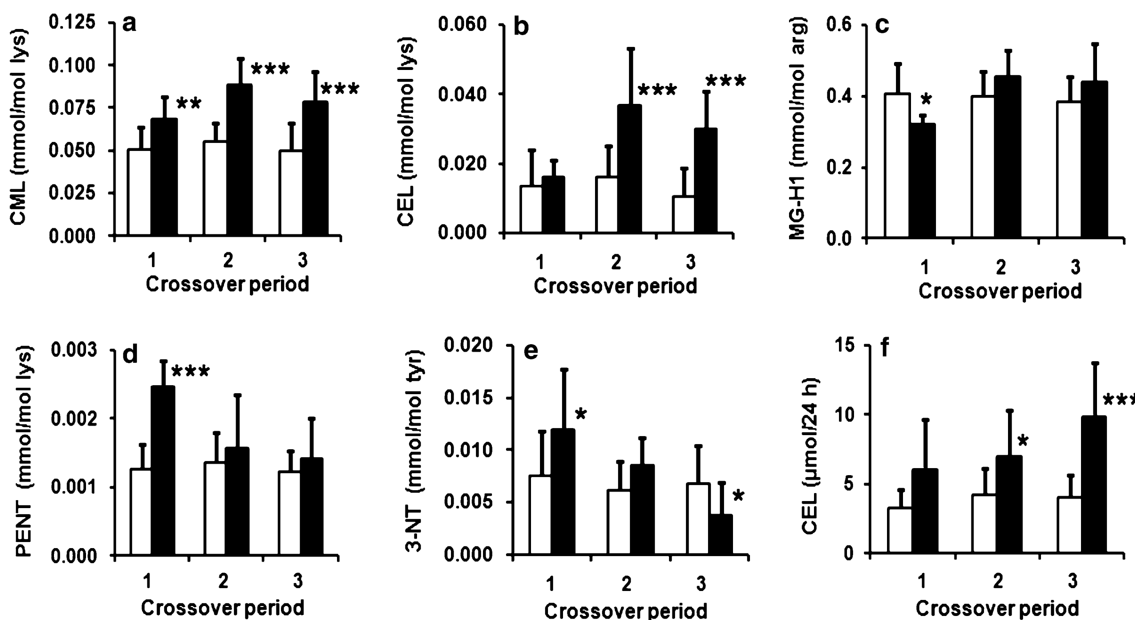


Fig. 4 Effect of crossover treatment period on the response of protein damage markers to Irbesartan treatment. **a** CML, **b** CEL, **c** MG-H1, **d** PENT and **e** 3-NT residue content of plasma protein—data are normalised to the amino acid residue modified. **f** CEL urinary free adduct: flux per 24 h. *Bars*: *unfilled* baseline, *filled* 300 mg o.d. Irbesartan (mean, error bar is SD). Crossover periods 1, 2 and 3 occurred after 10, 20 and 30 weeks Irbesartan treatment and had study subgroups of 17, 17 and 18 patients, respectively. Significance: $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ with respect to baseline (paired t test with Bonferroni correction)

