The brown fat-secreted adipokine neuregulin 4 is decreased in human and murine chronic kidney disease

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Abstract

Objective: Neuregulin 4 (NRG4) has recently been introduced as a novel brown adipose tissue (BAT)-secreted adipokine with beneficial metabolic effects in mice. However, regulation of Nrg4 in end-stage kidney disease (ESKD) and type 2 diabetes mellitus (T2DM) has not been elucidated, so far.

Design/methods: Serum NRG4 levels were quantified by ELISA in 60 subjects with ESKD on chronic hemodialysis as compared to 60 subjects with an estimated glomerular filtration rate >50 mL/min/1.73 m² in a cross-sectional cohort. Within both groups, about half of the patients had a T2DM. Furthermore, mRNA expression of *Nrg4* was determined in two mouse models of diabetic kidney disease (DKD) as compared to two different groups of non-diabetic control mice. Moreover, mRNA expression of *Nrg4* was investigated in cultured, differentiated mouse brown and white adipocytes, as well as hepatocytes, after treatment with the uremic toxin indoxyl sulfate.

Results: Median serum NRG4 was significantly lower in patients with ESKD compared to controls and the adipokine was independently associated with a beneficial renal, glucose and lipid profile. In mice with DKD, *Nrg4* mRNA expression was decreased in all adipose tissue depots compared to control mice. The uremic toxin indoxyl sulfate did not significantly alter *Nrg4* mRNA expression in adipocytes and hepatocytes, *in vitro*.

Conclusions: Circulating NRG4 is independently associated with a preserved renal function and mRNA expression of *-Nrg4* is reduced in adipose tissue depots of mice with DKD. The BAT-secreted adipokine is further associated with a beneficial glucose and lipid profile supporting NRG4 as potential treatment target in metabolic and renal disease states.

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Introduction

The prevalence of diabetes mellitus and chronic kidney disease (CKD) is increasing and results in a rise of death rates for these diseases (1). Besides diabetes mellitus and hypertension as cardiometabolic disease states, obesity has also been shown to be an independent risk factor for a decline in renal function and death in a recent

meta-analysis (2). Thus, patients with a BMI of 40 kg/m² had a hazard ratio of 2.02 for an estimated glomerular filtration rate (eGFR) decline as compared to subjects with a BMI of 25 kg/m² (2). Conversely, Shulman and coworkers demonstrate a long-term protection against end-stage kidney disease (ESKD) after bariatric surgery-induced

weight loss as compared to control patients in the Swedish Obese Subjects (SOS) study (3). Thus, circulating adipose tissue-secreted factors, so-called adipokines, potentially could mediate the association between obesity and CKD.

During the last years, brown adipose tissue (BAT) has emerged as a major factor contributing to weight homeostasis and several BAT-secreted cytokines have been introduced as brown adipokines/batokines and potential pharmacological targets (4, 5).

Neuregulin 4 (NRG4) is a novel and predominantly BAT-secreted adipokine which protects against diet-induced insulin resistance and hepatic steatosis in mice (6). In more detail, Wang and coworkers demonstrate that Nrg4-deficient mice have increased insulin resistance and hepatic steatosis after high-fat diet feeding (6). Conversely, when mice with a transgenic overexpression of NRG4 are exposed to high-fat diet, they have an improved metabolic status, that is less body weight gain, improved dyslipidemia and insulin sensitivity, as compared to controls (6). Based on these data, NRG4 appears to be a novel brown adipokine with beneficial effects on obesity and its metabolic complications.

However, the regulation of circulating NRG4 in patients with CKD is far from clear and the role of NRG4 as a potentially protective factor for the development of ESKD has not been investigated, so far. Furthermore, NRG4 regulation in ESKD due to severe diabetic kidney disease (DKD) has to be compared to non-diabetic ESKD causes addressing a potential elimination of the novel brown adipokine.

