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Znaczenie wybranych polimorfizmów genów syntaz tlenku azotu (*NOS*) oraz genu kodującego enzym konwertujący angiotensynę (*ACE*) w ocenie ryzyka wystąpienia cukrzycowej choroby nerek

The significance of selected polymorphisms of nitric oxide synthase (*NOS*) and the encoding angiotensin-converting enzyme (*ACE*) in assessing the risk of diabetic nephropathy

Rozprawa doktorska na stopień doktora
w dziedzinie nauk medycznych i nauk o zdrowiu
w dyscyplinie nauki farmaceutyczne

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„Najpiękniejszą rzeczą, jakiej możemy doświadczyć, jest oczarowanie tajemnicą. Jest to uczucie, które stoi u kolebki prawdziwej sztuki i prawdziwej Nauki. Ten, kto go nie zna i nie potrafi się dziwić, nie potrafi doznawać zachwytu, jest martwy, niczym zdmuchnięta świeczka."

Albert Einstein

Serdecznie dziękuję mojej Pani Promotor dr hab. inż. Marcie Kepinskiej, prof. UMW za wszelkie udzielone rady, wsparcie merytoryczne, cierpliwość, a także stałe motywowanie do dalszego rozwoju.

Dziękuję także mojemu mężowi oraz rodzinie za rozbudzenie i utrzymywanie we mnie ciągłego pragnienia zdobywania wiedzy.

Dziękuję moim przyjaciołom i znajomym za udzielone wsparcie oraz wyrozumiałość.

Spis treści

I. WYKAZ PUBLIKACJI STANOWIĄCYCH PRACĘ DOKTORSKĄ

1. Artykuł przeglądowy

Król Magdalena, Kepinska Marta: Human nitric oxide synthase – its functions, polymorphisms, and inhibitors in the context of inflammation, diabetes and cardiovascular diseases. *International Journal of Molecular Sciences*, **2021**, vol. 22, nr 1, art.56 [18 s.], DOI:10.3390/ijms22010056

IF = 6,208; MNiSW = 140 pkt; Liczba cytowań: 107

2. I artykuł oryginalny

Król-Kulikowska Magdalena, Abramenko Nikita, Jakubek Milan, Banasik Mirosław, Kepinska Marta: The role of angiotensin-converting enzyme (ACE) polymorphisms in the risk of development and treatment of diabetic nephropathy. *Journal of Clinical Medicine*, **2024**, vol. 13, nr 4, art.995 [17 s.], DOI: 10.3390/jcm13040995

IF = 3,000; MNiSW = 140 pkt; Liczba cytowań: 2

3. II artykuł oryginalny

Król-Kulikowska Magdalena, Banasik Mirosław, Kepinska Marta: The effect of selected nitric oxide synthase polymorphism on the risk of developing diabetic nephropathy. *Antioxidants*, **2024**, vol. 13, nr 7, art.838 [23 s.], DOI:10.3390/antiox13070838

IF = 6,000; MNiSW = 100 pkt

Całkowity IF = 15,208

MNiSW = 380 pkt

Liczba cytowań: 109

II. STRESZCZENIE

Wprowadzenie: Cukrzyca zaliczana jest do chorób cywilizacyjnych; może ona nie tylko znacznie obniżać jakość życia, ale także prowadzić do poważnych powikłań. Jednym z najczęstszych powikłań cukrzycy jest cukrzycowa choroba nerek, która często prowadzi do konieczności wykonania przeszczepu nerki. Coraz częściej w progresji tych chorób podkreśla się nie tylko rolę czynników środowiskowych, ale też genetycznych, w tym polimorfizmów genów kodujących białka i enzymy istotne w patofizjologii tych zaburzeń. W ich grono zalicza się między innymi izoformy syntazy tlenku azotu (NOS, ang. *nitric oxide synthase*), a także enzym konwertujący angiotensynę (ACE, ang. *angiotensyn-converting enzyme*).

W przebiegu chorób przewlekłych, w tym cukrzycowej choroby nerek, występuje zwiększona produkcja wolnych rodników. Chociaż są one niezbędne do przeprowadzenia pewnych procesów fizjologicznych, to ich nadmierna ilość może powodować stres oksydacyjny i/lub nitrozacyjny oraz uszkodzenie komórek. Cząsteczką powiązaną z tymi zjawiskami jest tlenek azotu (NO), który jest prekursorem reaktywnych form azotu (RNS, ang. *reactive nitrogen species*). Za syntezę tego związku odpowiadają izoformy NOS – NOS1, NOS2, NOS3.

W przebiegu cukrzycy oraz jej powikłań obserwujemy zwiększoną produkcję angiotensyny II (Ang II), za którą odpowiada ACE. W leczeniu cukrzycowej choroby nerek często stosuje się leki hipotensyjne, w tym środki z grupy inhibitorów ACE (ACEi), do których zaliczamy benazepryl, enalapryl, lizynopryl czy ramipryl. Dlatego też polimorfizmy w genie *ACE* mogą być związane ze skutecznością stosowania tego rodzaju terapii.

Cel pracy: Celem niniejszej rozprawy doktorskiej była ocena wybranych polimorfizmów: rs4343 i rs4646994 (w genie *ACE*); rs3782218 (w genie *NOS1*); rs1137933 (w genie *NOS2*); rs1799983, rs2070744 i rs61722009 (w genie *NOS3*) w kontekście zwiększonego ryzyka rozwoju cukrzycowej choroby nerek i/lub zwiększonego prawdopodobieństwa konieczności wdrożenia terapii nerkozastępczej. Istotnym było także zbadanie wpływu tych polimorfizmów na stężenie i aktywność ACE, stężenie izoform NOS (NOS1, NOS2, NOS3), a także stężenia wybranych parametrów istotnych w patogenezie cukrzycowej choroby nerek (stężenie glukozy, kreatyniny, CRP, wartość eGFR) oraz stężenia pierwiastków śladowych (cynk i miedź). Ważne było również wykonanie analizy *in silico* w celu zbadania interakcji pomiędzy dwiema domenami ACE – domeną N i C – a lekami z grupy ACEi wykorzystywanymi w terapii cukrzycowej choroby nerek: benazeprylem, enalaprylem, lizynoprylem oraz ramiprylem.

