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# Association of Angiotensin Converting Enzyme (insertion\deletion) and Angiotensin II Type 1 Receptor (A1166C) Gene Polymorphisms with Diabetic Nephropathy in Iraqi type 2 Diabetic Patients

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## Abstract

Renin-angiotensin-aldosterone system abnormalities are the most prevalent cause of renal hemodynamic abnormalities, and candidate genes in this system are involved in the etiology of diabetic nephropathy (DN). A polymorphism in the angiotensin converting enzyme (ACE) gene I(insertion)\D(deletion) has been correlated to plasma ACE levels. Furthermore, the Angiotensin II Type 1 Receptor AGT1R (A1166C) expression pattern is highly related to nephropathy. The objectives of this study involved evaluating the frequency of the ACE (I/D) and AGT1R (A1166C) gene polymorphisms and investigating the association of these polymorphism with the development of DN in Iraqi patients with Type 2 diabetes mellitus (T2DM) and evaluating the levels of several urinary and serum markers in relation to studied polymorphisms. This is a cross-sectional study that included 161 T2DM patients whom were divided into two groups: T2DM with DN(included 98 patients) and normalbuminuric T2DM (included 63 patients). ACE gene polymorphism analysis revealed that the D allele was far more prevalent in DN patients compared to normalbuminuric patients (60.2% vs. 50.8%), while the I allele frequency was 39.8% in DN and 49.2% in normoalbuminuric patients. In addition, DN patients carrying the DD genotype had higher serum kidney injury molecule 1 (KIM1), serum cystatin C (CysC), and HbA1C and lower glomerular filtration rate (GFR) compared to ID+II genotypes. For AGT1R (A1166C), both DN and normalbuminuric patients had a comparable high prevalence of the AA genotype, followed by the AC genotype, while the CC genotype had been seen only in 3 patients. In conclusion, among the studied T2DM patients, individuals with DD genotype had higher frequency of DN and had 2-fold risk for DN compared to II genotype which had the lowest risk for DN. Also the current study shows that the A1166C polymorphisms of AGT1R distribution frequency were similar for both study groups with higher frequency for A allele compared to C allele and not associated with risk of DN in Iraqi T2DM

**Keywords: Angiotensin Converting Enzyme (I\D) gene polymorphism, Angiotensin II Type 1 Receptor (A166C) gene polymorphisms, Cystatin C, Diabetic nephropathy, Kidney injury molecule1.**

# علاقة تعدد الاشكال الجيني للإنزيم المحول للأنجيوتنسين (إدخال D/I حذف) ومستقبلات الأنجيوتنسين ٢ من النوع الأول (A1166C) مع اعتلال الكلية السكري في مرضى السكري من النوع الثاني العراقيين

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## الخلاصة

الاضطرابات في نظام الرينين - أنجيوتنسين - الألدوستيرون هي السبب الأكثر انتشارًا لاضطرابات الدورة الدموية الكلوية ، وتشارك الجينات المعتمدة في هذا النظام في مسببات اعتلال الكلية السكري (DN).علاوة على ذلك ، يرتبط نمط التعبير عن مستقبل الأنجيوتنسين II من النوع الأول AGT1R (A1166C) بشكل كبير باعتلال الكلية. اشتملت أهداف هذه الدراسة على تقييم تواتر تعدد الأشكال الجيني ACE (I / D) و AGT1R (A1166C) والتحقيق في ارتباط هذه الأشكال المتعددة مع تطوراعتلال الكلية السكري في المرضى العراقيين الذين يعانون من النوع

الثاني من السكر وتقييم مستويات العديد من العلامات البولية وعلامات المصل فيما يتعلق بتعدد الأشكال الجينية المدروسة. هذه الدراسة مقطعية شملت ١٦١ مريضًا من مرضى السكري النوع الثاني تم تقسيمهم إلى مجموعتين ، مرضى السكري النوع٢ مع اعتلال الكلية السكري (شملت ٩٨ مريضًا) ومرضى السكري النوع ٢ البومينيورك الطبيعي(شملت ٦٣ مريضًا). أظهر تحليل تعدد الأشكال الجيني الإنزيم المحول للأنجيوتنسين أن الأليل D كان أكثر انتشارًا في مرضى اعتلال الكلية السكري مقارنة بالمرضى الألبومينوريك الطبيعي (٦٠,٢٪ مقابل ٥٠,٨٪) , بينما كان تردد الأليلI ٣٩,٨٪ في مرضى اعتلال الكلية السكري و ٤٩,٢٪ في مرضى الألبومينوريك الطبيعي. بالإضافة إلى ذلك ، كان لدى مرضى اعتلال الكلية السكري الذين يحملون النمط الوراثي DD ومصل االسيستاتين KIM1 واعلى مصل لجزيء اصابة الكلية CysC و HbA1C واقل بالنسبة GFR للمعدل الترشيح الكبيبي مقارنة بالأنماط الجينية (ID + II). بالنسبة لـ AGT1R (A1166C)، كان لدى كل من مرضى اعتلال الكلية السكري ومرضى البومين الطبيعي معدل انتشار مرتفع مماثل للنمط الجيني AA ، متبوعًا بالنمط الجيني AC ، بينما شوهد النمط الوراثي CC في ٣ مرضى فقط.. في الختام, من بين مرضى السكري النوع الثاني المدروسين، كان لدى الأفراد الذين يعانون من النمط الوراثي DD تردد أعلى في مجموعة اعتلال الكلية السكري وكان لديهم خطر ضعفي للإصابة باعتلال الكلية السكري مقارنة بالنمط الجيني II الذي كان لديه أقل خطر للإصابة باعتلال الكلية السكري .كما أظهرت الدراسة الحالية أن تعدد الأشكال الجيني AGT1R A1166C كان متشابهًا في كلتا مجموعتي الدراسة مع تردد أعلى للأليل A مقارنة بألاليل C ولم يرتبط بخطر اعتلال الكلية السكري للنوع الثاني من السكر في العراقي.