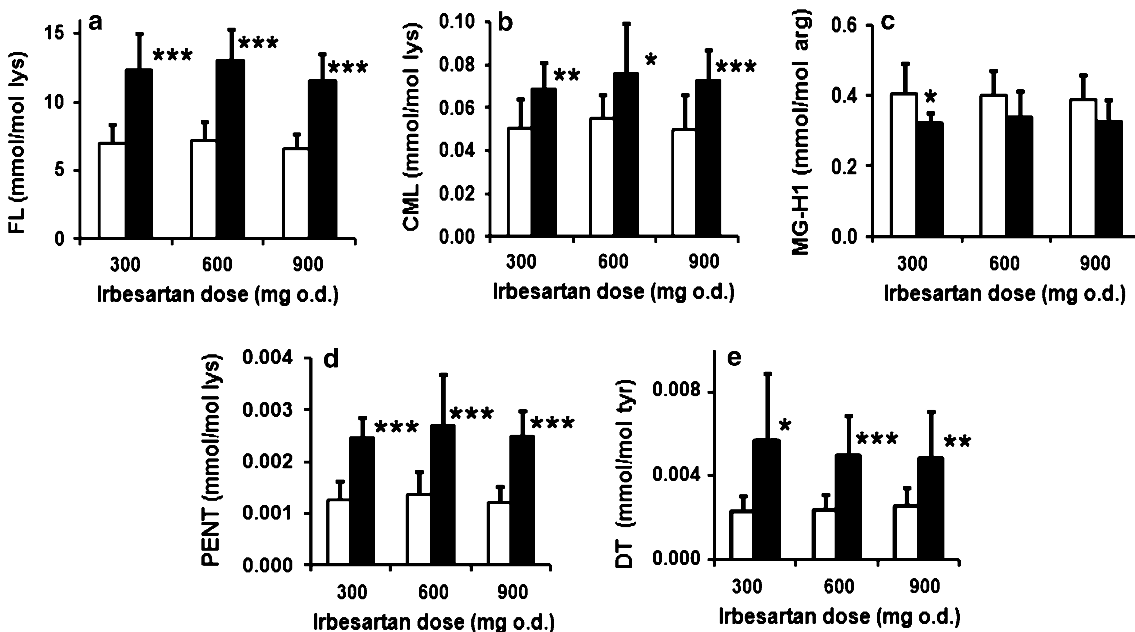


Fig. 5 Effect of Irbesartan dose on protein damage markers in the first treatment period. **a** FL, **b** CML, **c** MG-H1, **d** PENT and **e** DT residue content of plasma protein—data are normalised to the amino acid residue modified. *Bars*: *unfilled* baseline, *filled* Irbesartan treatment (mean; error bar is SD with $n = 17$ for 300 and 600 mg o.d. dose, and $n = 18$ for 900 mg o.d. dose). Significance: $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ with respect to baseline (paired t test with Bonferroni correction)

content of protein damage adduct residues and urinary fluxes of protein damage free adducts were re-examined. This revealed that for the first treatment period (any dose) where carryover effects do not apply Irbesartan produced increases in FL, CML, CEL, PENT and DT adduct residues and decreased 3DG-H adduct residue of plasma protein and decreased urinary flux of G-H1, MG-H1 and 3-NT free adducts (Figs. 6, 7).