Materiały i metody: W celu przygotowania rozprawy doktorskiej najpierw dokonano przeglądu najnowszej literatury dotyczącej roli izoform NOS w patogenezie wybranych chorób

przewlekłych, w tym cukrzycy oraz cukrzycowej choroby nerek. Analizie zostały poddane także prace poświęcone znaczeniu polimorfizmów genów kodujących te izoformy.

Z kolei w celu przygotowania prac oryginalnych została zebrana grupa badana składająca się z 232 osób, która została podzielona na następujące podgrupy: pacjenci z cukrzycową chorobą nerek (N=85), pacjenci z cukrzycową chorobą nerek i po przeszczepie nerki (N=97), grupa kontrolna (N=50). W surowicy wszystkich badanych zostały oznaczone następujące parametry: stężenie i aktywność ACE, stężenie NOS1, NOS2, NOS3, a także stężenie cynku i miedzi. Stężenia poszczególnych izoform ACE i NOS były oznaczane przy użyciu testów ELISA, aktywność ACE została oznaczona z wykorzystaniem testu kolorymetrycznego, a stężenia metali zostały oznaczone przy użyciu metody atomowej spektrometrii absorpcyjnej z atomizacją w płomieniu (FAAS, ang. *Flame Atomic Absorption Spectrometry*). Dodatkowo pozyskano wyniki stężeń glukozy, kreatyniny i CRP oraz wartości eGFR z Kliniki Nefrologii, Medycyny Transplantacyjnej i Chorób Wewnętrznych Uniwersyteckiego Szpitala Klinicznego im. Jana Mikulicza-Radeckiego we Wrocławiu. Ponadto zanalizowano wpływ siedmiu polimorfizmów na wyżej wymienione parametry; były to następujące polimorfizmy: rs4343 i rs4646994 (w genie *ACE*); rs3782218 (w genie *NOS1*); rs1137933 (w genie *NOS2*); rs1799983, rs2070744 i rs61722009 (w genie *NOS3*). Do tego celu wykorzystano techniki PCR oraz PCR-RFLP. Wykonano także analizę *in silico* przy użyciu oprogramowania AutoDock Vina. W celu przeprowadzenia analizy statystycznej wykorzystano pakiet SATISTICA 13.3.

Wyniki: Artykuł przeglądowy ukazał złożoną rolę NOS w patogenezie chorób wielu narządów i układów. Szczególnie zauważono zależności między polimorfizmami genów *NOS2* i *NOS3* o ryzykiem rozwoju insulinooporności, cukrzycy czy cukrzycowej choroby nerek. Ponadto zwrócono także uwagę na to, iż polimorfizmy w genie NOS1 były związane ze zwiększonym ryzykiem wystąpienia chorób sercowo-naczyniowych, w tym nadciśnienia tętniczego czy miażdżycy.

Podczas prowadzenia badań na materiale biologicznym zebranym od pacjentów nefrologicznych, wykazano, że polimorfizmy rs4343 i rs4646994 w genie *ACE* nie były powiązane ze zmianami stężeń samego enzymu. Natomiast zaobserwowano spadek aktywności ACE u pacjentów z cukrzycową chorobą nerek i genotypem A/A polimorfizmu rs4343 w porównaniu do grupy kontrolnej. Odnotowano także niższe stężenia cynku u pacjentów z cukrzycową chorobą nerek w porównaniu do grupy kontrolnej. Z kolei dzięki wykorzystaniu regresji logistycznej zauważono, iż genotyp G/G oraz sam allel G polimorfizmu rs4343 był związany ze zwiększonym ryzykiem rozwoju cukrzycowej choroby nerek. Podobne wyniki

uzyskano także u pacjentów, u których wykonano przeszczep nerki. Z kolei po wykonaniu analizy *in silico* zauważono, że benazepryl i lizynopryl wykazywały znaczną preferencję w wiązaniu dla domeny C, co ma znaczenie w kontekście polimorfizmu rs4646994 w genie *ACE*. Podobnych zależności nie zaobserwowano w przypadku enalaprylu i ramiprylu.

Nie wykazano wpływu polimorfizmów w genach *NOS1* i *NOS2* (odpowiednio rs3782218 i rs1137933) na zmiany stężeń NOS1 i NOS2. Zauważono jednak, że poszczególne genotypy w obrębie polimorfizmów *NOS3* (rs1799983, rs2070744 i rs61722009) były powiązane z różnicami w stężeniach NOS3 między grupą z cukrzycową chorobą nerek i grupą z cukrzycową chorobą nerek będącą po przeszczepie nerki a grupą kontrolną (w grupie kontrolnej zaobserwowano wyższe stężenia tego parametru). Dodatkowo podobnie jak w przypadku badań nad polimorfizmami *ACE* – zaobserwowano niższe stężenia cynku u pacjentów z cukrzycową chorobą nerek oraz pacjentów z cukrzycową chorobą nerek i po przeszczepie nerki w porównaniu do grupy kontrolnej. Było to niezależne od podziału ze względu na genotypy w obrębie badanych polimorfizmów. Ponadto wykazano, iż genotyp C/C i sam allel C polimorfizmu rs3782218 w genie *NOS1* były związane ze zwiększonym ryzykiem rozwoju cukrzycowej choroby nerek oraz zwiększonym prawdopodobieństwem konieczności wykonania przeszczepu nerki. Z kolei obecność allelu G polimorfizmu rs1137933 w genie *NOS2* zmniejszała to prawdopodobieństwo.

Wnioski: Zmiany genetyczne związane z izoformami NOS, ale również i ACE mają wpływ na rozwój cukrzycy oraz jej powikłań. Ich badanie, a zatem też lepsze zrozumienie, może przyczynić się do opracowania nowych parametrów diagnostycznych lub skuteczniejszych metod leczenia.

W ramach niniejszej rozprawy doktorskiej wykazano wpływ wybranych polimorfizmów genów *NOS1*, *NOS2*, *NOS3* i *ACE* na ryzyko rozwoju cukrzycowej choroby nerek, a także na zmiany w prawdopodobieństwie konieczności zastosowania terapii nerkozastępczej. Szczególnie interesującymi i wartymi uwagi wydają się być polimorfizmy rs4343 w genie *ACE*, rs3782218 w genie *NOS1* oraz rs1137933 w genie *NOS2*. Aby potwierdzić wyniki tych badań, konieczne jest jednak prowadzenie dalszych analiz, które uwzględniłyby większą populację. A choć rola polimorfizmów *NOS3* w ryzyku wystąpienia powikłań cukrzycy nie została zaobserwowana, to niższe stężenia NOS3 obserwowano u pacjentów z tymi powikłaniami.

