Serum WNT4 protein as an indicator of chronic glomerulonephritis but not a marker of inflammatory cell infiltration and fibrosis: A preliminary study

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Abstract

Background. The WNT signaling pathway contributes to renal fibrosis, which is a hallmark of chronic kidney disease (CKD). Serum concentration of WNT4 could be used to monitor the kidney disease; however, no data have yet been published on the subject.

Objectives. This study measures WNT4 protein in serum of CKD patients depending on the stage, type of nephropathy, the non-nephrotic (NNP) or nephrotic proteinuria (NP), inflammatory cell infiltration in kidney parenchyma (IIKP), interstitial fibrosis in biopsy and serum creatinine. We also evaluated the usefulness of the serum WNT4 as a marker of fibrosis and IIKP.

Materials and methods. The WNT4 protein level in serum of CKD patients and healthy individuals was measured using enzyme-linked immunoassay (ELISA). Patients' blood biochemical profiles and kidney biopsies were evaluated with common laboratory methods.

Results. The serum level of WNT4 protein was higher in CKD patients (i) regardless of the underlying etiology and at early stages of disease; (ii) with lupus nephritis and Immunoglobulin A (IgA) nephropathy; (iii) without or with a small area of IIKP; and (iv) with a small area covered with fibrosis. No difference was observed between NNP and NP patients. The utility of serum WNT4 as a marker of IIKP and fibrosis was not confirmed. Negative correlations with total and low-density lipoprotein (LDL)-cholesterol were found in CKD and IIKP patients. In patients with serum WNT4 above the median value, serum creatinine was higher. However, no correlation between serum WNT4 and creatinine level was found.

Conclusions. The observed increase in serum WNT4 protein in the early stages of CKD and in patients diagnosed with immune-mediated glomerular disease may suggest that WNT4 may act as a mediator of inflammation. A certain association with the dysregulation of serum lipid metabolism can also be suspected. Serum WNT4 protein may be considered as the indicator of chronic glomerulonephritis, but not a diagnostic marker of IIKP and fibrosis.

Key words: chronic kidney disease, serum, WNT4, kidney fibrosis, inflammatory cells infiltration

Background

Chronic kidney disease (CKD) develops as a consequence of age-related lesions, infections, metabolic disorders, episodes of acute kidney injury, autoimmune glomerulopathies, toxic injury, and many other insults that lead to progressive renal dysfunction. Infiltration of the tubulointerstitial compartment with inflammatory cells followed by interstitial fibrosis and tubular atrophy, as well as progressive glomerular sclerosis, constitute the histopathological appearance of late-stage nephropathies.[1] Chronic kidney disease is defined as abnormalities of kidney structure and/or function that persist for more than 3 months and significantly affect health status.[2] The CKD is becoming a disease of civilization, affecting up to 15% of the world population.[3] Thus, to improve the methods of prevention and detection, and to reduce the costs of treatment of the disease, there is an urgent need to establish molecular markers allowing for its early diagnosis.

The progression of CKD is associated with the acquisition of a mesenchymal phenotype by the epithelial cells of the convoluted tubules in a process called epithelial-mesenchymal transition (EMT). This process of dedifferentiation of highly specialized, polarized and basement membrane-embedded tubular epithelial cells into myofibroblast-like cells which can digest the tubular basement membrane, migrate into the interstitium and start producing matrix proteins, is a well-recognized mechanism of progression in several chronic nephropathies. The expansion of fibrosis is a consequence of chronic and not fully resolved inflammation. Both processes develop upon the activation of cell signal transduction pathways.[4–8]

The WNT signaling pathways are evolutionary conserved. When the canonical pathway (WNT/β-catenin signaling) is activated, it promotes regenerative processes. Uncontrolled, sustained WNT/β-catenin signaling activation induces tubular damage.[9] The WNT protein acting in a paracrine and an autocrine manner influences WNT/β-catenin signaling in fibroblasts, macrophages and mesangial cells.[1,10–13] It has been shown that among WNT family members, WNT4 protein plays a significant role in embryogenic morphogenesis and MET (mesenchymal-to-epithelial transition) occurring during the formation of renal tubules.[14–16] The activation of cellular signaling pathways through WNT4 protein is suppressed in a healthy adult kidney.[17] The reactivation of the WNT4 expression occurs in pathological states, as demonstrated in the mouse model.[17] The modulated expression of the *Wnt4* mRNA in the convoluted tubules is associated with renal fibrosis.[17] The expression of *Wnt4* gene was found to be reactivated in animal models of unilateral ureteral obstruction.[18] Moreover, WNT4 participates in the tissue regeneration process following experimental acute kidney injury.[19] After angiotensin II treatment, *WNT4* mRNA level increases in human kidney proximal epithelial cells (HK-2 cells) and podocytes.[20] The WNT4 activity can be blocked after binding to the Klotho protein, as demonstrated in the in vitro study in human proximal tubular epithelial cells.[5]

It has been shown that an elevated level of WNT1 protein in the blood correlates with an increased risk of myocardial infarction.[21] However, the serum concentrations of other WNT proteins have not yet been reported in humans. The WNT4 concentration has only been measured in human urine.[22]

Objectives

Assuming the contribution of WNT4 protein in kidney fibrosis and progression of CKD, the study aimed to evaluate whether the serum level of WNT4 protein might depend on CKD stage, underlying etiology and degree of proteinuria (nephrotic (NP) and non-nephrotic (NNP)). Moreover, we decided to verify the possible associations between serum WNT4 and creatinine, and the advancement of tubulointerstitial inflammation and fibrosis in kidney biopsy specimens obtained from stable patients with biopsy-confirmed nephropathies.

Materials and methods

All procedures performed in the studies involving human participants were in accordance with the ethical standards of the local Ethics Committee of the Medical Chamber (7/2013/V) and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. The blood was collected following a written informed consent by the participants of the study.

Patients

Stable CKD subjects (n = 63; mean age: 46.03 ±2.11 years, mean ± standard deviation (SD)) were recruited from patients treated in the Department of Nephrology, Hypertension and Internal Medicine of the Faculty of Medicine at the University of Warmia and Mazury in Olsztyn, Poland, between 2013 and 2016.