**الكلمات المفتاحية : اعتلال الكلية السكري ، تعدد الأشكال الجيني للانزيم المحول للأنجيوتنسين (I \ D) ، تعدد الأشكال الجيني لمستقبلات الأنجيوتنسين الثاني (A166C) ، جزيء إصابة الكلى ١ ، سيستاتين C.**

## Introduction

Diabetic nephropathy (DN) is the most severe micro vascular consequence of diabetes mellitus, which is linked to higher morbidity and mortality rates in diabetes patients, and consider the main factor for end-stage kidney disease (ESKD) [1, 2]. And about10–20% of people with type 2 diabetes (T2DM) die of kidney failure [3]. One of the complex mechanisms that controls both blood pressure and the internal pressure of the glomerulus is the renin-angiotensin-aldosterone system (RAAS) [4]. Metabolic and hemodynamic processes are crucial in the pathophysiology of DN, and the local RAAS which is known to be activated in proximal tubular epithelial cells, mesangial cells, and podocytes consider as part of the hemodynamic pathway[5]. As RAAS is involved in the onset and progression of DN, alterations in genes of the RAAS system may be involved in the development of DN [6]. Among the RAAS candidate genes, the angiotensin-converting enzyme (ACE), angiotensinogen (AGT), and angiotensin II type 1 receptor (AGT1R) appear to be the most physiologically and clinically important for renal disease [7]. So the genetic polymorphisms of these genes offer a basis for studying the link between genetic variations and the occurrence of cardiovascular disease and/or kidney damage [8]. The ACE gene encodes a zinc-dependent moderately nonspecific peptidase. The ACE protein can cleave a broad variety of substrates [9]. In spite of the massive number of research seeking for candidate genes, the ACE gene remains a distinct, well-characterized locus that is definitely linked to the pathophysiology and development of ESKD [10]. The ACE is encoded by a 21 Kb gene that consists of 26 exons and located on chromosome 17q23[11]. In 1990, an ACE (I\D) gene polymorphism was discovered, which was defined by the insertion (I) or deletion (D) of a 287 base pair (bp) segment [12].

Because the polymorphism is located in an intron, it has no effect on the enzyme's structure; yet, this polymorphism is significantly connected to plasma ACE level, with II, ID, and DD having low, medium, and high levels, respectively [13].

The AGT1R is extensively expressed in a variety of tissues, including the lung, vessel walls, and kidney. The AGT1R activation may influence renal function, and their activation not only causes water-sodium retention and high blood pressure, but it also contributes to microvascular abnormalities in T2DM [14]. Also, the AGT1R expression pattern is closely associated with nephropathy [15]. Angiotensin II (AngII) also involved, which exerts a variety of effects through the AGT1R, including overt albumin, glomerular fibrosis, and increased glomerular permeability [16]. The AGT1R gene exhibits a number of polymorphisms, including A1166C, T573C, A1062G, G1517T, and A1878G. The A1166C polymorphism, which is one of them and is situated in the gene's 3′ untranslated region, theoretically has no impact on how the AGT1R protein is encoded. However, it still has the capacity to affect the gene's mRNA expression's stability [17, 18]. The substantial correlation between the AGT1R (A1166C) single nucleotide polymorphism (SNP) and DN is supported by growing evidence [19-22]. In Iraq, only two studies about AGT1R (A1166C) SNP association with hypertension and acute coronary syndrome [23, 24] and there had been no previous Iraqi research on the AGT1R (A1166C) SNP with DN.

The objectives of the current study involve evaluating the frequency of the ACE (I/D) and AGT1R (A1166C) gene polymorphisms and to investigate the association of these SNPs with the development of DN in Iraqi patients with T2DM and to compare the levels of several urinary and serum markers in relation to these polymorphisms.

## Patients and Method

This across-sectional study was carried out on patients previously diagnosed to have T2DM who visit Diabetes and Endocrinology Center at Merjan Medical City in Babylon/Iraq. From March 2022 to the end of January 2023.

The study includes 161 participants divided into two groups according to urinary albumin-to-creatinine ratio (ACR), group1: T2DM with DN (ACR≥30 mg/g) include 98 patients and group2: normalbuminuric T2DM (ACR<30 mg/g) include 63 patients.

A standardized protocol was used to collect full medical histories and data from each study participant utilizing a researcher-made data collection sheet.

Diabetic nephropathy was defined as T2DM with ACR ≥30 mg/g [25]. This test was performed directly on each patient by taking a random spot urine and measuring urine creatinine using a colorimetric reaction (Jaffe reaction) of creatinine with "alkaline picrate" measured kinetically at 490 nm creatinine (Biolab kit) [26], while urine albumin was measured using an Abnova BCG Albumin kit based on a method that uses bromcresol green to form a colored complex specifically with albumin. The color's intensity as measured at 620 nm [27]. ACR (mg\g) is then computed by dividing urine albumin in mg/L by urine creatinine in g/L. Blood pressure was measured using a manometer apparatus in Diabetes and Endocrinology Center.

***Collection and analysis of blood samples***

After an overnight fast, venous blood samples were taken from each patient in a sitting position using disposable syringes. After using a tourniquet, ten milliliters of blood were drawn from each person, and 8 milliliters were slowly pushed into a gel and clot activator disposable tube without anticoagulant and left to coagulate for 10-15 minutes before being centrifuged to obtain serum for testing lipid profile and fasting blood sugar using fully automated Kromo Linear apparatus, and 2 milliliters were placed in an EDTA tube, and both the remaining serum and blood were stored at -80ºC. ELISA technique used for measuring cystatinC (CysC) and kidney injury molecule 1(KIM1) levels in serum. Glomerular filtration rate (GFR) was calculated using **CKD-EPI Creatinine-Cystatin Equation (2021) online calculator [28]**.