Ponadto badania te podkreśliły znaczenie wyboru leków z grupy ACEi w kontekście zmienności genetycznej występującej u pacjentów. Istnienie zależności między skutecznością tych preparatów a polimorfizmem rs4646994 w genie *ACE* wskazuje na to, iż prowadzenie

dalszych badań w tej tematyce mogłoby się przyczynić do indywidualizacji terapii, a tym samym skuteczniejszego leczenia chorych.

III. ABSTRACT

Introduction: Diabetes is classified as a disease of civilization; it can not only significantly reduce quality of life, but also lead to serious complications. One of the most common complications of diabetes is diabetic nephropathy, which often leads to the need for a kidney transplant. Increasingly, the role of not only environmental factors, but also genetic factors, including polymorphisms of genes encoding proteins and enzymes important in the pathophysiology of these disorders, is being emphasized in the progression of these diseases. They include nitric oxide synthase (NOS) isoforms, as well as angiotensin-converting enzyme (ACE).

In the course of chronic diseases, including diabetic nephropathy, there is an increased production of free radicals. Although they are essential for certain physiological processes, excessive amounts can cause oxidative and/or nitrosative stress and cell damage. A molecule associated with these phenomena is nitric oxide (NO), which is a precursor of reactive nitrogen species (RNS). NOS isoforms – NOS1, NOS2, NOS3 – are responsible for the synthesis of this compound.

In the course of diabetes and its complications, we observe increased production of angiotensin II (Ang II), for which ACE is responsible. The treatment of diabetic nephropathy often involves the use of hypotensive drugs, including agents from the ACE inhibitor group (ACEi), which include benazepril, enalapril, lisinopril or ramipril. Therefore, polymorphisms in *ACE* may be associated with the efficacy of this type of therapy.

Objective of the study: The purpose of this dissertation was to evaluate selected polymorphisms: rs4343 and rs4646994 (in *ACE*); rs3782218 (in *NOS1*); rs1137933 (in *NOS2*); rs1799983, rs2070744 and rs61722009 (in *NOS3*) in the context of increased risk of developing diabetic kidney disease and/or increased likelihood of needing to implement renal replacement therapy. It was also important to examine the effects of these polymorphisms on the concentration and activity of ACE, the concentrations of NOS isoforms (NOS1, NOS2, NOS3), as well as the concentrations of selected parameters relevant to the pathogenesis of diabetic nephropathy (glucose, creatinine, CRP, eGFR value) and trace element concentrations (zinc and copper). It was also important to perform an in silico analysis to study the interaction between two ACE domains – the N and C domains – and the ACEi drugs used in the treatment of diabetic kidney disease: benazepril, enalapril, lisinopril and ramipril.

Materials and Methods: To prepare the dissertation, the recent literature on the role of NOS isoforms in the pathogenesis of selected chronic diseases, including diabetes and diabetic

nephropathy, was first reviewed. Works on the significance of polymorphisms of genes encoding these isoforms were also analyzed.

In turn, for the preparation of original papers, a study group of 232 subjects was collected and divided into the following subgroups: patients with diabetic nephropathy (N=85), patients with diabetic nephropathy and after kidney transplantation (N=97), and a control group (N=50). The following parameters were determined in the serum of all subjects: concentration and activity of ACE, concentrations of NOS1, NOS2, NOS3, as well as concentrations of zinc and copper. Concentrations of individual ACE and NOS isoforms were determined using ELISA, ACE activity was determined using a colorimetric assay, and metal concentrations were determined using Flame Atomic Absorption Spectrometry (FAAS). In addition, results of glucose, creatinine and CRP concentrations and eGFR values were obtained from the Department of Nephrology, Transplant Medicine and Internal Medicine at the University Clinical Hospital in Wroclaw. In addition, the effects of seven polymorphisms on the aforementioned parameters were analyzed; these were rs4343 and rs4646994 (in *ACE*); rs3782218 (in *NOS1*); rs1137933 (in *NOS2*); rs1799983, rs2070744 and rs61722009 (in *NOS3*). PCR and PCR-RFLP techniques were used for this purpose. *In silico* analysis was also performed using AutoDock Vina software. The STATISTICA 13.3 package was used to perform the statistical analysis.

Results: The review article revealed the complex role of NOS in the pathogenesis of diseases of many organs and systems. In particular, the correlations between *NOS2* and *NOS3* polymorphisms with the risk of developing insulin resistance, diabetes or diabetic nephropathy were noted. In addition, it was also noted that polymorphisms in *NOS1* were associated with an increased risk of cardiovascular disease, including hypertension or atherosclerosis.

When conducting studies on biological material collected from nephrology patients, it was shown that the rs4343 and rs4646994 polymorphisms in *ACE* were not associated with changes in the concentrations of the enzyme itself. In contrast, a decrease in ACE activity was observed in patients with diabetic nephropathy and the A/A genotype of the rs4343 polymorphism compared to controls. There were also lower levels of zinc in patients with diabetic nephropathy compared to controls. In turn, through the use of logistic regression, it was noted that the G/G genotype and the G allele of the rs4343 polymorphism alone were associated with an increased risk of developing diabetic nephropathy. Similar results were also obtained in patients who underwent kidney transplantation. On the other hand, after performing *in silico* analysis, it was noted that benazepril and lisinopril showed a significant preference in

binding to the C domain, which is relevant in the context of the rs4646994 polymorphism in *ACE*. Similar relationships were not observed for enalapril and ramipril.

The effect of polymorphisms in *NOS1* and *NOS2* (rs3782218 and rs1137933, respectively) on changes in NOS1 and NOS2 concentrations was not demonstrated. However, it was noted that individual genotypes within the *NOS3* polymorphisms (rs1799983, rs2070744 and rs61722009) were associated with differences in NOS3 concentrations between the diabetic nephropathy group and the diabetic kidney transplant group and the control group (higher concentrations of this parameter were observed in the control group). In addition, similar to the study of the *ACE* polymorphisms – lower zinc concentrations were observed in patients with diabetic nephropathy and patients with diabetic nephropathy and kidney transplant patients compared to the control group. This was independent of the division by genotypes within the polymorphisms studied. In addition, it was shown that the C/C genotype and the C allele alone of the rs3782218 polymorphism in *NOS1* were associated with an increased risk of developing diabetic nephropathy and an increased likelihood of needing a kidney transplant. In contrast, the presence of the G allele of the rs1137933 polymorphism in *NOS2* decreased this likelihood.