The study was performed in patients with clinical suspicion of glomerular disease (primary or secondary) who underwent kidney biopsy, which has been performed based on the standard clinical indications, i.e. nephrotic syndrome, subnephrotic proteinuria, isolated (otherwise unexplained) hematuria, unexplained fall in glomerular filtration rate (GFR), as well as when symptoms of renal injury have occurred in the course of already diagnosed 'systemic' disease (i.e. systemic lupus). None of the patients suffered from diabetes or had a history of cardiovascular events. Well-controlled hypertension (1–3 blood pressure drugs) was observed in 67% of the patients included in the study. Biopsies were not performed intentionally for this study. Patients were excluded if they experienced an 'acute' illness (i.e. infection,

noninfectious inflammation, cardiovascular event) or a disease that had occurred within the last 30 days, and in the case of a known active malignancy, as well as drug or alcohol abuse.

The control group (n = 42; mean age 58.31 ±0.85 years, mean ±SD) was recruited from volunteers who did not suffer from any kidney disease and were otherwise healthy.

The demographic data were collected at the time of biopsy or blood collection. The diagnosis of nephropathy was established based on a clinical appearance, laboratory and immunological tests, and histopathological assessment.

Material collection and laboratory parameters

Blood samples were collected from overnight fasted patients. After centrifugation for 10 min at 3500 rpm, the serum samples were stored at –80°C. Immediately after the sample collection, serum biochemistry was assayed with a Cobas 6000 multi-analyzer (Roche Diagnostics, Basel, Switzerland) and Uro-dipcheck 400e (ERBA, Mannheim, Germany). The following parameters were analyzed: lipids, glucose, urea, and creatinine; estimated glomerular filtration rate (eGFR) was calculated using the modification of diet in renal disease (MDRD) formula.

Measurement of serum WNT4 concentration

The WNT4 levels in serum samples were determined using a commercially available enzyme-linked immunoassay (ELISA) kit, according to the manufacturer's protocol (CSB-EL026137HU; Cusabio, Wuhan, China). Briefly, reagents, working standards and samples were prepared according to the procedure. Standards and samples were added in a quantity of 100 µL per well. After 2-h incubation at 37°C and liquid removal, wells were filled with biotin antibody and were incubated for 1 h at 37°C. Antibodies were washed out with wash buffer and horseradish peroxidase (HRP)-avidin complexes were added to each well. After 1 h incubation at 37°C, aspiration and washing steps were repeated. The TMB substrate was added and after 30 min at 37°C, the reaction was stopped with the stop solution. All the above reagents were purchased from Cusabio (Wuhan, China). The optical density was established with ChroMate® Multichannel Microplate Reader (Awareness Technology, Inc., Wiener Neustadt, Austria). The detection range varied from 23.5 pg/mL to 1500 pg/mL. The sensitivity of the assay was less than 5.8 pg/mL. Each sample was measured in duplicate.

Renal tissue sampling, evaluation and classification

Paraffinized sections (5 µm) were used for routine histopathological assessment. Two pathologists classified and scored the biopsies separately, without knowing the medical history of the patients. The extent of inflammatory infiltration in the kidney parenchyma (IIKP) and renal fibrosis (Fibrosis) were determined separately, semi-quantitatively, using the following scale: 0 – absence; 1 – small clusters; 2 – mild clusters; 3 – moderately extensive clusters; 4 – wide clusters.

After considering the biochemical analysis and pathological evaluation, the test group was rearranged for research purposes. The data comparison was made between the control and: (i) CKD patients; (ii) CKD group divided into subgroups of patients with the NNP or NP; (iii) CKD group divided according to the Kidney Disease: Improving Global Outcomes (KDIGO) classification based on eGFR values[23]; (iv) pathomorphologically diagnosed glomerular kidney disease; (v) semi-quantitatively estimated degree of IIKP; and (vi) semi-quantitatively estimated degree of the kidney fibrosis. The association between the serum WNT4 protein and creatinine levels in the study population was also analyzed.

Statistical analyses

Statistical analyses were conducted using GraphPad Prism v. 7.05 software (GraphPad Software, Inc., San Diego, USA). The Kolmogorov–Smirnov test was used to check whether data were distributed normally (Supplementary Table 1–5). Demographic data and blood biochemistry of control and CKD patients were compared with a Mann–Whitney test. The results of the blood biochemical components were presented as mean ±SD. The comparison of serum WNT4 protein level in the CKD and control group was determined using the nonparametric equivalent of a Student's t-test for independent variables, i.e., Mann–Whitney test. The evaluation of the serum WNT4 protein level between the control group and CKD group divided into subgroups of patients with NNP and NP was conducted with the Kruskal–Wallis test followed by the post hoc corrected Dunn's test (Bonferroni correction). The comparison of serum WNT4 levels with CKD stages involved more than 2 groups of patients. Therefore, the Kruskal–Wallis test was followed by the post hoc corrected Dunn's test (Bonferroni correction). The serum WNT4 levels in patients with distinct types of glomerular disease were compared using the Mann–Whitney test. The association of the serum WNT4 protein with IIKP and fibrosis were estimated with the Kruskal–Wallis test, followed by the post hoc corrected Dunn's test (Bonferroni correction). The possibility of estimation of WNT4 protein in serum was analyzed using linear or multivariate regression depending if 1 (tubulointerstitial lesions) or more than 1 (age, fibrosis) variables were taken into consideration. The receiver operating characteristic (ROC) curves reflecting the sensitivity and specificity of the assay to detect IIKP and fibrosis, and their specific area under the curve (AUC) were calculated. The correlations between WNT4 protein level, blood biochemical parameters and pathomorphological features were calculated using a nonparametric Spearman's test. The analysis of the association of the serum WNT4 protein with serum creatinine was conducted using

the Mann–Whitney test. Potential associations of WNT4 and serum creatinine were analyzed with multivariate regression analysis. A value of 95% was used for the confidence intervals (95% CI). The WNT4 protein amounts are presented as median and interquartile range (IQR). The differences were considered statistically significant when $p < 0.05$.