Therefore, we quantified circulating NRG4 concentrations in 60 patients with ESKD on maintenance renal replacement therapy (RRT) treatment as compared to 60 subjects with an eGFR >50 mL/min/1.73 m². In both groups, about half of the patients had a type 2 diabetes mellitus (T2DM). Furthermore, the beneficial BAT-secreted adipokine was correlated with other markers of BAT function. Moreover, we investigated differential mRNA expression of *Nrg4* in two mouse models of DKD, that is, *eNOS*^{-/-}; *db/db* mice and *db/db* mice on a C57BLKS background, as compared to two different groups of non-diabetic control mice. Finally, mRNA expression of *Nrg4* was investigated in cultured, differentiated murine brown and white adipocytes, as well as in hepatocytes, after treatment with the uremic toxin indoxyl sulfate as compared to control treatment.

Our hypothesis was that the metabolically beneficial BAT-secreted adipokine is decreased in DKD and can serve as a potential treatment target in metabolic and renal disease states.

Methods

Subjects

The design of this cross-sectional study has been described previously (7, 8, 9, 10). Briefly, about 120 Caucasian subjects (women: $n=58$; men: $n=62$) were recruited by the Department of Endocrinology and Nephrology, University of Leipzig, from 2005 and 2007. Of the total cohort ($n=120$), about half ($n=60$) had an ESKD and were on maintenance RRT by hemodialysis. Study participants without CKD ($n=60$) had an eGFR >50 mL/min/1.73 m² according to the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (11) and, therefore, were coded as controls. In all participants, past medical history and anthropometric data were obtained by trained staff of the Department of Endocrinology and Nephrology, University of Leipzig. A T2DM was defined as a fasting glucose (FG) ≥ 126 mg/dL or use of insulin or oral hypoglycemic medications (12). Using these criteria, 32 of the 60 patients with ESKD on RRT and 30 out of 60 control patients were coded as T2DM. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as previously described (13). The following inclusion and exclusion criteria were applied for the present study: Inclusion criteria are age >18 years, non-pregnant, provided written informed consent; exclusion criteria: end-stage malignant diseases, acute generalized inflammation, acute infectious disease and history of drug abuse. The study was approved by the Ethics Committee of the University of Leipzig and all subjects gave written informed consent before taking part in the study.

Animal study

To determine NRG4 regulation in more detail, two animal models of CKD due to DKD on a C57BLKS background were compared to two groups of non-diabetic control mice. Thus, *eNOS*^{-/-}; *db/db* mice served as a model for severe DKD (14), whereas *db/db* mice were used as a model for mild DKD (15). Non-diabetic control animals included *eNOS*^{-/-} mice, as well as *db/+* mice on a C57BLKS background. All animal experiments were performed in the Medical Experimental Center at the University of Leipzig as described previously (10, 16, 17). All animal experiments were approved by the Local Ethics Committee of the State of Saxony (Landesdirektion Leipzig; approval nos. TVV 12/14 and TVV 65/15). Briefly, mice were maintained under pathogen-free conditions at $21 \pm 1^\circ\text{C}$ on a 12-h light/darkness cycle (6:00h–18:00h). All animals had free access to water and a normal pellet

diet. Spot urine was collected from all the mice in the morning at the age of 24 weeks. Afterward, the mice were killed by exsanguination under deep anesthesia with ketamine (WDT, Garbsen, Germany) and xylazine (Bayer Health Care, Wuppertal, Germany). Portions of the liver, kidney, as well as visceral (VAT), subcutaneous (SAT) and brown (BAT) adipose tissue, were snap-frozen in liquid nitrogen for RNA and protein isolation.