***Genetic analysis***

Genomic DNA was extracted from peripheral blood leukocytes from the frozen venous blood using Favor Prep Blood Genomic DNA Extraction Mini Kit. DNA purity and concentration was detected by Nano drop and the molecular weight (mw) of DNA extracted and the integrity of extracting DNA was detected by 1% agarose gel-electrophoresis.

ACE(I\D) polymorphisms (rs1799752) detected by high resolution melting real time (HRM-RT) [29]. The Rotor-Gene Q (Qiagen®) thermal cycler was used to carry out the reaction. Using three primers:

Primer 1ACE1 CATCCTTTCTCCCATTTCTC

Primer2ACE2TCGGATTACAGCCCTGATACAG

Primer3ACE3ATTTCAGAGCTGGAATAAAATT

The polymerase chain reaction (PCR) reaction was performed in a 0.1 ml micro tube containing 50 µL final volume, which included: 20µL SYBR Green PCR Master Mix, 3 µL a set of three primers, 2 µL of genomic DNA; 1 µL Mgcl2, and 24 µL DNAse-free water was added to completethe final reaction volume. The following temperature cycling conditions were used for the PCR reaction: First, denaturation at 95 °C for 5 minutes, then 35 cycles of denaturation at 95 °C for 20 seconds, followed by annealing and elongation at 55 °C for one minute. Following DNA amplification, the PCR products (amplicons) were submitted to the dissociation curve (melt curve); the samples were cooled to 60°C and then gradually raised in temperature by 0.1°C up to 95°C. The intensity of fluorescence (F) generated by the SYBR Green fluorophore was continuously recorded during temperature rise, and the signal was plotted as a function of temperature (T) to get the dissociation curve for each sample. The dissociation peaks were subsequently created by charting the negative derivative of fluorescence as a function of temperature (-dF/dT versus T). Genotyping was then performed by visual inspection of the dissociation curve and the results of some of the current study samples are demonstrated in Figure1, and 2.