Results

Clinical-pathological characteristics

Table 1 displays the demographic data and serum biochemistry of the whole studied population (control subjects and CKD patients) enrolled in the analysis. The statistical analysis showed significant differences between control subjects and CKD patients (Table 1). However, no correlation was observed between the amount of WNT4 protein and age ($p = 0.6550$; $r = -0.0574$).

Table 1. Demographic data and blood biochemistry of control and chronic kidney disease (CKD) patients enrolled in the analysis

Variable	Control	CKD
N	42	63
Age [years]	58.31 ±0.85	46.03 ±2.11****
Gender (M/F)	26/16	33/30
Total cholesterol [mg/dL]	170.7 ±6.46	220.7 ±8.91****
HDL [mg/dL]	54.89 ±3.24	55.18 ±2.57
LDL [mg/dL]	76.14 ±7.77	145 ±8.03****
TG [mg/dL]	61.98 ±9.38	175.4 ±9.65****
Glucose [mg/dL]	105.3 ±4.51	101.2 ±3.56
Urea [mg/dL]	33.21 ±1.18	55.15 ±4.16****
Creatinine [mg/dL]	0.76 ±0.02	1.87 ±0.22****
eGFR [mL/min/1.73 m^2]	103.1 ±2.60	62.92 ±4.37****

CKD – chronic kidney disease; HDL – high-density lipoprotein; LDL – low-density lipoprotein; TG – triglycerides; eGFR – estimated glomerular filtration rate. The asterisks indicate statistical significance (****$p < 0.0001$).

Associations of serum WNT4 levels with CKD stage and diagnosis of glomerular disease

The statistical comparison of the serum WNT4 levels was performed between the control (n = 42) and CKD patient (n = 63) groups, as well as between the subgroups of patients with NNP (n = 23) and NP (n = 27). A significantly higher serum WNT4 concentration was found in the CKD patients (21.71 pg/mL (IQR: 15.47–32.98 pg/mL)) as compared to the control group (16.62 pg/mL (IQR: 12.72–20.78 pg/mL); $p = 0.0074$; Fig. 1A). No statistically significant differences were found in serum WNT4 levels between control (16.62 pg/mL (IQR: 12.72–20.78 pg/mL) and NNP (19.19 pg/mL (IQR: 16.18–30.94 pg/mL) or NP (20.44 pg/mL (IQR: 14.15–32.98 pg/mL)) groups of CKD patients ($p = 0.0904$; Fig. 1B). A statistically significant inverse correlation between WNT4 and serum cholesterol levels was found in the whole CKD group ($p = 0.0084$; Table 2). However, serum WNT4 levels did not correlate with other biochemical parameters or eGFR (Table 2).

Patients with CKD were also grouped according to the KDIGO classification, based on their eGFR values (2013).[23] Patients who had an eGFR value ≥60 mL/min/1.73 m^2 were classified to CKD stage 1–2 (n = 33), and those with eGFR < 60 mL/min/1.73 m^2 – to CKD stage 3–5 (n = 30). The Kruskal–Wallis test followed by corrected Dunn's test showed a higher level of serum WNT4 protein in the blood of patients classified into CKD stage 1–2 (22.78 pg/mL (IQR: 15.32–39.56 pg/mL)) in comparison to control (16.62 pg/mL

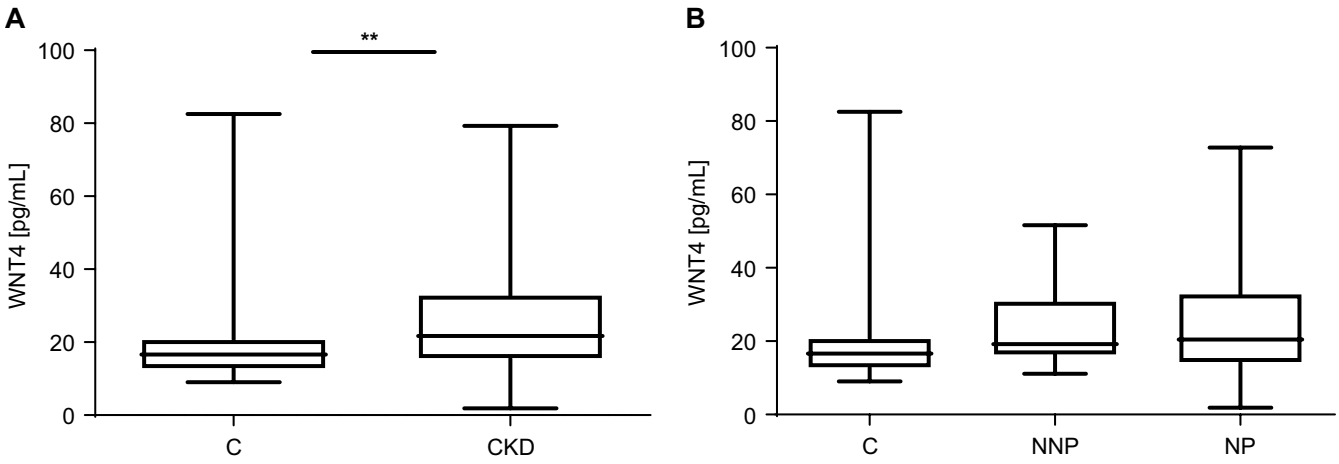

Fig. 1. A. Nonparametric Mann–Whitney test comparison of the concentration of WNT4 protein in the sera of control subjects (C; n = 42; 20.39 ±2.25 pg/mL) and the whole group of chronic kidney disease (CKD; n = 63; 26.86 ±2.03 pg/mL) patients presenting various stages and categories of kidney disease; B. Kruskal–Wallis test evaluation of the serum WNT4 protein level between the control group (C; n = 42; 20.39 ±2.25 pg/mL) and CKD group divided into subgroups of patients with non-nephrotic proteinuria (NNP; n = 23; 25.03 ±2.56 pg/mL) and nephrotic proteinuria (NP; n = 27; 25.15 ±3.11 pg/mL). For each box, the horizontal line inside the box shows the median. The ends of the boxes represent the 1st and 3rd quartiles. The whiskers extend to the highest and lowest values considered outliers (defined as 1.5 times the interquartile range (IQR)). Asterisks indicate statistically significant differences between the groups (**$p = 0.0074$)