Assays

In human subjects, all blood samples were taken after a fasting period of at least 8 h. In all ESKD patients, blood was obtained just before hemodialysis started. Serum levels of NRG4 (Phoenix Pharmaceuticals, Burlingame, CA, USA) were quantified using an ELISA according to the manufacturer's instructions. All other adipokines were quantified by ELISAs as described previously (7, 8, 9, 18). Routine serum parameters including creatinine, FG, fasting insulin (FI), triglycerides (TG), total, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol, as well as free fatty acids (FFA) were measured in a certified laboratory by standard methods.

In animal experiments, urinary albumin and creatinine were determined in spot urine using Albuwell M and Creatinine Companion kits (Exocell, Philadelphia, PA, USA) and albumin-creatinine ratios (ACR) were calculated, respectively.

Effect of the uremic toxin indoxyl sulfate on *Nrg4* mRNA expression in murine brown and white adipocytes, as well as hepatocytes, *in vitro*

Briefly, immortalized brown preadipocytes (19), 3T3-L1 cells (American Type Culture Collection) and AML12 cells (American Type Culture Collection) were grown as previously described (brown adipocytes: (19), 3T3-L1 white adipocytes: (7), AML12 hepatocytes: (17)). According to Stockler-Pinto and coworkers (20), after starvation, 1 mM indoxyl sulfate was added for a period of 24 h to brown and white adipocytes, whereas AML12 hepatocytes were treated with 0.5 mM indoxyl sulfate for 24 h. The experiments were performed according to the recommendations of the European Uremic Toxin Work Group (EUTox) (21) and control cells were treated with 1 mM or 0.5 mM potassium sulfate (Sigma), respectively.

Analysis of *Nrg4* and *ErbB4* mRNA production

In animal and in *in vitro* experiments, *Nrg4* and *ErbB4* mRNA expression was determined relative to acidic ribosomal

phosphoprotein P0 (36B4 (*Rplp0*)) using quantitative real-time RT-PCR. One microliter cDNA was quantified on a LightCycler 480 real-time PCR 96-well thermocycler using LightCycler 480 Probes Master Mix (Roche Diagnostics GmbH) essentially as described in (7). The following primer pairs were used: *Nrg4*, CACTTGTGAAACGCTGCATGT and GGCAAATGACCTGTGCCTG (antisense); *ErbB4*, CATGGACCGGGACCTGACAA (sense) and GGTAAGT GGAATGGCCCGT (antisense); *36B4*, AAGCGCGTCCTG GCATTGTCT (sense) and CCGCAGGGGCAGCAGTGGT (antisense).

Statistical analysis

SPSS software version 24.0 (IBM) was used for all statistical analyses of human data. Overall, group differences between the four subgroups (Control/T2DM−; Control/T2DM+; RRT/T2DM−; RRT/T2DM+) were assessed by non-parametric Kruskal–Wallis test with Bonferroni *post hoc* analysis for continuous parameters and χ^2 test for categorical parameters. Afterward, linear regression models with adjustment for age, sex and BMI were calculated for all parameters. Before performing multivariate linear regression analyses, all continuous parameters were logarithmically transformed. Univariate correlations of other BAT-related adipokines with NRG4 were analyzed by non-parametric Spearman's rank correlation method.

For animal and *in vitro* experiments, GraphPad Prism 6 (GraphPad Software Inc.) was used. Relative *Nrg4* and *ErbB4* mRNA expression in different tissues adjusted for 36B4, as well as ACR, were analyzed in mice with DKD, that is *eNOS*^{−/−}; *db/db* mice and *db/db* mice and were compared to control mice, that is *eNOS*^{−/−} and *db/+* mice. Results for animal and *in vitro* experiments are shown as mean ± s.d. All parameters were logarithmically transformed prior analyses. Group differences in the animal and *in vitro* experiments were analyzed by one-way ANOVA with Tukey's multiple comparisons test, as well as by Student's *t* test, respectively.

A *P* value of <0.05 was considered as statistically significant in all analyses.