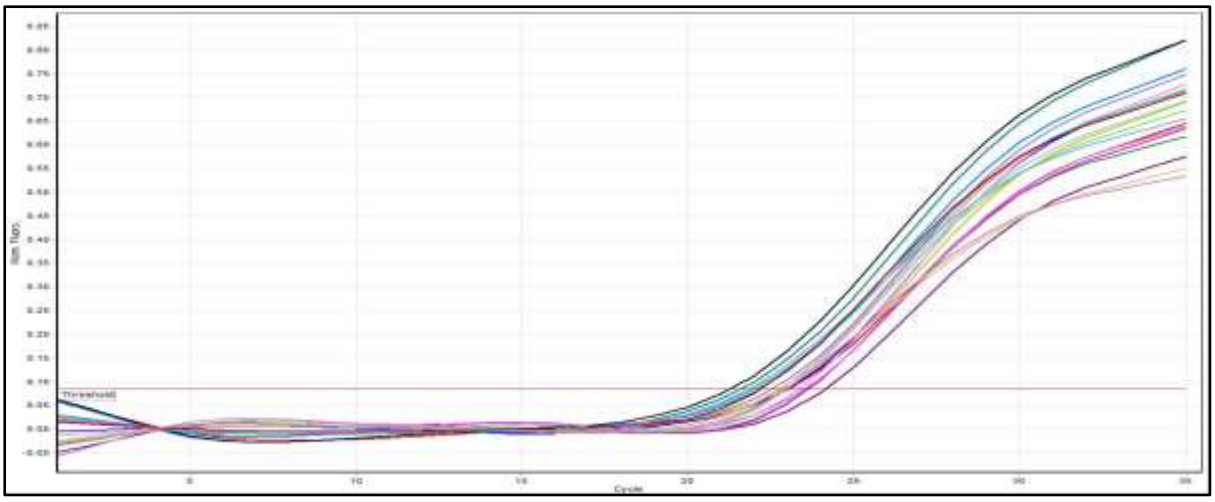

**Figure 1. HRM-RT amplification and melting curve raw data**

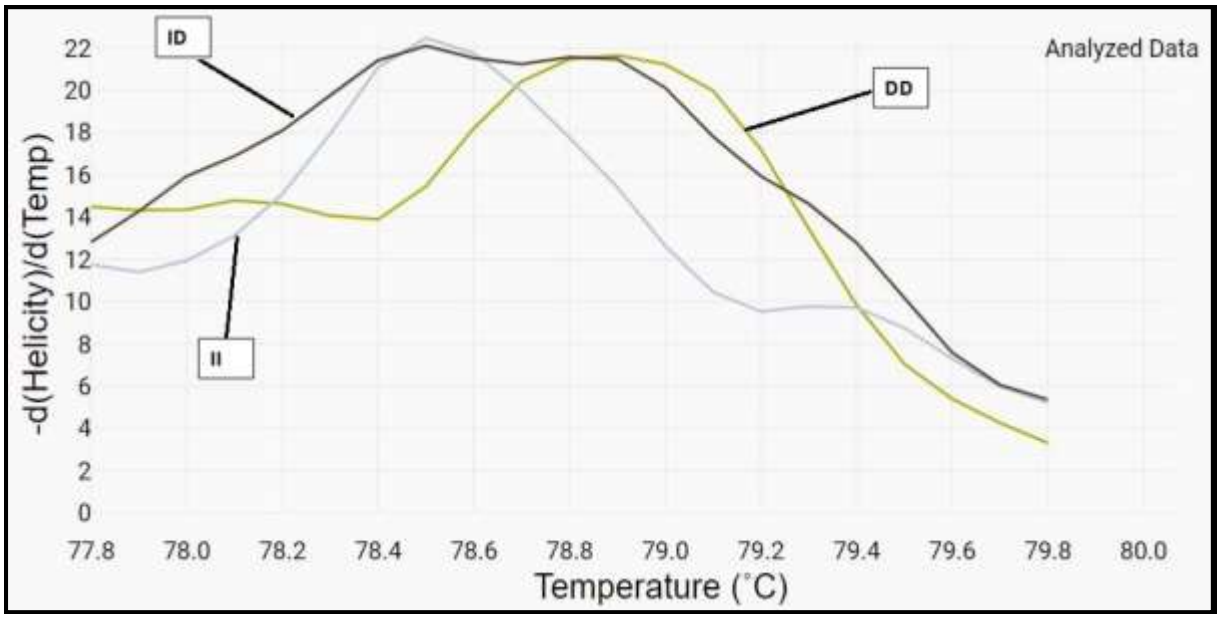


**Figure 2. HRM-RT analyzed data showed three different genotype according to their melting temperature (II, ID, DD).**

The AGT1R (A1166C) polymorphisms detected by the PCR and restriction fragment length polymorphism (PCR–RFLP) method [30]. In this study the primers were designed using the NCBI-primer BLAST online software that include:
Forward primer ATGAGCACGCTTTCCTACCG
Reverse primer TTCTTCGAGCAGCCGTCATC

The PCR amplification has been carried out in a final volume of 20µL reaction mixture containing 2µL of genomic DNA, 1µL of each primer, 8µL master mix 0.5µL $MgCl_2$, 7.5µL DNAse - free water. After optimization process, selection the best conditions for PCR at which the DNA was amplified for 35 cycles with each cycle consisting of denaturation at 94°C for 5 min, then 94°C for 30 seconds, annealing at 60°C for 30 seconds and elongation at 72°C for 30 seconds and final extension 94°C for 5 min. By 2% agarose gel electrophoresis with ethidium bromide (EtBr) staining and direct visualization in UV light, the PCR products were examined. Only those PCR products that had a single amplification product with no sign of non-specific amplification was taken for restriction by appropriate restricted enzyme which selected by the aid of SnapGene viewer software (V6.0.5). The restriction reaction carried out by using the following mixture: 3µL of PCR product, 0.25µL of the selected restriction enzyme (DdeI), restriction buffer 1.5µL (each restriction enzyme has its restriction buffer supplied by the manufacturer), the reaction mixture then completed to 13 µL by 8.5µL DNAse-free water, then 25 µL of Vaseline oil added over the reaction mixture to reduce evaporation since the reaction mixture incubated at 60°C in water bath overnight. Then, using non-denaturing polyacrylamide gel electrophoresis, the

length of the PCR product, the specificity of the PCR reaction, and the restricted PCR product were examined, and the results of gel electrophoresis image for samples of the current study are shown in Figure 3.

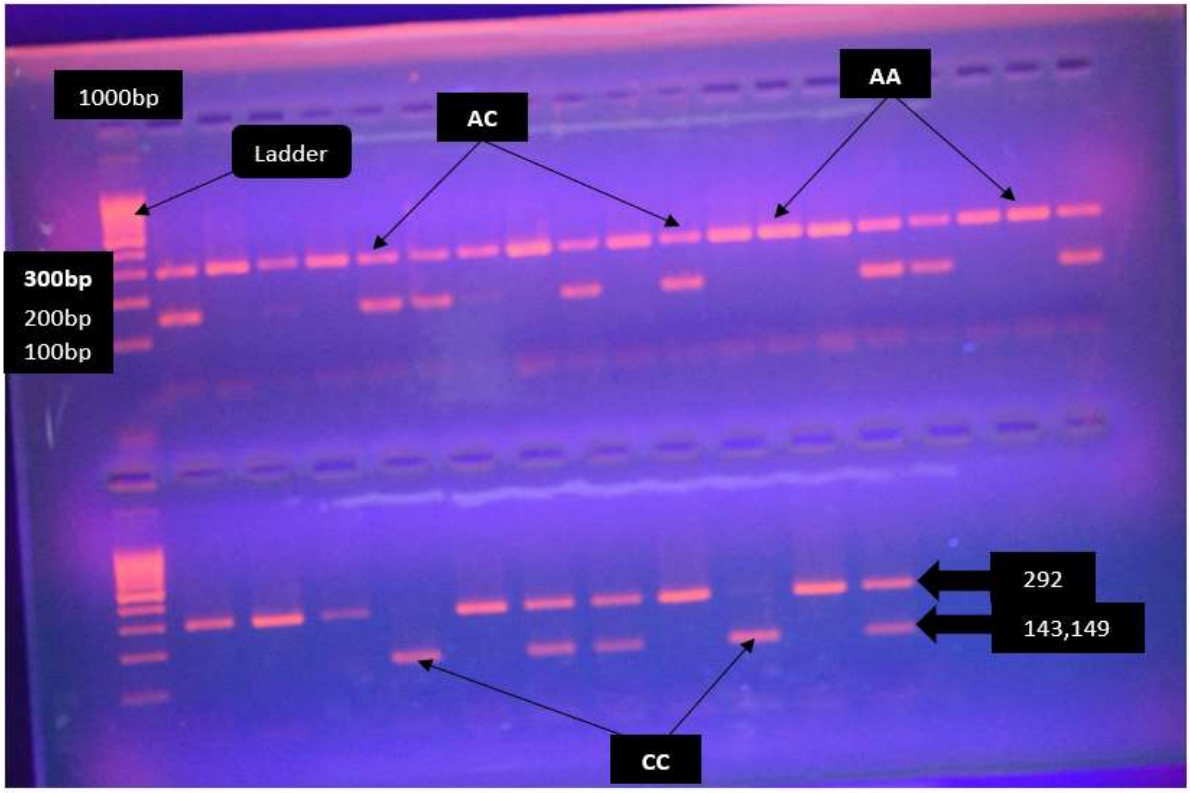


**Figure 3. Agaroes gel electrophoresis image show RFLP-PCR product analysis for AGT1Rrs5186 gene stained with EtBr in 2% agarose gel at 70 volt for 1 hour, 1000 bp DNA ladder, showing AA, AC and CCgenotype.**