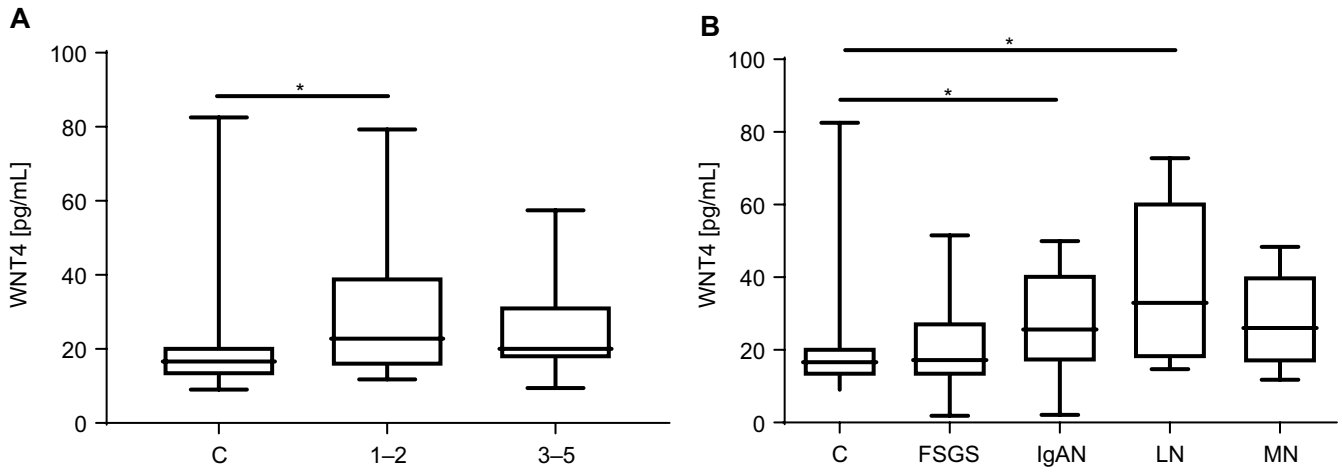

Fig. 2. A. Kruskal–Wallis test comparison of the concentration of WNT4 protein in the serum of control subjects (n = 42) and patients qualified to groups representing different stages of kidney disease depending on Kidney Disease: Improving Global Outcomes (KDIGO) classification (stage 1–2 (n = 33), stage 3–5 (n = 30)); B. Various types of kidney diseases (focal segmental glomerulosclerosis (FSGS) (n = 14), IgA nephropathy (IgAN) (n = 14), lupus nephritis (LN) (n = 5), and membranous nephropathy (MN) (n = 6)). The Kruskal–Wallis analysis showed statistically significant differences between the level of serum WNT4 protein in the control group (n = 42; 20.39 ±2.25 pg/mL) and patients classified into chronic kidney disease (CKD) stages 1–2 (29.31 ±3.00 pg/mL; *p = 0.0118) or patients with LN (37.95 ±10.5 pg/mL; *p = 0.0481) or IgAN (28 ±5.62 pg/mL; *p = 0.0408) diagnosis. For each box, the horizontal line inside the box shows the median. The ends of the boxes represent the 1st and 3rd quartiles. The whiskers extend to the highest and lowest values considered outliers (defined as 1.5 times the interquartile range (IQR))

Table 2. Correlation coefficients (r) between biochemical parameters of blood and WNT4 protein level in the serum of chronic kidney disease (CKD) patients, patients presenting inflammatory cell infiltration in the kidney parenchyma (IIKP) and patients presenting renal fibrosis (Fibrosis)

Groups	N	Total cholesterol	HDL	LDL	TG	Glucose	Urea	Creatinine	eGFR
Units	–	mg/dL	mg/dL	mg/dL	mg/dL	mg/dL	mg/dL	mg/dL	mL/min/1.73 m^2
CKD	63	**–0.33** p = 0.0084	–0.04	–0.22	–0.15	0.01	–0.07	–0.05	0.08
IIKP	49§	**–0.41** p = 0.0036	–0.17	**–0.31** p = 0.0285	0.08	–0.14	–0.16	–0.1	0.14
Fibrosis	56§	**–0.32** p = 0.0159	–0.10	–0.19	–0.14	–0.03	–0.07	–0.06	0.10

HDL – high-density lipoprotein; LDL – low-density lipoprotein; TG – triglycerides; eGFR – estimated glomerular filtration rate. Statistical significance (p) is presented above the r-value. Paragraph (§) indicates that the group of patients was reduced. Elimination consisted of patients not showing the examined feature. Bold denotes statistical significance.

(IQR: 12.72–20.78 pg/mL); p = 0.0118; Fig. 2A). No statistical significance was observed between the control (16.62 pg/mL (IQR: 12.72–20.78 pg/mL)) and CKD stage 3–5 group (20.07 pg/mL (IQR: 17.28–31.7 pg/mL)), neither between CKD stage 1–2 (22.78 pg/mL (IQR: 15.32–39.56 pg/mL)) and CKD stage 3–5 group (20.07 pg/mL (IQR: 17.28–31.7 pg/mL); p = 0.9999).

Four distinct types of glomerular disease could be diagnosed in the patients: focal segmental glomerulosclerosis (FSGS; n = 14), Immunoglobulin A (IgA) nephropathy (IgAN; n = 14), lupus nephritis (LN; n = 5) and membranous nephropathy (MN; n = 6). However, the nonparametric analysis of independent variables with a Mann–Whitney test showed that patients diagnosed with LN (32.98 pg/mL (IQR: 17.57–60.83 pg/mL); p = 0.0481) or IgAN (25.59 pg/mL (IQR: 16.63–40.89 pg/mL); p = 0.0408) had significantly higher serum WNT4 protein levels as compared to the control group (16.62 pg/mL (IQR: 12.72–20.78 pg/mL); Fig. 2B).

No correlation was found between serum WNT4 protein level and biochemical parameters within the group of patients with CKD or those diagnosed with LN or IgAN (data not shown).

Association of the serum WNT4 protein level with tubulointerstitial lesions in kidney biopsy samples

The evaluation of IIKP of CKD patients revealed 30 subjects in whom the kidney tissue fragments did not show any inflammation or that presented with only small areas of inflammation (group 0–1). The mild, medium and extensive areas of inflammation were observed in biopsies of 33 patients (group 2–4).