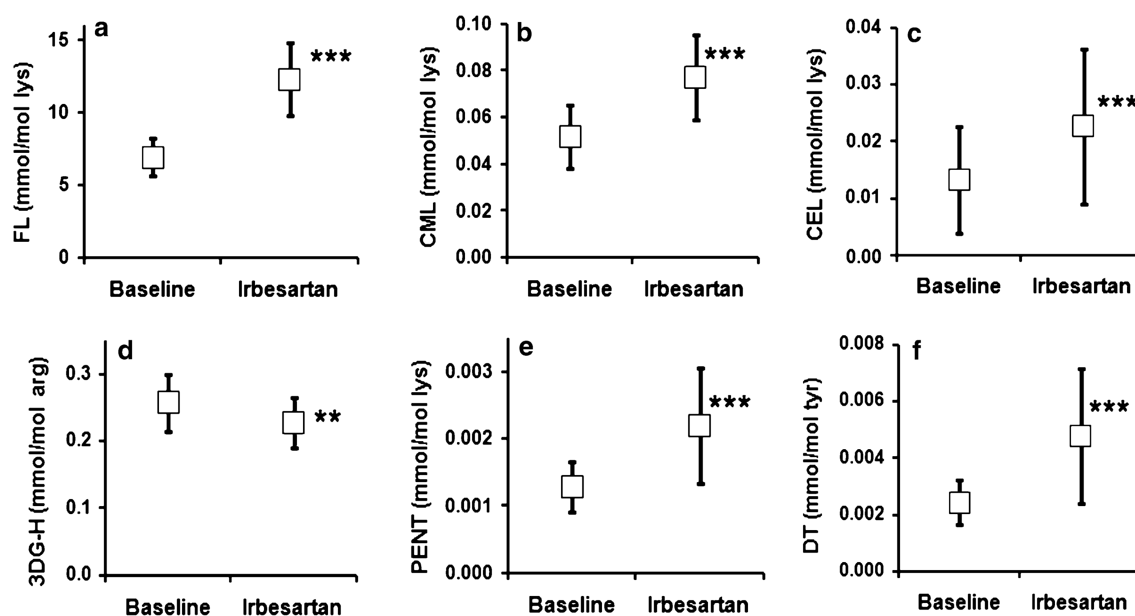


Fig. 6 Effect of all Irbesartan (all doses combined) on protein damage markers of plasma protein in the first treatment period. **a** FL, **b** CML, **c** CEL, **d** DG-H, **e** PENT and **f** DT. Data are normalised to the amino acid residue modified. Data are mean ± SD. Significance: $^{**}P < 0.01$ and $^{***}P < 0.001$ with respect to baseline (paired t test with Bonferroni correction)

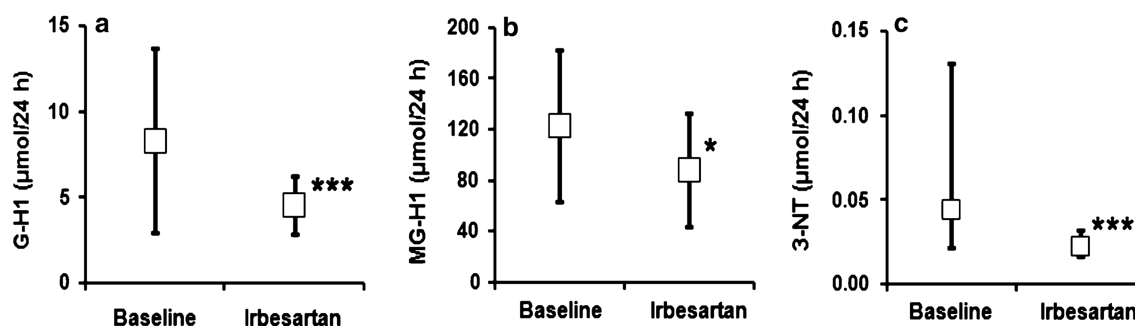


Fig. 7 Effect of all Irbesartan (all doses combined) on protein damage markers of urine in the first treatment period. **a** G-H1, **b** MG-H1 and **c** PENT. Data are urinary flux (µmol per 24 h); mean ± SD for **a** and **b** and median (upper–lower quartile) for **c**. Significance: $^{*}P < 0.05$, and $^{***}P < 0.001$ with respect to baseline [paired t test with Bonferroni correction, (**a**, **b**), Wilcoxon's signed ranks test (**c**)]

Discussion

In this study, we found that treatment of patients with type 2 diabetes with Irbesartan increased plasma protein FL residues. This increase was independent of change in glycaemic control since HbA_{1c} was unchanged and there was minor decrease in fasting plasma glucose. This effect was maintained when carryover effects were excluded. The explanation for this may be related to the effect of Irbesartan on the glomerular filter. Irbesartan treatment tightens the glomerular filter and thereby decreases protein leakage into glomerular filtrate. The glomerular filter has been found to exhibit preferential retention of FL-modified albumin in patients with type 2 diabetes (Cha et al. 1991). This may be exacerbated further by Irbesartan. This effect of Irbesartan may be expected as the mean molecular size of FL-modified albumin, as judged by Stoke's radii, is greater than that of the mean glomerular filter pore size of patients treated with Irbesartan (4.2 vs. 3.8 nm) (Remuzzi et al. 1999; Sattarahmady et al. 2007). Glomerular transit of FL-modified albumin may therefore be impaired with Irbesartan therapy. In contrast, modification of albumin by methylglyoxal and glyoxal forming MG-H1 and G-H1, respectively, did not increase the Stoke's radius of albumin (Sattarahmady et al. 2007; Westwood and Thornalley 1995) and MG-H1 and G-H1 content of plasma protein was not increased with Irbesartan treatment.