Conclusions: Genetic changes related to NOS isoforms, but also to ACE, affect the development of diabetes and its complications. Their study, and therefore a better understanding, may contribute to the development of new diagnostic parameters or more effective treatments.

Within the framework of this dissertation, the influence of selected polymorphisms of *NOS1*, *NOS2*, *NOS3* and *ACE* on the risk of developing diabetic nephropathy, as well as on changes in the likelihood of needing renal replacement therapy, was demonstrated. The rs4343 polymorphisms in *ACE*, rs3782218 in *NOS1* and rs1137933 in *NOS2* seem to be particularly interesting and worthy of attention. However, further analyses that include a larger population are needed to confirm the results of these studies. And while the role of *NOS3* polymorphisms in the risk of diabetes complications has not been observed, lower NOS3 levels were observed in patients with these complications.

In addition, these studies underscored the importance of choosing ACEi drugs in the context of the genetic variability present in patients. The existence of a correlation between the efficacy of these preparations and the rs4646994 polymorphism in *ACE* indicates that conducting further research on this topic could contribute to the individualization of therapy, and thus more effective treatment of patients.

IV. WSTĘP

1. Cukrzycowa choroba nerek

Cukrzycowa choroba nerek, zwana dawniej nefropatią cukrzycową, jest jednym z najczęstszych powikłań cukrzycy typu 1 (T1D, ang. *type 1 diabetes*) i 2 (T2D, ang. *type 2 diabetes*) oraz najczęstszą przyczyną schyłkowej niewydolności nerek (ESRD, ang. *end-stage renal disease*) [1,2]. Niestety związana jest ona także ze zwiększoną śmiertelnością pacjentów chorujących na cukrzycę [3]. W ostatnich latach można zaobserwować wzrost częstości występowania cukrzycy na świecie, szczególnie w krajach rozwijających się [4]. W tym wypadku oznacza to, że wraz ze wzrostem częstości występowania tej choroby, przewiduje się także zwiększenie występowania cukrzycowej choroby nerek [5]. Zapobiec temu można poprzez kontynuację badań nad patofizjologią cukrzycowej choroby nerek oraz opracowanie nowych lub dopracowanie obecnych strategii klinicznych.

Cukrzycową chorobę nerek opisuje się jako strukturalne oraz czynnościowe uszkodzenie miąższu nerek wywołane bezpośrednio przez utrzymującą się w przebiegu cukrzycy hiperglikemię [6,7]. Do czynników sprzyjających jej powstawaniu można zaliczyć: nieleczenie lub nieprawidłowe leczenie cukrzycy, długi czas trwania choroby, płeć męską, nadciśnienie tętnicze, wysokie stężenia cholesterolu i triglicerydów, palenie tytoniu, ale także predyspozycje genetyczne [8,9]. Pomimo tego, iż choroba ta została opisana już w 1936 roku [10], to jej patogeneza wciąż uchodzi za złożoną, co utrudnia określenie celów terapeutycznych [11,12]. Rozwój cukrzycowej choroby nerek jest wieloczynnikowy, zależny od wielu ścieżek, mechanizmów i mediatorów [13]. Obejmuje on między innymi zaburzenia metaboliczne, tworzenie zaawansowanych produktów glikacji, tzw. AGEs (ang. *advanced glycation end-products*) oraz reaktywnych form tlenu (ROS, ang. *reactive oxygen species*) i reaktywnych form azotu (RNS, ang. *reactive nitrogen species*), czego efektem będą zjawiska odpowiednio stresu oksydacyjnego i stresu nitrozacyjnego. Za odgrywające ważną rolę w procesie patogenezy cukrzycowej choroby nerek uznaje się także czynniki, takie jak angiotensyna II (Ang II), układ renina-angiotensyna-aldosteron (RAAS, ang. *renin-angiotensin-aldosterone system*) i procesy zapalne [14,15].

Zmiany, do jakich dochodzi w przebiegu cukrzycowej choroby nerek, mają charakter mikroangiopatii, które wynikają z przyłączania glukozy do białek budujących naczynia, błon komórkowych i struktur narządu w procesie glikacji. Tak zmienione białka tracą swoje funkcje, w wyniku czego dochodzi do zmian sztywności naczyń krwionośnych, ale także zmian

strukturalnych obserwowanych w kłębuszkach i cewkach nerkowych oraz śródmiąższu [16]. W przebiegu cukrzycowej choroby nerek następuje upośledzenie procesu filtracji kłębuszkowej, co można zaobserwować poprzez obniżenie wartości wskaźnika filtracji kłębuszkowej (GFR, ang. *glomerular filtration rate*). Występuje także białkomocz [17].

2. Syntazy tlenku azotu

2.1 Podział syntaz tlenku azotu oraz ich rola w organizmie

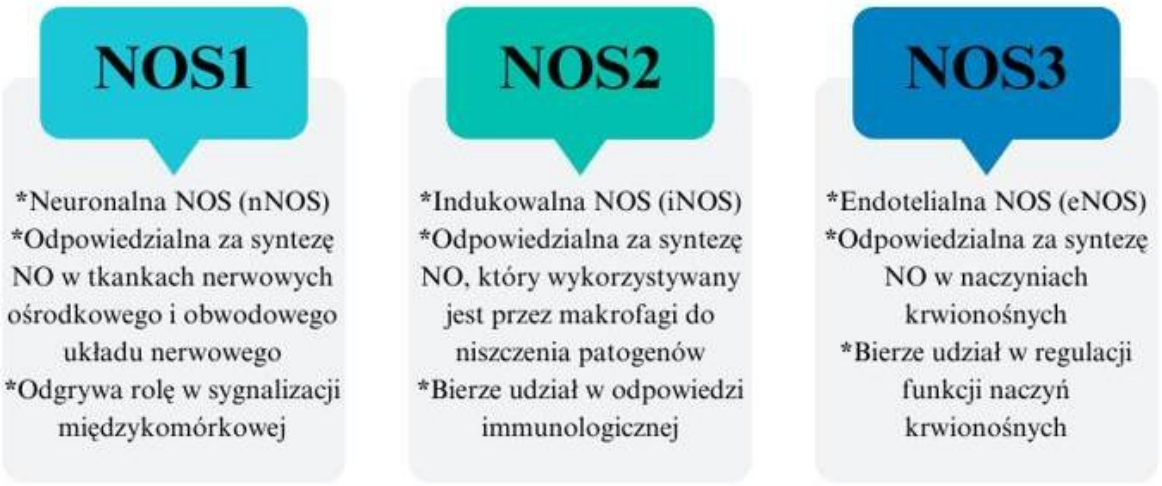
Hiperglikemia przyczynia się do powstawania wolnych rodników i ROS, powodując tym samym stres oksydacyjny [18,19]. Analogicznym zjawiskiem jest stres nitrozacyjny wywołany przez RNS, które są pochodnymi tlenku azotu (NO, ang. *nitric oxide*). RNS charakteryzują się wysoką reaktywnością chemiczną, a ich nadmierna ilość może doprowadzić do uszkodzeń białek, lipidów, a nawet DNA, co może indukować apoptozę [20]. Z kolei silny stres nitrozacyjny powoduje zmniejszenie puli adenozynotrójfosforanu (ATP), co uniemożliwia komórce wejście na ścieżkę kontrolowanej, apoptotycznej śmierci, powodując jej nekrozę [21].