Results

Baseline characteristics of the human cohort (*n* = 120)

Basic clinical characteristics of the study population divided into the four subgroups (i.e. Control/T2DM−; Control/T2DM+; RRT/T2DM−; RRT/T2DM+) are shown

in Table 1. Median (interquartile range) serum NRG4 levels were 2.3 (0.7) µg/L in the total sample. In the total cohort, circulating NRG4 was significantly higher in female (2.3 (0.7) µg/L) as compared to male (2.1 (0.6) µg/L) subjects ($P=0.026$). In contrast, patients with T2DM (2.1 (0.8) µg/L) did not have different serum concentrations of the adipokine as compared to non-diabetic subjects (2.3 (0.7) µg/L) ($P=0.059$).

When patients were stratified by the four subgroups, that is, Control/T2DM–; Control/T2DM+; RRT/T2DM–; RRT/T2DM+, circulating NRG4 was significantly different between the four subgroups ($P=0.010$) (Table 1).

Association of NRG4 with metabolic parameters in the total cohort ($n = 120$)

Linear regression analysis revealed that serum concentrations of NRG4 remained independently and negatively associated with FI, HOMA-IR, TG and

creatinine (all $P<0.05$; Table 2) after adjustment for age, sex and BMI. There was a significant, independent and positive association between NRG4 and systolic blood pressure, HDL cholesterol and eGFR (all $P<0.05$; Table 2). In contrast, the BAT-secreted adipokine was not independently associated with markers of obesity, LDL cholesterol, FFA and inflammation (Table 2). After excluding patients on metformin treatment, the associations between NRG4 and FI, HOMA-IR, TG, HDL cholesterol and renal function remained significant (data not shown).

Correlation of NRG4 with other BAT-secreted adipokines and markers of BAT function in the total cohort ($n = 120$)

In the total cohort, NRG4 serum levels were positively correlated to irisin ($P=0.004$) and adiponectin levels ($P=0.016$) (Table 3). Furthermore, NRG4 was negatively

Table 1 Baseline characteristics of the study population. Baseline characteristics of the study population stratified by the four subgroups, i.e. Control/T2DM–, Control/T2DM+, RRT/T2DM– and RRT/T2DM+. Values for median (interquartile range) or total numbers (percentage) are shown. Categorical parameters were analyzed using the χ^2 test. Continuous parameters were analyzed by Kruskal–Wallis test followed by Bonferroni *post hoc* analysis. Overall P values of the Kruskal–Wallis test are shown and significant P values (<0.05) are depicted in bold.