In sera of patients with at least small areas of inflammatory cell infiltrates in the biopsy samples (group 0–1), the serum level of WNT4 protein was higher than that of the control group (24.91 pg/mL (IQR: 17.36–34.19 pg/mL) compared to 16.62 pg/mL (IQR: 12.72–20.78 pg/mL), respectively; p = 0.0142; Fig. 3A), as confirmed with a Kruskal–Wallis test followed by corrected Dunn's test. No statistical significance was observed between the control group (16.62 pg/mL (IQR: 12.72–20.78 pg/mL)) and group 2–4 (19.19 pg/mL (IQR: 14.8–34.83 pg/mL); p = 0.2594), neither between group 0–1 (24.91 pg/mL (IQR: 17.36–34.19 pg/mL))

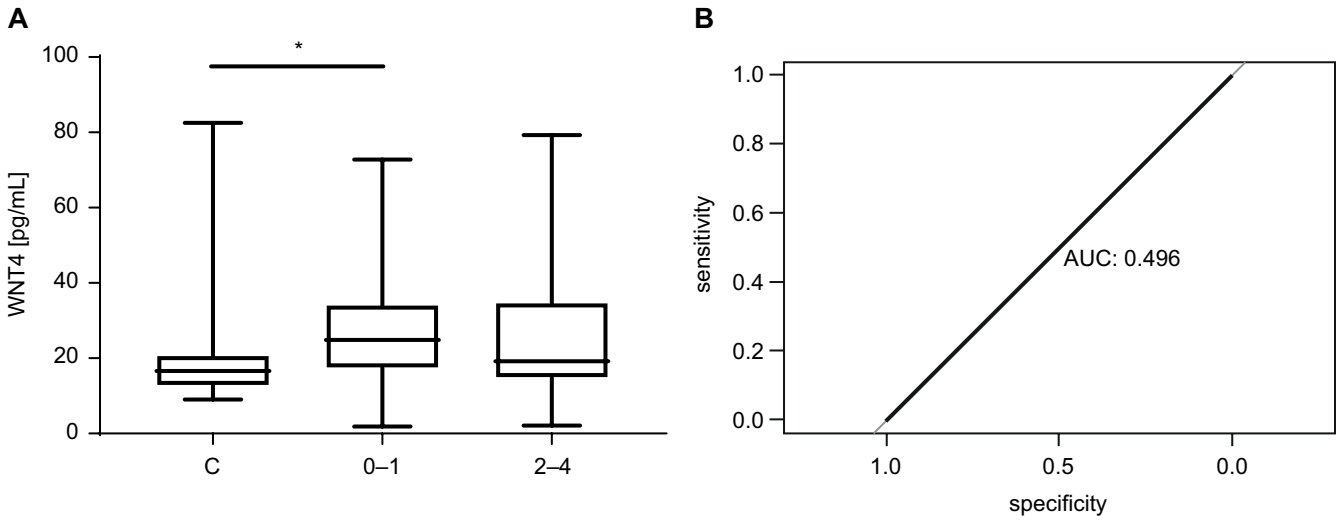

Fig. 3. A. Kruskal–Wallis test comparison presented statistically significant difference between the WNT4 protein level in serum of patients who were classified as chronic kidney disease (CKD) patients without inflammatory cells or with a small area of inflammatory cells infiltration in the biopsied tissue (n = 30; 27.83 ±2.78 pg/mL; group 0–1) in comparison to serum WNT4 level in control subjects (20.39 ±2.25 pg/mL). Asterisks indicate differences between groups (*p = 0.0142; A). The receiver operating characteristic (ROC) curve presenting the area under the curve (AUC) value was estimated to establish sensitivity and specificity of the WNT4 protein in blood measurement for setting the severity of the tubulointerstitial lesions (B). For each box, the horizontal line inside the box shows the median. The ends of the boxes represent the 1st and 3rd quartiles. The whiskers extend to the highest and lowest values considered outliers (defined as 1.5 times the interquartile range (IQR))

and group 2–4 (19.19 pg/mL (IQR: 14.8–34.83 pg/mL); p = 0.8205; Fig. 3A), in terms of serum WNT4.

Results of linear regression analysis showed that the serum level of WNT4 protein in the blood could not be determined based on the estimation of tubulointerstitial lesions (p = 0.7507; r^2 = 0.0022). The AUC value for the sensitivity and specificity ROC curve was 0.4960 (Fig. 3B).

The negative correlation was observed between the serum WNT4 protein and the cholesterol level (p = 0.0036) as well as the low-density lipoprotein (LDL) level (p = 0.0285) in the IIKP group of CKD patients (Table 2).

Association of the serum WNT4 protein level with histological features of kidney fibrosis

No fibrosis or small clusters of fibrosis (group 0–1) were observed in kidney sections of 25 patients. Kidney tissue samples from 38 CKD patients showed mild, moderately increased or large areas of the interstitium, which were occupied by fibrosis (group 2–4).

The comparison of serum WNT4 protein levels between patients with CKD but displaying almost no fibrosis (group 0–1; 27.02 pg/mL (IQR: 17.99–35.45 pg/mL)) and control, healthy subjects (16.62 pg/mL (IQR: 12.72–20.78 pg/mL) showed higher values of WNT4 in the CKD 0–1 group (p = 0.0062; Fig. 4A). No statistical significance was observed between the healthy group (16.62 pg/mL (IQR: 12.72–20.78 pg/mL) and group 2–4 (19.35 pg/mL (IQR: 14.74–33.23 pg/mL); p = 0.3034), neither between group 0–1 (27.02 pg/mL (IQR: 17.99–35.45 pg/mL)) and group 2–4 (19.35 pg/mL (IQR: 14.74–33.23 pg/mL); p = 0.3300; Fig. 4A).

The estimation of the serum WNT4 protein level could not be determined based on age (p = 0.3549; r^2 = 0.0140) and fibrosis (p = 0.3299; r^2 = 0.0158). The AUC value for the sensitivity and specificity ROC curve was 0.5940 (Fig. 4B).