Increase of plasma protein FL content independent of change in HbA_{1c} uncouples assessment of glycaemic control by HbA_{1c} and plasma fructosamine. The difference between measured HbA_{1c} and predicted HbA_{1c}—the latter based on measurement of plasma fructosamine and the

usual linear relationship between HbA_{1c} and plasma fructosamine—has been called the "glycosylation gap" and is increased in DN (Cohen et al. 2003). The increase of plasma FL with Irbesartan treatment herein suggests that therapeutic agents tightening the glomerular filter may also contribute to the glycosylation gap. This deserves further investigation.

An expected consequence of increased FL content of plasma protein for patients with Irbesartan treatment is increased rate of degradation of FL to CML and CEL residues in plasma protein, leading to increased urinary excretion of CML and CEL free adducts. FL degrades oxidatively to CML and is a minor source of methylglyoxal—the precursor of CEL (Ahmed et al. 1986; Thornalley et al. 1999). Increased rate of FL degradation may also lead to increased formation of pentosidine (Dyer et al. 1991). This may explain the concurrent increase of plasma protein CML, CEL and pentosidine with increased FL with Irbesartan therapy. Irbesartan treatment also produced ca. twofold increases in NFK and DT adduct content of plasma protein. This suggests that NFK and DT-modified plasma protein may also be retained by the tightened glomerular filter with Irbesartan treatment and/or the rate of NFK and DT formation was increased by increased oxidation degradation of plasma protein FL.

In this study we found, for the first time, that Irbesartan therapy produced significant decreases in urinary excretion of selected AGEs MG-H1 and G-H1 and 3-NT. This was also found when carryover effects were excluded (see later). In diabetes, urinary AGE and 3-NT free adducts are thought to originate mainly from proteolysis of AGE-modified and nitrated tissue proteins, respectively (Karachalias et al. 2010). Urinary excretion of protein damage free adducts is related to total body exposure to protein damage, relating to flux of formation of glycation, oxidation and nitration adducts within tissues and body fluids except where there is significant sourcing of free adducts from others sources and/or significant metabolism and repair of free adducts (Karachalias et al. 2010; Thornalley et al. 2003). Repair is significant for FL free adduct, undergoing de-glycation to lysine by fructosamine 3-phosphokinase (Szwergold et al. 2001), and metabolism significant for 3-NT to 3-nitro-4-hydroxyphenylacetic acid and 3-nitro-4-hydroxyphenylacetic acid (Ohshima et al. 1990). These changes may therefore reflect decreased exposure to AGEs, particularly MG-H1 and G-H1, and protein nitration damage that likely contribute to health benefits of Irbesartan. Decrease in urinary MG-H1 and G-H1 is consistent with the recently discovered involvement of angiotensin blockade in regulation of glyoxalase 1 (Miller et al. 2010) which detoxifies methylglyoxal and glyoxal (Thornalley 1993). The flux of urinary MG-H1 free adduct is sourced mainly from the proteolysis of MG-H1 modified proteins in tissues (Karachalias et al. 2010). Increase in glyoxalase 1 expression in tissues will decrease tissue levels of methylglyoxal, decreasing MG-H1 residue content of tissues proteins and export for MG-H1 free adduct there from following proteolysis. This may occur without decrease in plasma protein MG-H1 content, although patients receiving 300 mg o.d. Irbesartan in the first phase of the crossover study had decreased plasma protein MG-H1 residue content with respect to baseline level (Fig. 4c).

A high flux of MG-H1 urinary free adduct excretion was found for patients with type 2 diabetes at baseline herein. This was found previously in patients with type 1 diabetes (Ahmed et al. 2005) and reflects the relatively high levels of protein damage caused by glycation by methylglyoxal in clinical diabetes (Thornalley 2005). Reversal of increased urinary excretion of MG-H1 by Irbesartan reflects decreased formation of MG-H1 in tissues and body fluids (Karachalias et al. 2010). Accumulation of MG-H1 in vascular cell mitochondrial proteins in diabetes is associated with increased mitochondrial dysfunction, oxidative stress and related metabolic dysfunction (Rosca et al. 2005). Increased formation of MG-H1 in vascular extracellular matrix type IV collagen was associated with endothelial detachment (Dobler et al. 2006)—a risk predictor of cardiovascular disease (Segal et al. 2002). Partial reversal of MG-H1 formation by Irbesartan therapy is, therefore, of likely pharmacological benefit.