	Control/T2DM–	Control/T2DM+	RRT/T2DM–	RRT/T2DM+	Overall P
n	30	30	28	32	–
Sex (m/f)	11/19	16/14	15/13	20/12	0.239
Nrg4 (µg/L)	2.5 (0.7)*	2.1 (1.0)	2.2 (0.2) [†]	2.1 (0.8)	0.010
Age (years)	63 (19)	63 (16)	59 (23)	68 (12)	0.051
BMI (kg/m ²)	28.2 (5.6)	29.1 (5.2)*	25.2 (6.5) [‡]	27.9 (6.6)	0.004
WHR	0.88 (0.12) [§]	0.94 (0.10)	0.96 (0.18)	1.00 (0.14) [†]	<0.001
WHR	0.57 (0.08) [§]	0.62 (0.10) [§]	0.57 (0.16)	0.66 (0.11) ^{†,‡}	0.006
SBP (mmHg)	125 (21)	126 (20)	125 (38)	120 (25)	0.339
DBP (mmHg)	77 (10)	73 (15)	77 (20)	70 (18)	0.095
FG (mmol/L)	5.1 (1.3) [‡]	7.6 (3.2)*, ^{†,§}	4.6 (1.2) [‡]	5.2 (3.3) [‡]	<0.001
FI (pmol/L)	45.1 (33.3)	47.9 (62.6)	28.2 (47.6)	50.1 (91.6)	0.230
HOMA-IR	1.4 (1.2)	2.8 (3.0)*	0.8 (1.4) [‡]	1.4 (3.3)	0.003
Cholesterol (mmol/L)	5.3 (0.9)*, [§]	4.9 (1.5)	4.4 (1.1) [†]	4.2 (1.3) [†]	<0.001
HDL cholesterol (mmol/L)	1.4 (0.4)*, [§]	1.2 (0.5) [§]	1.0 (0.5) [†]	1.0 (0.3) ^{†,‡}	<0.001
LDL cholesterol (mmol/L)	3.5 (1.1)*, ^{†,§}	2.9 (0.9) [†]	2.7 (0.9) [†]	2.1 (1.4) [†]	<0.001
TG (mmol/L)	1.1 (0.8)*, [§]	1.4 (0.9)	1.6 (0.9) [†]	1.8 (1.4) [†]	<0.001
FFA (mmol/L)	0.5 (0.2)	0.6 (0.4)	0.6 (0.5)	0.7 (0.5)	0.173
Creatinine (µmol/L)	76 (17)*, [§]	72 (22)*, [§]	829 (431) ^{†,‡}	717 (221) ^{†,‡}	<0.001
eGFR (mL/min/1.73 m ²)	78.8 (24.4)*, [§]	85.2 (23.0)*, [§]	5.0 (3.2) ^{†,‡}	5.7 (2.7) ^{†,‡}	<0.001
hsIL-6 (ng/L)	1.87 (2.11)*, [§]	2.07 (1.25)*, [§]	6.14 (4.91) ^{†,‡}	7.48 (7.30) ^{†,‡}	<0.001
Leptin (µg/L)	17.8 (25.4)	16.8 (22.3)	11.4 (35.1)	28.0 (54.4)	0.065
Metformin treatment (%)	0 (0)	21 (70)	0 (0)	0 (0)	<0.001

Superscript numbers (*, †, ‡, §) indicate subgroup comparisons with Bonferroni *post hoc* analysis.

*,^{†,‡,§}Indicates $P < 0.05$ as compared to *RRT/T2DM–, [†]Control/T2DM–, [‡]Control/T2DM+, and [§]RRT/T2DM+, respectively.

BMI, body mass index; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; FFA, free fatty acids; FI, fasting insulin; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; hsIL-6, high-sensitivity interleukin-6; LDL, low-density lipoprotein; RRT, maintenance renal replacement therapy; SBP, systolic blood pressure; T2DM, type 2 diabetes mellitus; TG, triglycerides; WHR, waist-to-hip ratio; WHtR, waist-to-height ratio.

Table 2 Association of serum neuregulin 4 concentrations with anthropometric parameters and markers of glucose metabolism, serum lipids, inflammation and renal function. Associations were assessed in linear regression models adjusted for age, sex and BMI, respectively. All parameters were logarithmically transformed prior to analyses. Standardized β -coefficients and P values are given.

	β	P
Age (years)	–	n.s.
BMI (kg/m ²)	–	n.s.
WHR	–	n.s.
WHtR	–	n.s.
SBP (mmHg)	0.203	0.030
DBP (mmHg)	–	n.s.
FG (mmol/L)	–	n.s.
FI (pmol/L)	–0.240	0.013
HOMA-IR	–0.222	0.021
Cholesterol (mmol/L)	–	n.s.
HDL cholesterol (mmol/L)	0.441	<0.001
LDL cholesterol (mmol/L)	–	n.s.
TG (mmol/L)	–0.452	<0.001
FFA (mmol/L)	–	n.s.
Creatinine (μ mol/L)	–0.276	0.004
eGFR (mL/min/1.73 m ²)	0.277	0.003
hsIL-6 (ng/L)	–	n.s.
Leptin (μ g/L)	–	n.s.

n.s., non-significant model.

related to fibroblast growth factor 21 (FGF21) ($P=0.003$) (Table 3). In contrast, there was no significant correlation between angiopoietin-like protein 8 and NRG4 (Table 3).