The WNT4 protein serum level was negatively correlated with total cholesterol in CKD patients with renal fibrosis in biopsy specimens (p = 0.0159; Table 2).

Association between the serum concentration of WNT4 protein and creatinine

The association between serum WNT4 protein and creatinine was analyzed based on the assumption that WNT4 protein can be considered a novel biomarker for the the early detection of kidney disease (which has been suggested by Zhao et al.).[22] The method presented earlier by Goliasch et al. was followed to study this assumption.[21] The entire group (control subjects and CKD patients; n = 105) was divided into 2 subgroups, according to serum WNT4 median value (16.62 pg/mL) in the control group, to assess the relationship between the serum levels of WNT4 protein and creatinine in the study sample. Statistical analysis showed that serum creatinine was higher in patients who had WNT4 protein concentration above median as compared to those with the serum WNT4 protein concentration below the median value (1.48 ±0.18 pg/mL compared to 0.99 ±0.09 mg/dL, respectively; p = 0.0354; Fig. 5A). However, the results of linear regression analysis showed that the serum WNT4 protein level could not be determined based on creatinine level (p = 0.8429; r^2 = 0.0006).

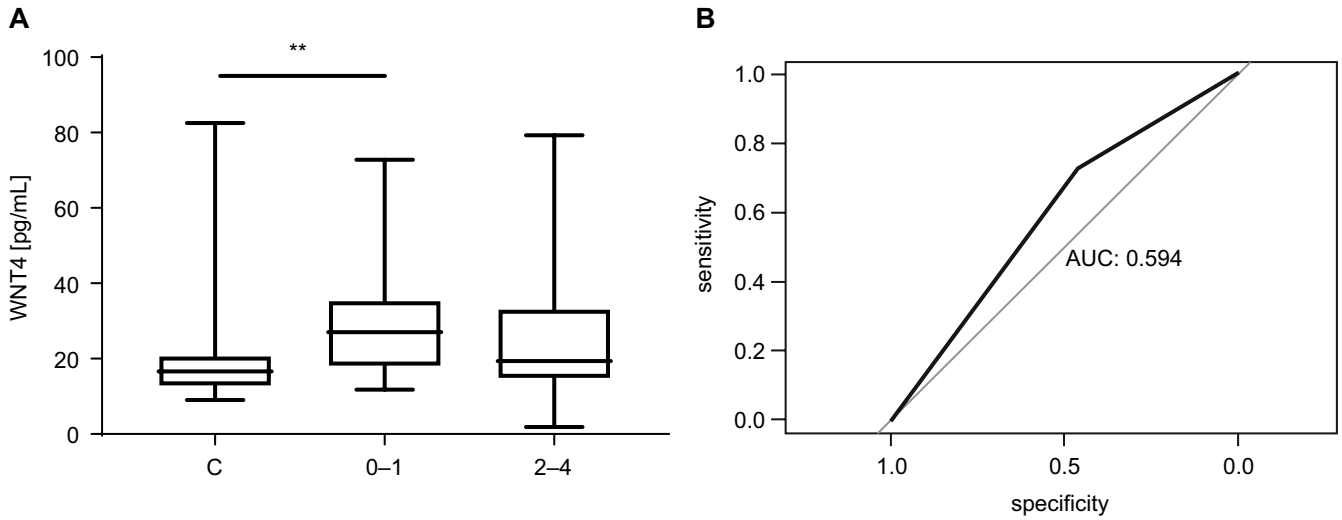

Fig. 4. Serum level of WNT4 protein compared using Kruskal–Wallis test was significantly higher in patients with a small area of fibrosis (group 0–1; 27.83 ±2.78 pg/mL) than in the group of control subjects (20.39 ±2.25 pg/mL). Asterisks indicate differences between groups (**p = 0.0062; A). The receiver operating characteristic (ROC) curve presenting the area under the curve (AUC) value was estimated to establish sensitivity and specificity of the serum WNT4 protein for setting the advancement of fibrosis (B). For each box, the horizontal line inside the box shows the median. The ends of the boxes represent the 1st and 3rd quartiles. The whiskers extend to the highest and lowest values considered outliers (defined as 1.5 times the interquartile range (IQR))