There were very few correlations of protein damage markers with GFR and UAE. The lack of correlation with GFR may be due to the small 4–8% decrease in GFR from baseline induced by Irbesartan (Rossing et al. 2005) and limited glomerular filtration of most damaged plasma proteins (Rabbani et al. 2007). The lack of correlation with UAE is consistent with limited effect of increased interaction of albumin with the renal tubular epithelium on plasma protein glycation or tissue protein glycation and proteolysis. Irbesartan therapy changed multiple correlations of protein damage markers, however. In future studies of protein damage in DN it will be important to carefully match RAAS blockade therapy in patient study groups as ARB therapy may affect the protein damage assessment outcomes, particularly in correlation analysis.

A potentially confounding factor in this crossover study is the effect of carryover responses to Irbesartan from prior treatment periods. This may be expected particularly for protein damage markers of plasma protein where the half-life of the major plasma protein, albumin, is ca. 20 days (Peters 1996). There was no washout period in this three-way crossover design but rather a 2-week period during which the dose of Irbesartan was either gradually increased or decreased as appropriate for the following treatment dose. If there is no response of protein damage by

Irbesartan treatment or the response is stable and independent of Irbesartan dose, no crossover effect is expected. There were significant carryover effects for plasma protein CML, CEL, MG-H1, PENT and 3-NT and urinary flux of CEL. The effect of Irbesartan on protein damage markers was generally not dose-dependent and hence carryover effects are likely linked to the duration of Irbesartan treatment than difference in dose. This indicates that there are on-going changes in the formation and removal of damaged proteins proteolysis after 10 weeks of treatment that are likely linked to the long-term changes in renal structure and function associated with prolonged treatment with Irbesartan. In future crossover studies of this type, it will be important to include washout periods between each dosing period to eliminate carryover effects where ethically justifiable. A washout period of 3–4 half-lives of the affected marker is typical and for damage to albumin in plasma the washout period would be 8–11 weeks from metabolic considerations. Nevertheless, re-examination of protein damage markers in the first treatment period where carryover effects do not apply, there remained significant effects of protein damage markers in plasma protein and urine.

This study provides novel insights on the effect of Irbesartan on protein damage in DN provided by comprehensive assessment of glycation, oxidation and nitration adducts by LC–MS/MS. Previous studies of effect of Irbesartan on protein damage markers have been performed. Serum "AGE peptides", a fluorescence/absorbance ratio of deproteinised extract, were estimated in 269 type 2 diabetic patients with microalbuminuria receiving placebo or 300 mg o.d. Irbesartan for 2 years. No effect of treatment was found (Persson et al. 2006). Serum protein CML residues and total serum pentosidine were determined in 135 type 2 diabetic patients with overt nephropathy after treatment with 300 mg o.d. Irbesartan or placebo for 23 months. No significant difference was found between treatment and placebo groups (Busch et al. 2008). Plasma protein 3-NT residue content was estimated by enzyme-linked immunosorbent assay (ELISA) in 20 type 2 diabetic patients treated with 300 mg o.d. Irbesartan for 1 week and then given a high fat test meal, 75 g glucose or both. Irbesartan decreased both plasma 3-NT estimates before, during and after the tests meals (Ceriello et al. 2005). Since the estimates of plasma 3-NT by the custom ELISA used were 50–100 higher than expected values (Rabbani and Thornalley 2008), the estimates and changes of plasma 3-NT residue content of this study are insecure. The effect of Irbesartan on putative glomerular filtration retention of glycated and oxidised albumin found in this study is unprecedented and merits further investigation in future studies. A major weakness of previous studies was the assay methodology and also lack of estimates of the major AGE, MG-H1, and urinary AGEs. Where analytical methods were secure, as in the study of Busch et al. (2008), no comparison of Irbesartan effect can be made due to the difference in duration of Irbesartan treatment.

In summary, changes found in plasma protein damage and urinary free adduct excretion of diabetic patients receiving Irbesartan therapy herein are consistent with decreased exposure to protein damage by methylglyoxal, glyoxal and reactive nitrating species and the tightening of the glomerular filtration barrier.

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