***Statistical analysis***

The Statistical Package for the Social Sciences (SPSS) software for Windows version 26.0 (IBM Corp., Armonk, NY, U.S.) was used to do the statistical analysis. The Shapiro-Wilk test was used to test the normality of the continuous variables. For normally distributed data, the continuous variables are given as mean ± standard deviation (SD) and for skewed data, as median (range). Independent T test or Mann-Whitney test were used for analyzing data. The categorical variables are expressed as numbers and frequency and were analyzed using the chi-square test. The risk association between the genotypes and DN was estimated through the calculation of odds ratio (OR) and 95% confidence intervals (CI) using chi-square test and the adjusted OR using logistic regression analysis considering (gender, age and body mass index (BMI)) as independent variables. The chi-square test was used to assess genotype deviation from the Hardy-Weinberg equilibrium (HWE). Statistical significance was defined as a P value 0.05.

## Results

***Study group characteristics***

Demographic, clinical, and biochemical details of the patients enrolled in the study are given in Table 1. That demonstrates a significant difference between males and females, with a higher prevalence of females in DN patients. No significant difference in BMI, smoking history, diabetic (DM) duration, hypertension(HT) duration, chronic heart disease(CHD), systolic blood pressure(SBP), diastolic blood pressure(DBP), and living place. While a significant difference was seen in retinopathy. In relation to biochemical variables, no significant difference in glycemic and lipid profile except for HbA1c and total cholesterol (TC) that were significantly difference between study groups with higher levels in DN patients. Renal function indices revealed no significant differences in serum creatinine (SCr) while a significant difference in ACR, serum CystC, KIM1 and GFR.

**Table 1. Demographic, Clinical and Biochemical data in normoalbuminuric and DN patients**

| Type of variables | Variable | normoalbuminuric patients N=63 | DN Patients N=98 | P value |
|---|---|---|---|---|
| **Demographic variables** | **Age(years)** | 60.17±7.87 | 59.9±8.31 | 0.83 |
| | **Gender(male: female)** | 34:29 | 35:63 | **0.022*** |
| **Clinical variables£** | | | | |
| | **BMI(kg\m$^2$)** | 30.54±4.23 | 30.82±5.19 | 0.72 |
| | **Smoking history** | 4(6.3%) | 5(5.1%) | 0.738 |
| | **DM.duration(years)** | | | |
| | **5-14** | 48(76.2%) | 77(78.5%) | |
| | **15-24** | 13(20.6%) | 17(17.3%) | 0.758 |
| | **≥25** | 2(3.2%) | 9(9.2%) | |
| | **HT.duration(years)** | | | |
| | **1-10** | 43(68.2%) | 73(74.5%) | |
| | **11-20** | 18(28.6%) | 24(24.5%) | 0.357 |
| | **21-30** | 2(3.2%) | 1(1%) | |
| | **Retinopathy** | 19(30.2%) | 91(92.9%) | **<0.001*** |
| | **CHD** | 18(28.6%) | 38(38.8%) | 0.185 |
| | **SBP(mmHg)** | 131.11±13.81 | 131.89±15.17 | 0.743 |
| | **DBP(mmHg)** | 82.30±7.50 | 80.26±9.20 | 0.142 |
| | **Living place** | | | |
| | **Urban** | 35(56%) | 55(56%) | 0.944 |
| | **Rural** | 28(44%) | 43(44%) | |
| **Biochemical variables** | | | | |
| **Glycemic indices** | **FBS(mmol\l)** | 10.36±3.86 | 10.58±4.30 | 0.74 |
| | **HbA1C (%)** | 8.37±1.71 | 9.16±1.97 | **0.010*** |
| | **Hb(g\dl)** | 13.52±1.53 | 13.13±1.48 | 0.108 |
| **Lipids profile** | **TC(mmol\l)** | 4.14±1.33 | 4.65±1.27 | **0.011*** |
| | **TG(mmol\l)** | 2.08±1.08 | 2.15±1.18 | 0.734 |
| | **HDL(mmol\l)** | 0.94±0.22 | 0.99±0.24 | 0.185 |
| **Renal function indices** | **ACR(mg\g)** | 24.28±5.54 | 148.64±143.94 | **<0.001*** |
| | **S. creatinine(mmol\l)** | 77.29±19.02 | 82.76±24.94 | 0.117 |
| | **S. cystatin C(mg\dl)** | 1.19±0.53 | 1.60±1.38 | **0.009*** |
| | **S.KIM1(ng\ml)** | 0.80±0.21 | 1.13±1.06 | **0.004*** |
| | **GFR(ml\min\1.73m$^2$)** | 98.33±17.74 | 85.17±23.9 | **<0.001*** |

P value for continuous data (BMI, FBS, HbA1C, TC, TG, HDL, Hb, ACR, S.cr, S.cys, S.KIM1, GFR)calculated using two-sided t-test, £: fisher exact test used for clinical variable except for gender, retinopathy, and CHD used Pearson $\chi 2$ test; £: Proportional data is displayed as the number of positive outcomes and their proportion, for normally distributed as mean±SD. BMI, body mass index; DN, diabetic nephropathy; SBP,systolic blood pressure; DBP, diastolic blood pressure; FBS, fasting blood sugar;HbA1C,glycated hemoglobin; Hb, hemolglobin; HDL, high-density lipoprotein; TC total cholesterol ; TG, triglyceride; DM, diabetes mellitus; ACR, albumin creatinine ratio; KIM1,kideny injury molecule1**;** CHD: chronic heart disease; GFR:glomerular filtration rate.*significant ($p< 0.05$)