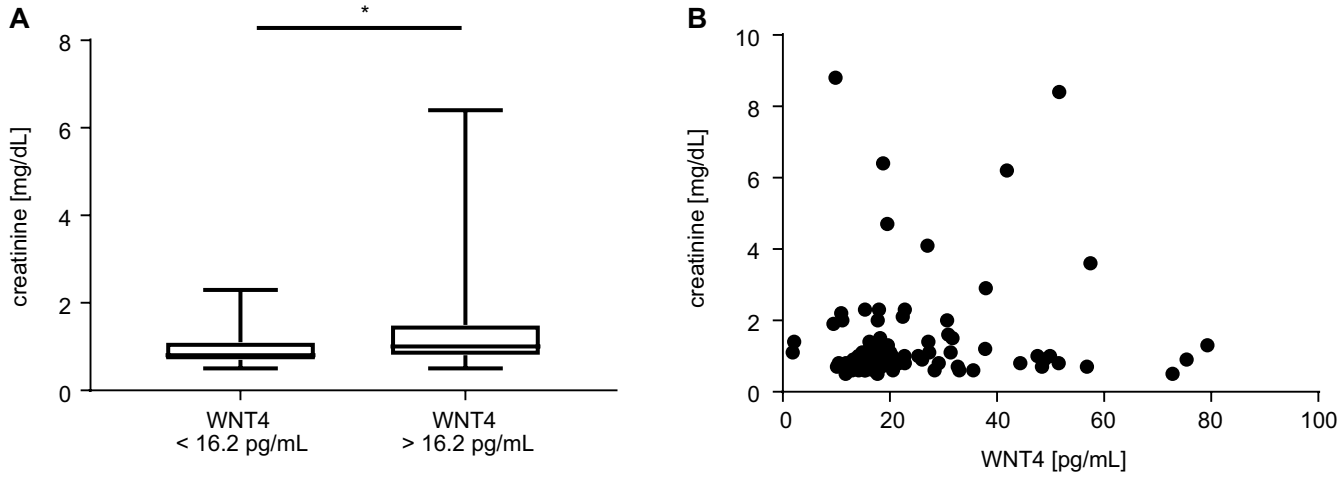

Fig. 5. Serum creatinine concentration in patients divided into 2 groups according to the median WNT4 protein concentration (16.62 pg/mL) in the sera of the total studied population (n = 105) which included control subjects and patients with chronic kidney disease (CKD). Mann–Whitney test comparison showed that serum creatinine was higher in patients who had WNT4 protein concentration higher than 16.62 pg/mL (1.48 ±0.18 pg/mL) in comparison to those with a WNT4 protein concentration lower than 16.62 pg/mL (0.99 ±0.09 pg/mL). Asterisks indicate the differences between the groups (*p = 0.0354; A). Lack of correlation was observed between serum WNT4 protein and creatinine levels (r = 0.12; B). For each box, the horizontal line inside the box shows the median. The ends of the boxes represent the 1st and 3rd quartiles. The whiskers extend to the highest and lowest values considered outliers (defined as 1.5 times the interquartile range (IQR))

Discussion

This novel study found that serum WNT4 protein level is higher in patients diagnosed with CKD, classified according to the eGFR value or the type of glomerulopathy, but not according to the presence of NP or NNP. A higher level of serum WNT4 protein in CKD patients as compared to control subjects suggests that mechanisms activating the WNT pathway and influencing WNT4 may become apparent at any stage of the disease and may depend on the underlying pathology, but not on the degree of proteinuria. The potential dependence on the glomerular filtration rate may be a limitation for WNT4 to be a marker and an indicator of podocyte injury. In our study, the elevated serum WNT4 protein levels in the subgroups of both primary (IgAN) and secondary (LN) glomerular disease might result from the dysregulation of the immune system. Therefore, it seems that the elevated serum WNT4 level, leading to the development of certain glomerular and tubular lesions (as presented in experimental studies),[17,18,20] reflects also the activation of the immune system.

The increase of serum WNT4 protein level in patients with different kinds of glomerular disease and different

(but not necessary more advanced) stages of CKD may represent a novel and common denominator of not yet identified pathological event in chronic kidney injury. Our findings demonstrate that serum WNT4 protein level is higher in patients with CKD. Moreover, it is also higher in patients without IIKP or those with only minor advancement of IIKP, as well as in those without advanced renal fibrosis. Inspired by the study by Chang et al., we suspect, that WNT4 considered as a marker of podocyte injury can display an identical pattern of expression as inflammatory mediators and act in a comparable way.[24] Furthermore, as the WNT4 triggers tissue infiltration with neutrophils[25] and facilitates the transformation of stem cells into the fibrogenic fate,[26] it cannot be ruled out that the identical mechanism is in action during CKD progression. It would be worth answering this question by conducting studies in a larger group of patients with biopsy-confirmed glomerular disease.

The elevated serum WNT4 protein level may accompany CKD but cannot be considered a marker of IIKP and fibrosis. It is supported by our results showing that serum WNT4 protein correlated with serum total and LDL-cholesterol. Changes in serum lipids such as free fatty acids, triglycerides and glycophospholipids develop in the course of CKD (especially when proteinuria is present), but also significantly impact the course of CKD (although to date lipid-lowering therapies did not show to be nephroprotective).[27,28] Additionally, the accumulation of lipids can lead the palmitate retention.[29] It has been shown that the lipidation of WNT proteins (including coupling of palmitate) is required for their secretion and function. Palmitoylation accounts for the hydrophobicity and poor solubility of WNT proteins, which modulate their ability to activate WNT/β-catenin signaling.[30–33] It remains to be addressed whether, in this way, WNT4 can act as the primary 'switch' involved in the development of kidney dysfunction and the initiation of fibrosis. It would be of particular interest given that we observed the elevated serum WNT4 protein level mainly in patients in early stages of CKD (stages 1–2), and with early IIKP (stages 0–1) and fibrosis (stages 0–1).

The current study has shown that serum creatinine concentration was higher in a group with serum WNT4 protein concentration above the WNT4 median value (16.62 pg/mL). However, these parameters were not correlated. As far as we know, no other studies measuring the level of WNT4 protein in serum have yet been published. In a single study presenting the WNT4 protein expression as a biomarker for podocyte integrity in the rat model of renal ischemia-reperfusion injury, the serum level of WNT4 protein correlated with serum creatinine.[24] Another research showed an increase in WNT4 protein in the urine of patients with minimal change disease and coexisting tubular injury; however, no correlation with serum creatinine level was observed,[22] as in our study.

Limitations

We are aware that empirical results presented herein should be considered in light of certain limitations. The observed results are based on a relatively small group of patients (nevertheless, even the 'large' trials in the field of glomerular disease usually do not exceed 100 patients). Thus, to not rely solely on the division of CKD depending on the stages (which is an imprecise classification since each class contains patients with the same kidney function, but diverse underlying pathology and clinical presentation), the particular diagnoses of kidney disease were considered, compared with the control group, with CKD group as a whole, but also with the CKD patients divided based on the degree of proteinuria. With this approach, the course of future research has been delineated. On the other hand, primary and immune-mediated secondary glomerular diseases are considered rare and most of the clinical research is performed rather on dozens than hundreds of patients.

Conclusions

The preliminary data presented here suggest that elevated levels of serum WNT4 protein may be associated with the immune system response. Moreover, it accompanies the dysregulation of serum lipids that are associated with CKD. It is observed in the early stages of CKD, IIKP and fibrosis, and may trigger CKD progression. Therefore, it would be interesting to investigate the involvement of WNT4 in the molecular mechanisms responsible for the development of CKD, considering that WNT4 is only an indicator of chronic glomerulonephritis and not a diagnostic marker of IIKP and fibrosis.

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Supplementary Table 1. Verification of parametric test assumption for control (A) and chronic kidney disease (CKD) (B) group

A.