***Nrg4* and *ErbB4* mRNA expression in mice with DKD**

Figure 1 summarizes animal data on NRG4 regulation in two mouse models of DKD, that is *eNOS*^{–/–}/*db/db* mice and

db/db mice as compared to two different groups of non-diabetic control mice, that is, *eNOS*^{–/–} mice and *db/+* mice.

In all adipose tissue depots investigated, that is BAT, VAT and SAT, *Nrg4* mRNA expression was significantly reduced in 24-week-old mice with DKD. The lowest expression of *Nrg4* was observed in mice with severe DKD, that is *eNOS*^{–/–}/*db/db* mice, in BAT and SAT (Fig. 1A and C), as well as in mice with mild DKD, that is *db/db* mice, in VAT (Fig. 1B). In contrast, *Nrg4* mRNA expression in the liver was significantly increased in mice with DKD as compared to two groups of non-diabetic controls (Fig. 1D), whereas *Nrg4* mRNA expression in the kidney was unaltered (Fig. 1E).

Urinary ACR was increased in mice with severe DKD, that is, *eNOS*^{–/–}/*db/db* mice (Fig. 1F).

In the kidneys, *mErbB4* mRNA expression was significantly higher in *eNOS*^{–/–} mice as compared to *db/db* mice (Supplementary Fig. 1, see section on [supplementary data](#) given at the end of this article). In contrast, renal *mErbB4* mRNA expression was not different with control mice, that is, *db/+* mice, compared to mice with severe DKD, that is *eNOS*^{–/–}/*db/db* mice (Supplementary Fig. 1).

Effect of the uremic toxin indoxyl sulfate on *Nrg4* mRNA expression in murine brown adipocytes, 3T3-L1 adipocytes and AML12 hepatocytes *in vitro*

Treatment of murine brown adipocytes (Fig. 2A), murine 3T3-L1 white adipocytes (Fig. 2B), as well as murine AML12 hepatocytes (Fig. 2C), with the uremic toxin indoxyl sulfate for 24 h did not significantly alter the mRNA expression of *Nrg4* as compared to control cells treated with potassium sulfate.

Table 3 Univariate correlations of several BAT-secreted adipokines and markers of BAT function with serum neuregulin 4.

Univariate correlations of serum levels of the respective adipokines with each other in the total study population ($n = 120$). r and P values are given.

		Neuregulin 4	Irisin	Angptl8	Adiponectin	FGF21
Neuregulin 4 (μ g/L)	r	–				
	P	–				
Irisin (μ g/L)	r	0.261	–			
	P	0.004	–			
Angptl8 (μ g/L)	r	0.125	0.258	–		
	P	0.174	0.004	–		
Adiponectin (mg/L)	r	0.220	0.001	–0.100	–	
	P	0.016	0.976	0.275	–	
FGF21 (ng/L)	r	–0.266	–0.008	–0.150	0.238	–
	P	0.003	0.849	0.101	<0.001	–

Bold values indicate significant correlation as assessed by Spearman's correlation method.

Angptl8, angiopoietin-like protein 8; BAT, brown adipose tissue; FGF21, fibroblast growth factor 21.

Figure 1

Neuregulin 4 regulation in 24-week-old mice with severe DKD (*eNOS^{-/-}db/db*; black bars) and mild DKD (*db/db*; dark grey bars) as compared to two groups of non-diabetic control mice, i.e. *eNOS^{-/-}* mice (light grey bars) and *db/+* mice (white bars). (A, B, C, D and E) *Nrg4* mRNA expression normalized to *36B4* in (A) brown adipose tissue (BAT), (B) visceral adipose tissue (VAT), (C) subcutaneous adipose tissue (SAT), (D) liver, as well as (E) kidney. (F) Urinary albumin-creatinine ratio (ACR) in spot urine. Results are shown as means $\pm$ standard deviation. *P* values as assessed by one-way ANOVA with Tukey's multiple comparisons test. $n \geq 3$ per group. *Indicates $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Discussion