**Table 2. Distribution and odd ratio of ACE (I/D) genotypes and alleles in diabetic patients with and without nephropathy.**

| | Patients groups | | OR(CI95%) | P-value | Adjusted OR (CI95%) | P-value |
|---|---|---|---|---|---|---|
| | T2DM with DN N=98 | T2DM without DN N=63 | | | | |
| ACE genotypes | 40(40.7%) | 20(31.7%) | 1.9(0.832-4.34) | 0.145 | 1.8(0.76-4.21) | 0.181 |
| DD | 38(38.7%) | 24(38.1%) | 1.5(0.669-2.38) | 0.323 | 1.5(0.64-3.40) | 0.369 |
| ID | 20(20.4%) | 19(30.2%) | reference group | | reference group | |
| II | | | | | | |

**Continued table 2.**

| | | | | | | |
|---|---|---|---|---|---|---|
| Dominant | | | | | | |
| DD | 40(40.8%) | 20(31.7%) | 1.5(0.762-2.886) | 0.316 | 1.5(0.71-2.81) | 0.320 |
| ID+II | 58(59.2%) | 43(69.3%) | | | | |
| Recessive | | | | | | |
| II | 20(20.4%) | 19(30.2%) | 0.6(0.287-1.23) | 0.188 | 0.62(0.29-1.32) | 0.212 |
| ID+DD | 78(79.6%) | 44(69.8%) | | | | |
| ACE allele | | | | | | |
| D | 118(60.2%) | 64(50.8%) | 1.5(0.933-2.302) | 0.096 | | |
| I | 78(39.8%) | 62(49.2%) | | | | |

Pearson's chi square $\chi 2$ test, adjusted OR using logistic regression. * Significant ($p< 0.05$), DN: diabetic nephropathy OR: odd ratio, CI95%: confidence interval at 95%.

Table 3 illustrates AGT1R (A1166C) genotypes and allele's distribution and OR in nephropathic and normoalbuminuric patients. Both DN and normalbuminuric patients had comparable high prevalence of AA genotype followed by AC genotype, while CC genotype seen only in 3 patients. The frequency of A allele was 81.1% in DN and 81.7% in normoalbuminuric patients while C allele frequency was 18.9% in DN and 18.3% in normoalbuminuric patients (P = 0.888). Computation of the OR (95% CI) as an estimate of relative risk for nephropathy with and without adjustment revealed that individuals with CC genotype have 0.8-fold and 1.2-fold increment risk for DN, respectively.

**Table 3. Distribution and odd ratio of AGT1R (A1166C) genotypes and alleles in diabetic patients with and without nephropathy.**

| | **Patients groups** | | **OR (CI95%)** | **P-value** | **Adjusted OR (CI95%)** | **P-value** |
|---|---|---|---|---|---|---|
| | **T2DM with DN N=98** | **T2DM without DN N=63** | | | | |
| AGT1R genotypes | | | | | | |
| AA | 63(64.3%) | 41(65.1%) | Reference group | | Reference group | |
| AC | 33(33.7%) | 21(33.3%) | 0.98(0.498-1.918) | 0.948 | 1.04(0.52-2.08) | 0.905 |
| **CC** | 2(2%) | 1(1.6%) | 0.77(0.067-8.748) | 1.000f | 1.24(0.10-14.85) | 0.868 |
| Dominant | | | | | | |
| AA | 63(64.3%) | 41(65.1%) | | | | |
| AC+CC | 35(35.7%) | 22(34.9%) | 0.97(0.498-1.874) | 0.918 | 1.05(0.53-2.08) | 0.885 |
| AGT1R allele | | | | | | |
| **A** | 159(81.1%) | 103(81.7%) | | | | |
| **C** | 37(18.9%) | 23(18.3%) | 0.96(0.539-1.708) | 0.888 | | |

Pearson's chi square test, adjusted OR using logistic regression, DN: diabetic nephropathy. * Significant ($p< 0.05$), f: fisher exact test

***Haplotype distribution***

Haplotype analysis, done by SHEsis software(31) used for the determination of the association of ACE with AGT1R variants. Non-significant difference was noted between these two variants in DN and normalbuminuric cases, with DA has the highest risk and IA has the lowest risk for DN, however these were not significant.

**Table 4. Haplotype analysis for ACE (I\D) and AGT1R (A1166C)**

| | **T2DM with DN** | **T2DM without DN** | **Chi2** | **Fisher's p** | **OR [CI95%]** |
|---|---|---|---|---|---|
| **D A** | 99.79(0.509) | 52.66(0.418) | 2.557 | 0.109881 | 1.444 [0.920-2.268] |
| **D C** | 18.21(0.093) | 11.34(0.090) | 0.008 | 0.928937 | 1.036 [0.476-2.255] |
| **I A** | 59.21(0.302) | 50.34(0.399) | 3.241 | 0.071862 | 0.651 [0.407-1.040] |
| **I C** | 18.79(0.096) | 11.66(0.093) | 0.010 | 0.921450 | 1.039 [0.482-2.239] |

***Genotype–phenotype correlation***

Table 5a shows the differences in the levels of studied biochemical markers in DN patients with different ACE (I\D) genotypes revealing that the DD genotype had higher KIM1, CysC, and HbA1C values that were significant only for CysC and HbA1C, also the DD genotype was associated with a significant drop in GFR value when compared to (II+ID) genotypes. While ACR and SCr was lower in DD genotype but the differences were not significant.

Table 5b demonstrates no significant differences between the levels of all studied biochemical markers in different ACE (I\D) genotypes in normalbuminuric patients.