NO powstaje w wyniku reakcji katalizowanej przez syntazę tlenku azotu (NOS, ang. *nitric oxide synthase*). Wyróżnia się trzy izoformy tego enzymu, a każda z nich jest związana z innym miejscem ekspresji oraz działania w organizmie [22]. NO jest syntetyzowany z reszty azotowej aminokwasu L-argininy w obecności fosforanu dinukleotydu nikotynoamidoadeninowego (NADPH) i tlenu cząsteczkowego. NOS wiąże się z dinukleotydem flawinoadeninowym (FAD), mononukleotydem flawinowym (FMN), hemem, tetrahydrobiopteryną (BH_4) i kalmoduliną [23].

Dwie z trzech izoform NOS są zwane konstytutywnymi, a ich aktywność zależy od kompleksu Ca_2^+/kalmodulina. Są to NOS1 – związana z transdukcją sygnału w neuronach ośrodkowych i obwodowych – oraz NOS3 – związana z syntezą NO w naczyniach krwionośnych [23]. NOS1 pełni kluczową rolę dla mózgu oraz obwodowego układu nerwowego, gdzie NO pełni funkcję neuroprzekaźnika [24]. Z kolei NOS3 odpowiada za syntezę NO w śródbłonku naczyniowym, gdzie odpowiada za regulację napięcia naczyń, proliferację komórkową, adhezję leukocytów i agregację płytek krwi [25,26].

Trzecia z izoform NOS – NOS2 – zwana jest indukowalną NOS. Bierze ona udział w odpowiedzi immunologicznej i odpowiada za syntezę NO, który jest istotnym czynnikiem prozapalnym. NOS2 może być wytwarzana w większości jądrzastych komórek organizmu

i odgrywa kluczową rolę w eliminowaniu wewnątrzkomórkowych patogenów, w tym wirusów [27]. Podział i krótki opis każdej z izoform NOS zaprezentowano na Rycinie 1.



Rycina 1. Charakterystyka izoform NOS [28,29].

Wszystkie trzy izoformy katalizują jednak tę samą reakcję. W pierwszym jej etapie enzym katalizuje utlenianie L-argininy, dzięki czemu powstaje związek pośredni – N-hydroksy-L-arginina, która następnie utlenia się do L-cytruliny, w efekcie czego powstaje NO. Sam NO jest również odpowiedzialny za regulację ekspresji i aktywności enzymu, który katalizuje jego syntezę. Poprzez reakcję z resztami aminokwasowymi i tworzenie grupy S-nitrozowej, NO może odwracalnie hamować aktywność NOS [30,31].

2.2 Znaczenie syntaz tlenku azotu i ich polimorfizmów w rozwoju cukrzycowej choroby nerek

Cukrzycowa choroba nerek jest dla współczesnej medycyny wyzwaniem, zarówno z powodu jest powszechności, ale też z powodu jej złożonej patogenezy [32]. W celu zrozumienia tych skomplikowanych zależności, konieczne jest odniesienie się do zaburzeń równowagi pro-/antyoksydacyjnej, jakie towarzyszą wielu chorobom przewlekłym, w tym cukrzycy oraz jej powikłaniom [21,33]. Jak wcześniej zostało wspomniane, stan hiperglikemii powoduje zwiększoną produkcję wolnych rodników, ROS oraz RNS, które są pochodnymi NO. W komórkach β trzustki ekspresjonowane są wszystkie trzy izoformy, a NOS1 występuje w ziarnistościach wydzielniczych insuliny [34]. Dodatkowo NOS2 uczestniczy w deregulacji procesów metabolicznych, co powoduje dysfunkcję śródbłonka poprzez tworzenie się lokalnego środowiska zapalnego [35].

W literaturze można znaleźć przykłady znaczenia oraz wpływu polimorfizmów genów kodujących izoformy NOS na zwiększone ryzyko rozwoju cukrzycy czy też cukrzycowej choroby nerek, chociaż większość badań skoncentrowanych jest na polimorfizmach występujących w *NOS2* oraz *NOS3* [36,37]. Praktycznie brak jest informacji na temat polimorfizmów w genie *NOS1*, co najprawdopodobniej spowodowane jest łączeniem tej izoformy z innymi schorzeniami i przypadłościami [38,39]. W metaanalizie przeprowadzonej przez Dobrijević i wsp. [40] posłużono się wynikami zebranymi z 36 różnych badań. Wykazano, iż polimorfizmy rs2070744 i rs1799983 w genie *NOS3* wiążą się ze zwiększoną podatnością na rozwój cukrzycowej choroby nerek. Istotny okazał się tutaj również czynnik etniczny, co może wskazywać na to, że zachorowalność na tę chorobę związana jest nie tylko z czynnikami środowiskowymi i genetycznymi, ale również może mieć związek z pochodzeniem. W badaniu zauważono także wpływ polimorfizmu rs869109213 w genie *NOS3* na zwiększone ryzyko rozwinięcia się cukrzycowej choroby nerek [40]. Podobne wyniki uzyskano w innej metaanalizie. Polimorfizmy rs2070744, rs869109213 i rs1799983 w genie *NOS3* również wykazywały związek ze zwiększoną zachorowalnością na cukrzycową chorobę nerek [41].