In the current study, we show that the novel BAT-secreted adipokine NRG4 is significantly decreased in patients with ESKD on RRT as compared to controls and eGFR is positively and independently associated with circulating NRG4. Furthermore, the BAT-secreted adipokine is associated with a beneficial glucose and lipid profile and other BAT-related adipokines. In two different mouse models of DKD, *Nrg4* mRNA expression is significantly reduced in adipose tissue as compared to two groups of non-diabetic control mice. Moreover, the uremic toxin indoxyl sulfate does not reduce the *Nrg4* mRNA expression in adipocytes and hepatocytes *in vitro*.

Our results indicate that patients with ESKD have lower circulating levels of the metabolically beneficial brown adipokine NRG4 as compared to control patients with an eGFR >50 mL/min/1.73 m². In contrast to NRG4, other metabolic cytokines including leptin (22), adiponectin (23), retinol-binding protein-4 (8), progranulin (24), adipocyte fatty acid-binding protein (25), FGF21 (26) and follistatin-like 3 (17) are significantly elevated in ESKD. Interestingly, lower concentrations in ESKD have also been reported for the adipokines irisin (18, 27) and fetuin B (10). To investigate the regulation of NRG4 in CKD in more detail, we, therefore, have quantified mRNA expression of *Nrg4* in two mouse models with CKD due to DKD as compared to two groups of non-diabetic control mice. Interestingly, *Nrg4* mRNA expression in adipose tissue which is the main source

of NRG4 (6) is significantly downregulated in mice with DKD as compared to non-diabetic control mice in all adipose tissue depots, i.e. BAT, VAT and SAT. Based on our results, uremic and/or metabolic disturbances observed in CKD and DKD might influence *Nrg4* expression in renal dysfunction on a transcriptional level similar to irisin (27). We, therefore, have treated murine brown and white adipocytes, as well as murine hepatocytes, with the uremic toxin indoxyl sulfate. Here, the uremic toxin indoxyl sulfate does not alter the mRNA expression of *Nrg4* in all three cell types suggesting that this major uremic toxin is not responsible for reduced circulating NRG4 in ESKD. It should be noted that in addition to indoxyl sulfate other uremic toxins are increased in CKD (reviewed in (28)) potentially contributing to the observed effects. Thus, p-cresyl sulfate, another protein-bound uremic toxin, directly induces cardiometabolic effects, i.e. insulin resistance in CKD (29). Further studies, therefore, are needed to investigate whether NRG4 has direct and causal effects on renal function and to identify the pathophysiological mechanisms of NRG4 regulation in CKD. It has to be pointed out that Nugroho and coworkers have convincingly demonstrated that NRG4 directly induces angiogenesis and angiogenic function in adipose tissue thereby ameliorating adipose tissue hypoxia (30, 31). Furthermore, ERBB4 signaling is involved in the activation of eNOS in cardiac myocytes (32). Thus, future studies also need to investigate whether vascular effects of NRG4 directly contribute to the development of CKD. Importantly, our translational findings suggest

Figure 2

Neuregulin 4 regulation in (A) differentiated, immortalized murine brown adipocytes, (B) murine 3T3-L1 white adipocytes and (C) murine AML12 hepatocytes after treatment with 1 mM (A and B) or 0.5 mM (C) indoxyl sulfate for 24 h as compared to control cells treated with 1 mM (A and B) or 0.5 mM (C) potassium sulfate for 24 h. The mRNA expression of *Nrg4* normalized to *36B4* is depicted. Results are shown as means $\pm$ standard deviation. *P* value as assessed by Student's *t* test. $n \geq 5$ per group.

that markers of renal function, for example, creatinine or eGFR, should be included in all future studies on *Nrg4* physiology.