**Table 5a. Levels of studied biochemical parameters in different ACE (I/D) genotypes in DN patients.**

| Variables | DN patients n=98 | | P value |
|---|---|---|---|
| | DD median(range) n=40 | ID+II median(range) n=58 | |
| Albumin:creatinine ratio | 80.7(36.5-578.3) | 105.1(38.5-632.7) | 0.49 |
| Serum. creatinine | 76.5(45-149) | 82(37-151) | 0.43 |
| Kidney injury molecule 1 | 1.4(0.68-7.52) | 1.04(0.41-8.34) | 0.051 |
| Cystatin C | 0.95(0.43-6.23) | 0.82(0.28-5.95) | **0.007*** |
| Glomerular filtration rate | 86.5(19-121) | 92.5(21-142) | **0.047*** |
| HbA1C | 9.7(5.8-15) | 8.45(5.6-13.7) | **0.019*** |
| Total cholesterol | 4.45(2.4-7.5) | 4.4(2.5-8.6) | 0.62 |

*significant ($p < 0.05$), Mann –Whitney U test.

**Table 5b. Levels of studied biochemical parameters in different ACE (I/D) genotypes in normalbuminuric patients**

| Variables | normalbuminuric patients n=63 | | P value |
|---|---|---|---|
| | DD median(range) n=20 | ID+II median(range) n=43 | |
| Albumin:creatinine ratio | 26.35(9.8-29.8) | 26.9(14.2-29.9) | 0.81 |
| Serum. creatinine | 75(50-140) | 78(40-116) | 0.53 |
| Kidney injury molecule 1 | 1.07(0.48-2.12) | 1.18(0.06-2.85) | 0.77 |
| Cystatin C | 0.77(0.24-1.2) | 0.83(0.29-1.18) | 0.37 |
| Glomerular filtration rate | 98(69-141) | 97(68-150) | 0.81 |
| HbA1C | 8.05(6-11.3) | 8.1(6.1-12.8) | 0.91 |
| Total cholesterol | 4.35(2-6.1) | 4.2(1.7-7.1) | 0.69 |

*significant ($p < 0.05$), Mann –Whitney U test.

Table 6a displays the levels of studied biochemical parameters in different AGT1R(A1166C) genotypes, showing non-significant higher value for ACR, CysC, HbA1C, TC and lower SCr, KIM1, and GFR in DN patients carrying AA genotypes compared to (AC+CC) genotypes.

Table 6b indicates that normalbuminuric patients who carried C allele either in heterozygote AC or in homozygote CC had elevated levels for all studied biochemical parameters except for HbA1C and GFR which are higher in AA genotype, however these differences still not significant between these different AGT1R(A1166C) genotypes.

**Table 6a. Levels of studied biochemical parameters in different AGT1R (A1166C) genotypes in DN patients.**

| Variables | DN patients n=98 | | P value |
|---|---|---|---|
| | AA median(range) n=63 | AC+CC median(range) n=35 | |
| Albumin:creatinine ratio | 93.6(36.5-530.1) | 81.5(38.6-632.7) | 0.99 |
| Serum. creatinine | 77(44-151) | 82(37-144) | 0.29 |
| Kidney injury molecule 1 | 1.18(0.41-8.34) | 1.19(0.66-4.72) | 0.93 |
| Cystatin C | 0.87(0.43-6.23) | 0.85(0.28-3.81) | 0.38 |
| Glomerular filtration rate | 87(21-121) | 92(19-142) | 0.45 |
| HbA1C | 9.1(5.8-13.9) | 8.3(5.6-15) | 0.18 |
| Total cholesterol | 4.5(3-7.3) | 4.2(2.4-8.6) | 0.6 |

*significant ($p < 0.05$), Mann –Whitney U test.

**Table 6b. Levels of studied biochemical parameters in different AGT1R (A1166C) genotypes in normalbuminuric patients**

| Variables | normalbuminuric patients n=63 | | P value |
|---|---|---|---|
| | AA median(range) n=41 | AC+CC median(range) n=22 | |
| Albumin:creatinine ratio | 26.1(9.8-29.8) | 28.9(14.2-29.8) | 0.38 |
| Serum. creatinine | 75(40-140) | 77.5(47-116) | 0.81 |
| Kidney injury molecule 1 | 1.0(0.06-2.85) | 1.2(0.18-2.12) | 0.77 |
| Cystatin C | 0.79(0.24-1.08) | 0.84(0.44-1.2) | 0.12 |
| Glomerular filtration rate | 97(69-150) | 96(68-123) | 0.24 |
| HbA1C | 8.1(6.1-12.8) | 7.95(6-12.5) | 0.63 |
| Total cholesterol | 4.1(1.7-6.1) | 4.7(2-7.1) | 0.06 |

*significant ($p< 0.05$), Mann –Whitney U test.

# Discussion

Nephropathy in diabetic people is recognized as a complex illness since it is the consequence of the interaction of several hereditary and environmental factors. To identify those who are at risk, genetic differences might be used as a marker. Numerous research have been conducted to identify the genetic risk factors that trigger certain diseases [32]. According to the current Iraqi population sample, the majority of T2DM patients who developed DN were female. Also retinopathy was shown to be considerably higher in DN groups, while other demographic and clinical parameters revealed no significant differences between study groups. Biochemical variables show significant differences only in HbA1C and TC, with higher levels in DN patients. While renal measures show highly significant differences between study groups, with higher levels for all renal parameters and lower GFR in nephropathic patients except for SCr, which was higher in DN but not significant.

The RAAS is a potent regulator of arterial blood pressure via angiotensin II, and candidate genes in the system are involved in the etiology of diabetic renal problems [33]. It has been demonstrated that the ACE level is highly associated with the ACE (I/D) polymorphism. Because the ACE (I/D) gene polymorphism occurs in the noncoding gene area, the base insertion or deletion may modify the splicing process of the ACE precursor mRNA, influencing the stability of ACE mRNA, and eventually affecting the expression or stabilization of ACE [34]. The current study showed that DD genotype was the most frequent, followed by ID and then II of total cases of DN, with no significant difference in these genotypes between DN and normalbuminuric patients, however the current study revealed that DD genotype and D allele was associated with higher risk of DN (1.5 fold) compared to (ID+II) and I allele, respectively, these results were approximately consistent with a recent study by Zeng W-l *et al*. regarding the risk but their *P*-value was significant [35]. There are two studies in Iraq about association of ACE (I/D) with DN, first study by Hussein M A *et al*. which was in accordance with the present study but found higher risk, that found DD genotype carriers have 2.9 folds risk for development of DN when compared with the reference II genotype after adjustment for age, BMI, age, and sex [13]. While the results of the other study, revealed that all genotypes were related to an elevated risk of nephropathy, including the II genotype, and these finding were inconsistent with the current findings [36]. Another Iraqi study by Al-Radeef *et al.* on ACE (I\D) in renal failure patients show similar frequency for alleles and genotypes with the present study [7].