Pomimo szerokiego przebadania polimorfizmów w genie *NOS3,* polimorfizmy w genie *NOS2*, a tym bardziej w genie *NOS1*, zdają się być ciągle otwartym tematem, gdzie w celu poczynienia kompleksowej analizy należałoby przeprowadzić wielkoformatowe badanie. Dalsze prowadzenie badań w tym obszarze jest zatem jak najbardziej uzasadnione i potrzebne.

3. Enzym konwertujący angiotensynę

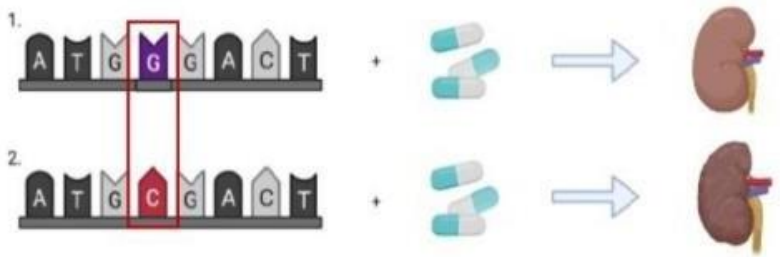
3.1 Rola enzymu konwertującego angiotensynę

Enzym konwertujący angiotensynę, konwertaza angiotensyny (ACE, ang. *angiotensin-converting enzyme*) jest istotnym elementem układu renina-angiotensyna. Odpowiada on za przekształcanie angiotensyny I (Ang I) w Ang II, która odgrywa kluczową rolę w homeostazie ciśnienia krwi poprzez zwężanie naczyń krwionośnych. ACE jest również częścią układu kinina–kalikreina, w którym odpowiada za rozkład bradykininy i innych peptydów wazoaktywnych [42]. Z biochemicznego punktu widzenia ACE jest metaloproteinazą cynkową, dlatego też aktywność tego enzymu może być hamowana przez środki chelatujące metale [43].

3.2 Znaczenie enzymu konwertującego angiotensynę, jego inhibitorów i polimorfizmów w rozwoju cukrzycowej choroby nerek

Inhibitory ACE (ACEi) są szeroko stosowane jako standardowa terapia u pacjentów z cukrzycową chorobą nerek ze względu na działanie ochronne na nerki. Przyjmują je także pacjenci cierpiący na nadciśnienie tętnicze, niewydolność serca oraz cukrzycę typu 2 [44]. Jednak odpowiedź na leczenie ACEi różni się u poszczególnych pacjentów, a przyczynami tego zjawiska mogą być między innymi czynniki genetyczne. Ich rola jest związana przede wszystkim z obecnością polimorfizmów, w tym polimorfizmów pojedynczego nukleotydu (SNP, ang. *single nucleotide polymorphism*), polimorfizmów typu insercja/delecja oraz zmiennej liczby powtórzeń tandemowych (VNTR, ang. *variable number tandem repeat*) [45]. Podstawowy mechanizm działania ACEi polega na hamowaniu konwersji Ang I do Ang II. ACE posiada dwie homologiczne domeny: N i C, które są zdolne do rozszczepiania Ang I i bradykininy [46,47], a domena C jest w tym skuteczniejsza [48]. Polimorfizm rs4646994 w genie *ACE* powoduje przedwczesną terminację, w efekcie czego enzym ma tylko jedno miejsce aktywne w domenie N, ograniczając w ten sposób wiązanie leku do jednego miejsca [45]. Ten fakt został też zauważony u pacjentów chorujących na COVID-19, u których enzymy proteolityczne mogą selektywnie wpływać na domeny ACE, co prowadzi do zmiennej aktywności enzymu w zależności od genotypu w obrębie polimorfizmu rs4646994 [49].

Wtórny mechanizm działania ACEi jest związany ze zwiększoną produkcją Ang I, w efekcie czego dochodzi do wzmożonego rozszerzania się naczyń krwionośnych, co czyni leki z tej grupy skutecznymi środkami terapeutycznymi w leczeniu nadciśnienia tętniczego. Proces ten uwarunkowany jest działaniem NO [50]. Silva i wsp. [51] zaobserwowali, że pacjenci z allelem C polimorfizmu rs2070744 w genie *NOS3* wykazywali lepszą odpowiedź hipotensyjną na enalapryl w porównaniu do pacjentów z allelem T. Na Rycinie 2 przedstawiono graficzny przykład skuteczności terapii w zależności od wariantu genetycznego.



Rycina 2. Schematyczne przedstawienie zróżnicowanej odpowiedzi na ten sam rodzaj terapii w zależności od wariantu genetycznego.

Legenda: 1 – pacjent z allelem G prawidłowo reaguje na terapię; 2 – pacjent z allelem C nie reaguje na terapię, co prowadzi do schyłkowej niewydolności nerek.

Ponadto istnieją także przesłanki mówiące o wpływie polimorfizmów w genie *ACE* na zwiększone prawdopodobieństwo rozwoju i progresję cukrzycowej choroby nerek [52,53]. Ismail i wsp. [54] zaobserwowali, że częstość występowania genotypu DD polimorfizmu rs4646994 w genie *ACE* oraz allelu D była istotnie wyższa u pacjentów z cukrzycową chorobą nerek w porównaniu do pacjentów z cukrzycą, ale bez powikłań.

V. CEL I ZAŁOŻENIA PRACY

Głównym celem niniejszej pracy była ocena wpływu wybranych polimorfizmów w genach *ACE* oraz *NOS1, NOS2, NOS3* na ryzyko wystąpienia cukrzycowej choroby nerek lub konieczności wprowadzenia terapii nerkozastępczej. Celami szczegółowymi były:

a) Ocena związku pomiędzy występowaniem określonego genotypu w badanych polimorfizmach – rs4343 i rs4646994 (*ACE*); rs3782218 (*NOS1*); rs1137933 (*NOS2*); rs1799983, rs2070744 i rs61722009 (*NOS3*) – a rozwojem cukrzycowej choroby nerek oraz koniecznością wprowadzenia terapii nerkozastępczej.

b) Ocena wpływu badanych polimorfizmów na stężenie i aktywność ACE, a także na stężenia NOS1, NOS2 i NOS3, a tym samym sprawdzenie, czy procesy, w których biorą udział te enzymy, zależą od czynników genetycznych.

c) Ocena wpływu badanych polimorfizmów na stężenia parametrów oznaczanych rutynowo u pacjentów chorujących na cukrzycową chorobę nerek (glukoza, kreatynina, eGFR, CRP).

d) Ocena wpływu badanych polimorfizmów na potencjalne zaburzenia metabolizmu pierwiastków śladowych, takich jak cynk i miedź, które mogą wystąpić podczas rozwoju powikłań cukrzycowych.

e) Zbadanie i porównanie interakcji między dwiema domenami ACE (domeną N i domeną C) a wybranymi inhibitorami ACE (benazeprylem, enalaprylem, lizynoprylem, ramiprylem), a także ukazanie wpływu wybranych polimorfizmów *ACE* na skuteczność terapii ACEi.