Besides renal function, NRG4 is independently and positively associated with HDL cholesterol, as well as negatively correlated to TG, in our cohort. Interestingly, these results are virtually the same when patients with ESKD and subjects without CKD are analyzed separately (data not shown). When the association between NRG4 with HDL cholesterol and TG are investigated in the four subgroups separately, correlations remain significant only in patients with T2DM (data not shown). These data suggest a beneficial metabolic regulation of NRG4 in patients with ESKD. In accordance with this hypothesis, Wang *et al.* demonstrate that mice with a transgenic overexpression of NRG4 show lower TG levels in both the liver and plasma (6). Furthermore, Yan and coworkers have shown an independent and positive association between NRG4 and HDL cholesterol in 311 Chinese patients with newly diagnosed T2DM supporting our subgroup analyses (33). Thus, future studies need to investigate whether NRG4 causally regulates plasma lipoprotein particles. Furthermore, NRG4 is positively correlated with irisin and adiponectin in our cohort. For both adipokines, a beneficial correlation with markers of dyslipidemia has been reported previously (34, 35). The negative association of NRG4 with FGF21 might also be mediated by the well-known upregulation of FGF21 in cardiometabolic disease states (36).

It is interesting to note that in our cohort, patients with T2DM have unaltered NRG4 serum levels as compared to non-diabetic controls. This is in contrast to previous data from our group demonstrating reduced NRG4 levels in women with gestational diabetes mellitus as compared to controls (37). Furthermore, data on circulating levels of *Nrg4* in patients with T2DM are inconsistent, so far and some studies suggest decreased (38) or increased (39) NRG4 levels in patients with T2DM as compared to controls. Mechanistically, Wang and coworkers elegantly demonstrate that mice with a transgenic overexpression of NRG4 have had an improved insulin sensitivity as compared to controls (6). In more detail, the adipokine exerts its beneficial metabolic effects by binding to the ERBB4 receptor (6). Zeng and coworkers convincingly demonstrate that heart rescued ERBB4 deletion mice (*ErbB4*^{-/-}*ht*⁺) have increased HOMA-IR levels (40). Furthermore, treatment of epididymal adipocytes from WT mice with recombinant NRG4 has increased glucose uptake to levels comparable to insulin treatment, whereas there is no effect of NRG4 in adipocytes isolated from *ErbB4*^{-/-}*ht*⁺ mice (40).

It is worth noting that we have shown a beneficial and negative association of the adipokine with markers of insulin resistance, for example, HOMA-IR, in our cohort supporting the rodent data from Zeng *et al.* (40). Based on our data in mice with DKD, there is no similar pattern of renal *ErbB4* mRNA expression as compared to *Nrg4* expression in adipose tissue depots. Thus, a disturbed ERBB4 regulation does not seem to be causally involved in the observed renal associations.

We have further found a positive association of NRG4 with systolic blood pressure in our human cohort. This is in contrast to data from Cai and coworkers showing decreased NRG4 levels in patients with hypertension as compared to normotensive controls (41). However, statistical significance has been lost after adjustment for confounders in their Chinese study (41). Thus, future studies need to assess the causal role of NRG4 on hypertension in human metabolic disease states.

Taken together, circulating NRG4 is independently associated with a preserved renal function and mRNA expression of *Nrg4* is reduced in adipose tissue depots of mice with DKD. The BAT-secreted adipokine is further associated with a beneficial glucose and lipid profile supporting NRG4 as potential treatment target in metabolic and renal disease states.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/EJE-19-0017>.

Declaration of interest

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

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Author contribution statement

S K, A H, M F and T E wrote the manuscript and researched data. N K, A F, H K, M N, S P and A B researched data and reviewed/edited the manuscript. M B, M-Z Z, R C H and M S contributed to the discussion and reviewed/edited the manuscript. Guarantor: Dr Thomas Ebert is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. S K, A H, M F and T E equally contributed to this work.

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