Out of several SNPs within the AGT1R gene, the best-studied polymorphism within the AGT1R gene is AGT1R (A1166C), where the nucleotide Adenine (A) is changed to Cytosine (C). The C allele has the enzyme-restriction site (DdeI) at nucleotide position 1166, hence it produces a smaller fragment than the A allele which lacks the restriction enzyme site [37]. There are limited investigations on the association of the AGT1R (A1166C) polymorphism with the risk of diseases in Iraq. There are only two studies published on the correlation of this polymorphism with hypertension and acute coronary syndrome [23, 24], and no studies are available on the association with DN, therefore this current study is the first one in this field which revealed that wild type AA has higher frequency compared to other genotypes, in addition the mutant CC carrier appear only in 3 individuals and these were approximately consistent with Asian studies that gathered in Zhuang Y *et al*. metanalysis [14].

Also current study found that C allele has 1 fold risk for DN compare to A allele with no significant difference. While Zhuang Y *et al.* metananlysis on T2DM found non-significant higher risk for CC genotype (5 folds) compare to AA genotype, also C allele has relative risk (1.5 folds) compared to A allele which also higher than current investigation results [(14)]. The variations in population, inclusion criteria, and DN definition between studies may be the cause of the inconsistencies.

Haplotype analysis revealed that both D allele-containing haplotypes (DA, DC) were linked with an elevated risk of DN, identifying the D allele as the strongest risk allele, and that this stronger D allele-containing haplotype eliminated the protection provided by the A allele-containing haplotype. This study adds to the evidence that the D allele is a key risk factor for DN. There have been no previous investigations on the haplotype of these two SNPs in DN.

In terms of ACE (I/D) polymorphism and biochemical markers, the current study found that DD polymorphism was related with an increase in serum KIM1, CysC, HbA1C and a considerably lower level for GFR, but no significant difference between ACE genotypes for ACR in the DN patients group. I. Ezzidi *et al*. discovered significantly greater levels of ACR and SCr in the DD group compared to other genotypes, which contradicted the current study's result of higher but non-significant ACR and SCr levels in the (II+ID) group [(38)]. While according to AAdS, Reis *et al.*, patients with DN carrying the DD genotype had greater HbA1C, fasting plasma glucose, and SCr while having a lower GFR compared to other genotypic profiles for the ACE (I\D) polymorphism, which was consistent with the current findings regarding HbA1C and GFR [(39)]. There has been no prior research on the correlation of other biochemical parameters such as KIM1 and CysC with ACE (I\D) polymorphism. However, recently on March 2023 Taha MM *et al.* found that serum CysC was higher in the ID genotype compared to the DD and II genotypes [(40)].

The presence of the C allele may be related to increased angiotensin II receptor affinities or higher levels of AGT1R gene expression. By modifying the function of renal cells or by causing abnormalities in systemic or renal hemodynamics, the elevated angiotensin II activity increases kidney vulnerability to the effects of hyperglycemia resulting in systemic and glomerular hypertension, proteinuria, and the development of DN [(41, 42)]. In the current study, the association of AGT1R (A1166C) with biochemical markers showed no significant differences in the measured markers between various AGT1R (A1166C) genotypes in DN patients or normalbuminuric patients. There are limited studies available about association of biochemical markers with this polymorphism, F. Razi *et al.* found that (AC+CC) genotypes had significantly higher ACR compared to AA genotype [(43)]. Halder K and Purkait P studied different serum markers according to AGT1R(A1166C) polymorphism and found that SCr level higher but not significant in AA genotype compared to others genotype [(44)].

## Conclusion

In conclusion, among T2DM, patients with DD genotype had higher frequency in DN patients and had 2 fold risk for DN compared to II genotype which had the lowest risk for DN. In addition DN patients whom carrying DD genotype had higher serum KIM1, CysC, HbA1C, and lower GFR compared to (ID+II) genotypes. Also the present study reveals that the A1166C polymorphism of AGT1R distribution frequency were similar in both study groups with higher frequency for A allele compared to C allele and not associated with risk of DN in Iraqi T2DM.

## Limitations

This study was limited by its small sample size that hindered meaningful association of these genetic polymorphism with DN, also localize on a single endocrinology center in one city (Babylon city/Iraq). Therefore, care must be taken when extrapolating the findings of this study to the entire country. Moreover, there are no measurements of plasma angiotensinogen levels, angiotensin II or other RAS activation indicators to be available to connect directly with the genetic variants examined here.

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## Conflicts of interest

There are no conflicts of interest among the writers

## Ethics Statements

Ethical committee approval was gained from Research Ethics Committee of the University of Baghdad – College of Pharmacy (Approval number: RECAUBCP4102021A on 4/10/2021), and also from the medical institutions that the research be officially accepted by the research unit of the Center for Training and Human Development of Babylon Health Directorate in Babylon province (Decision number: 26 on 8/3/2022). Verbal

informed consent from patients included in the study before specimens were been taken.

## Author Contributions

The work was designed, supervised, and performed by Ansam, Dheyaa, and Nassar. Ansam and Dheyaa wrote this manuscript's initial draft. Sample collection and data gathering by Ansam and Nassar. Ansam analyzed and interpreted the data. All authors reviewed and approved the final article.

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