VI. MATERIAŁY I METODY

1. Materiał badany i charakterystyka pacjentów

I i II artykuł oryginalny

Materiał badany pochodził od pacjentów ze zdiagnozowaną cukrzycową chorobą nerek będących pod opieką Katedry i Kliniki Nefrologii, Medycyny Transplantacyjnej i Chorób Wewnętrznych Uniwersytetu Medycznego im. Piastów Śląskich we Wrocławiu, a za ich kwalifikację do badań odpowiadał prof. dr hab. Mirosław Banasik. Dobór pacjentów został dokonany na podstawie wywiadu lekarskiego, przeprowadzonych badań laboratoryjnych oraz badań obrazowych (np. USG). Ocenie poddano następujące parametry: stężenie kreatyniny oraz estymowany wskaźnik filtracji kłębuszkowej (eGFR, ang. *estimated glomerular filtration rate*) – obliczony według skróconego wzoru MDRD (ang. *Modification of Diet in Renal Disease*), stężenie glukozy, stężenie sodu i potasu, morfologię krwi obwodowej, badanie ogólne moczu oraz albuminurię. Kryteriami włączającymi do badań były: zdiagnozowana cukrzyca, a także stwierdzona albuminuria, białkomocz lub podwyższone stężenie kreatyniny. Na podstawie przeprowadzonych badań obrazowych wykluczono pacjentów z innymi uszkodzeniami nerek. Dodatkowo każdy uczestnik badań wypełnił ankietę zawierającą informacje o danych antropometrycznych (wzrost, masa ciała), wieku, płci, występowaniu innych chorób przewlekłych, stosowanych używkach (palenie tytoniu, spożywanie alkoholu) oraz przyjmowanych lekach.

Grupę badaną stanowiło 232 osób, które zostały podzielone na trzy podgrupy: grupę pacjentów ze zdiagnozowaną cukrzycową chorobą nerek (N=85), grupę pacjentów ze zdiagnozowaną cukrzycową chorobą nerek i będących po przeszczepie nerki wynikającym z tej choroby (N=97) oraz grupę kontrolną (N=50). Wszyscy uczestnicy zostali poinformowani o celach badań i wyrazili pisemną zgodę na pobranie materiału biologicznego. Projekt został też pozytywnie zaopiniowany przez Komisję Bioetyczną przy Uniwersytecie Medycznym we Wrocławiu, uzyskując zgodę na prowadzenie badań pod warunkiem zachowania anonimowości uzyskanych danych (nr KB 835/2021).

Pacjentom przebywającym pod opieką Katedry i Kliniki Nefrologii, Medycyny Transplantacyjnej i Chorób Wewnętrznych pobrano próbki krwi żylnej do dwóch próbówek: jednej z aktywatorem krzepnięcia oraz drugiej zawierającej kwas wersenowy (EDTA, ang. *ethylenediaminetetraacetic acid*). Po pobraniu probówki zostały przetransportowane do Katedry i Zakładu Biochemii Farmaceutycznej, gdzie z probówki z EDTA pobrano 500 µL

krwi pełnej i rozpipetowano do probówek typu Eppendorf (w celu oznaczenia stężeń cynku i miedzi). Następnie probówki z aktywatorem krzepnięcia oraz probówki z EDTA poddano wirowaniu w warunkach 1 500 x g przez 15 minut w temperaturze 4°C w celu uzyskania surowicy oraz osocza. W celu uniknięcia wielokrotnych cykli rozmrażania i zamrażania materiału biologicznego surowicę oraz osocze poporcjowano. Dodatkowo została pobrana warstwa kożuszka leukocytarno-płytkowego z probówki zawierającej EDTA (w celu późniejszej izolacji DNA). Kożuszek leukocytarno-płytkowy został przeniesiony do probówki typu Eppendorf, zawieszony i przemyty w PBS, a następnie odwirowany w warunkach 16 000 x g przez 3 minuty w celu usunięcia PBS. Materiał biologiczny był przechowywany w temperaturze -80°C do czasu jego wykorzystania.

Z kolei materiał biologiczny oraz niezbędne informacje o osobach stanowiących grupę kontrolną zostały uzyskane dzięki współpracy z Biobankiem Siei Badawczej Łukasiewicz – PORT Polskiego Ośrodka Rozwoju Technologii. Wszystkie próbki materiału biologicznego (krew pełna, surowica, wyizolowane DNA) były przechowywane w temperaturze -80°C do czasu ich wykorzystania.

2. Metody

Artykuł przeglądowy

W celu sporządzenia tego artykułu dokonano kompleksowego przeglądu i systematycznej analizy literatury opublikowanej do listopada 2020 roku z wykorzystaniem wyszukiwarki PubMed oraz przeglądarki Google Scholar. Podczas przeglądania aktualnej literatury stosowano następujące słowa kluczowe: „NOS1", „NOS2" i „NOS3", „*nitric oxide synthases*" i „*polymorphism of nitric oxide synthases*", a także następujące kombinacje słów: „*nitric oxide synthases* AND *inflammation*", „*nitric oxide synthases* AND *diabetes*" i „*nitric oxide synthases* AND *cardiovascular diseases*".

I i II artykuł oryginalny

Oznaczenie aktywności ACE oraz stężeń ACE, glukozy, kreatyniny i CRP

W ramach badań opisanych w **I artykule oryginalnym** wykonano pomiar aktywności ACE w surowicy krwi za pomocą zestawu ACE1 Activity Assay Kit (Colorimetric) (nr kat.: ab273308, Abcam, Cambridge, Wielka Brytania). Zmierzono także stężenie ACE w surowicy krwi za pomocą zestawu Human ACE (Angiotensin I Converting Enzyme) ELISA Kit (nr kat.: EH0026, Fine Biotech Co., Ltd., Wuhan, Chiny). Z kolei pomiar stężeń glukozy, kreatyniny

i białka C-reaktywnego (CRP, ang. *C-reactive protein*) został wykonany w laboratorium Uniwersyteckiego Szpitala Klinicznego im. Jana Mikulicza-Radeckiego we Wrocławiu. Wartości eGFR zostały wyliczone z wykorzystaniem skróconego wzoru MDRD.