Control	Age	Total cholesterol	HDL	LDL	TG	Glucose	Urea	Creatinine	eGFR
Minimum	41	105	23	0	0	76	20	0.5	91
25% percentile	55	156	43	0	0	85.5	29	0.7	92
Median	59	166	53.5	53	74	94	32	0.8	99
75% percentile	62	198.5	66.75	95.5	105.5	110	37.75	0.9	109.8
Maximum	70	235	92	156	275	226	46	0.9	144
Lower 95% CI of mean	56.6	157.5	48.25	36.11	43.16	96.23	30.8	0.7143	97.75
Upper 95% CI of mean	60.02	184	61.54	64.72	80.8	114.3	35.63	0.8071	108.4
KS normality test									
KS distance	0.1017	0.1236	0.091	0.2962	0.2819	0.2353	0.1486	0.2001	0.2457
p-value	>0.1000	>0.1000	>0.1000	<0.0001	<0.0001	<0.0001	>0.1000	0.0056	0.0001
Passed normality test (α = 0.05)?	yes	yes	yes	no	no	no	yes	no	no

B.

CKD	Age	Total cholesterol	HDL	LDL	TG	Glucose	Urea	Creatinine	eGFR
Minimum	18	62	11	10	53	66	14	0.5	5
25% percentile	30	175	39	103	115	84	32	0.8	32.6
Median	48	210	52	124	169	93	42	1.2	59
75% percentile	59	255	68	187	235	106	64	2	87.7
Maximum	78	470	113	367	408	226	183	8.8	151.9
Lower 95% CI of mean	41.82	202.9	50.06	129	156.1	94.09	46.85	1.429	54.2
Upper 95% CI of mean	50.25	238.5	60.31	161.1	194.6	108.3	63.46	2.309	71.65
KS normality test									
KS distance	0.117	0.1568	0.08205	0.1635	0.07769	51.34	25.63	49.91	3.189
p-value	0.0319	0.0002	>0.1000	<0.0001	>0.1000	<0.0001	<0.0001	<0.0001	0.2030
Passed normality test (α = 0.05)?	no	no	yes	no	yes	no	no	no	yes

HDL – high-density lipoprotein; LDL – low-density lipoprotein; TG – triglycerides; eGFR – estimated glomerular filtration rate; 95% CI – 95% confidence interval; KS – Kolmogorov–Smirnov.

Supplementary Table 2. Verification of parametric test assumption for study groups

Study groups	Control	CKD	NP	NNP	1–2	3–5	FSGS	IgAN	LN	MN
Minimum	9.01	1.88	1.88	11.16	11.76	9.46	1.88	2.12	14.7	11.76
25% percentile	12.72	15.47	14.15	16.18	15.32	17.28	12.7	16.63	17.57	16.39
Median	16.62	21.71	20.44	19.19	22.78	20.07	17.18	25.59	32.98	26.04
75% percentile	20.78	32.98	32.98	30.94	39.56	31.7	27.82	40.89	60.83	40.46
Maximum	82.58	79.33	72.79	51.64	79.33	57.48	51.5	49.92	72.79	48.41
Lower 95% CI of mean	15.83	22.8	18.77	19.72	23.19	20.41	13.61	18.19	8.803	13.54
Upper 95% CI of mean	24.94	30.92	31.54	30.33	35.43	30.81	27.53	37.05	67.1	42.46
KS normality test										
KS distance	0.26	0.171	0.1508	0.2046	0.1744	0.1819	0.1623	0.1275	0.1839	0.2098
p-value	<0.0001	<0.0001	>0.1000	0.0136	0.0102	0.0221	>0.1000	>0.1000	>0.1000	>0.1000
Passed normality test (α = 0.05)	no	no	yes	no	no	no	yes	yes	yes	yes

95% CI – 95% confidence interval; CKD – chronic kidney disease; NP – nephrotic proteinuria; NNP – non-nephrotic proteinuria; FSGS – focal segmental glomerulosclerosis; IgAN – IgA nephropathy; LN – lupus nephritis; MN – membranous nephropathy; KS – Kolmogorov–Smirnov. 1–2 and 3–5 – CKD group divided according to the Kidney Disease: Improving Global Outcomes (KDIGO) classification based on estimated glomerular filtration rate (eGFR) values.

Supplementary Table 3. Verification of parametric test assumption for groups presenting tubulointerstitial lesions in kidney biopsy samples

IIKP	Control	0–1	2–4
Minimum	9.01	1.88	2.12
25% percentile	12.72	17.36	14.8
Median	16.62	24.91	19.19
75% percentile	20.78	34.19	34.83
Maximum	82.58	72.79	79.33
Lower 95% CI of mean	15.83	22.1	19.95
Upper 95% CI of mean	24.94	33.55	32
KS normality test			
KS distance	0.26	0.1351	0.2107
p-value	<0.0001	>0.1000	0.0007
Passed normality test (α = 0.05)?	no	yes	no

95% CI – 95% confidence interval; IIKP – inflammatory cell infiltration in kidney parenchyma; KS – Kolmogorov–Smirnov.

Supplementary Table 4. Verification of parametric test assumption for groups presenting fibrosis in kidney biopsy samples

Fibrosis	Control	0–1	2–4
Minimum	9.01	11.76	1.88
25% percentile	12.72	17.99	14.74
Median	16.62	27.02	19.35
75% percentile	20.78	35.45	33.23
Maximum	82.58	72.79	79.33
Lower 95% CI of mean	15.83	23.01	19.76
Upper 95% CI of mean	24.94	35.66	30.69
KS normality test			
KS distance	0.26	0.1659	0.1923
p-value	<0.0001	0.0740	0.0011
Passed normality test (α = 0.05)?	no	yes	no

95% CI – 95% confidence interval; KS – Kolmogorov–Smirnov.

Supplementary Table 5. Verification of parametric test assumption for groups compared to associate the serum WNT4 protein with serum creatinine

Creatinine	WNT4 < 16.2 pg/mL	WNT4 > 16.2 pg/mL
Minimum	0.5	0.5
25% percentile	0.7	0.8
Median	0.8	1
75% percentile	1.1	1.5
Maximum	2.3	6.4
Lower 95% CI of mean	0.8139	1.119
Upper 95% CI of mean	1.173	1.835
KS normality test		
KS distance	0.2531	0.2713
p-value	<0.0001	<0.0001
Passed normality test (α = 0.05)?	no	no

95% CI – 95% confidence interval; KS – Kolmogorov–Smirnov.

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