Oznaczenie stężeń NOS1, NOS2, NOS3, glukozy, kreatyniny, eGFR i CRP

W ramach badań opisanych w **II artykule oryginalnym** wykonano pomiar stężenia NOS1 w surowicy z wykorzystaniem zestawu Human Nitric Oxide Synthase, Brain, NOS1 ELISA Kit (nr kat.: E0924Hu, Bioassay Technology Laboratory, Szanghaj, Chiny). Stężenie NOS2 w surowicy zmierzono z wykorzystaniem zestawu Human Nitric Oxide Synthase, Inducible, INOS-NOS2 ELISA Kit (nr kat.: E4710Hu, Bioassay Technology Laboratory, Szanghaj, Chiny). Z kolei stężenie NOS3 w surowicy zmierzono za pomocą zestawu Human Endothelial Nitric Oxide Synthase (eNOS) ELISA Kit (nr kat.: MBS265088, MyBioSource, Inc., San Diego, Kalifornia, USA). Natomiast pomiar stężeń pozostałych parametrów (glukozy, kreatyniny i CRP) został przeprowadzony podobnie jak w przypadku **I artykułu oryginalnego** – w laboratorium Uniwersyteckiego Szpitala Klinicznego im. Jana Mikulicza-Radeckiego we Wrocławiu. Wartości eGFR zostały wyliczone z wykorzystaniem skróconego wzoru MDRD.

Oznaczenie stężeń cynku i miedzi

W ramach badań stanowiących część **I i II artykułu oryginalnego** 500 µL krwi pełnej zostało przekazane do Pracowni Spektrometrii Absorpcji Atomowej, Katedry i Kliniki Chorób Wewnętrznych, Zawodowych, Nadciśnienia Tętniczego i Onkologii Klinicznej Uniwersytetu Medycznego im. Piastów Śląskich we Wrocławiu. Tam zostały oznaczone stężenia cynku i miedzi w surowicy krwi z wykorzystaniem spektrofotometru absorpcji atomowej SOLAAR M6 (Thermo Elemental Solaar House, Cambridge, Wielka Brytania) w. Do tego celu użyto metody atomowej spektrometrii absorpcyjnej z atomizacją w płomieniu (FAAS, ang. *Flame Atomic Absorption Spectrometry*). Dokładność i powtarzalność metody zweryfikowano na podstawie pomiaru stężeń metali w próbkach kontrolnych.

Analiza polimorfizmów ACE (rs4343 i rs4646994)

DNA wyizolowano z kożuszka leukocytarno-płytkowego z wykorzystaniem gotowego zestawu Syngen Blood/Cell DNA Mini Kit (nr kat.: SY221012, Syngen Biotech, Wrocław, Polska). Następnie dokonano pomiaru czystości wyizolowanego DNA, odczytując wartość jego absorbancji przy λ=260 nm i λ=280 nm.

W celu analizy polimorfizmu rs4343 wykorzystano technikę łańcuchowej reakcji polimerazy – polimorfizmu długości fragmentów restrykcyjnych (PCR-RFLP, ang. *polymerase chain reaction – restriction fragment length polymorphism*). Z kolei polimorfizm insercyjno-delecyjny – rs4646994 – oznaczono techniką PCR. Startery zostały zaprojektowane z wykorzystaniem programu Primer-BLAST w oparciu o sekwencje genów pochodzące z GenBank (*National Center for Biotechnology Information*). Sekwencje starterów, warunki reakcji oraz enzym restrykcyjny wykorzystany w tym badaniu przedstawiono w Tabeli 1.

Tabela 1. Warunki przeprowadzonych reakcji (PCR-RFLP, PCR oraz trawienia enzymem restrykcyjnym) dla polimorfizmów rs4343 i rs4646994 w genie *ACE* [55].

SNP (*Gen*)	**Sekwencje starterów**	**Warunki reakcji**
rs4343 (*ACE*)	Forward: 5′ CTG ACG AAT GTG ATG GCC GC 3′ Reverse: 5′ TGA GTT CCA CGT ATT TCG 3′	wstępna denaturacja: 95°C przez 5 min denaturacja: 95°C przez 40 s hybrydyzacja: 58,4°C przez 35 s elongacja: 72°C przez 40 s końcowa elogacja: 72°C przez 10 min
	Enzym restrykcyjny	**Warunki trawienia enzymem restrykcyjnym**
	BstUI	37°C przez 16 godz.
rs4646994 (*ACE*)	Forward: 5′ CTG GAG ACC ACT CCC ATC CTT TCT 3′ Reverse: 5′ GAT GTG GCC ATC ACA TTC GTC AGA T 3′	wstępna denaturacja: 95°C przez 5 min denaturacja: 95°C przez 40 s hybrydyzacja: 60°C przez 35 s elongacja: 72°C przez 40 s końcowa elongacja: 72°C przez 10 min

Fragmenty DNA po przeprowadzeniu restrykcji właściwymi enzymami uwidoczniono na 2% żelu agarozowym (nr kat.: SY 521011, Syngen Biotech, Wrocław, Polska) z dodatkiem Green DNA Gel Stain (nr kat.: SY 521031, Syngen Biotech, Wrocław, Polska).

Analiza polimorfizmów NOS1 (rs3782218), NOS2 (rs1137933) i NOS3 (rs1799983, rs2070744, rs61722009)

W celu analizy wybranych polimorfizmów użyto próbek wyizolowanego DNA z kożuszka leukocytarno-płytkowego. Dane dotyczące izolacji zostały opisane w sekcji odnoszącej się do **I artykułu oryginalnego**.

Cztery z pięciu badanych polimorfizmów (rs3782218, rs1137933, rs1799983, rs2070744) oceniono z wykorzystaniem techniki PCR-RFLP. Z kolei polimorfizm typu VNTR – rs61722009 – oceniano z wykorzystaniem techniki PCR. Startery zostały zaprojektowane

przy użyciu programu Primer-BLAST w oparciu o sekwencje genów pobrane z GenBank (*National Center for Biotechnology Information*).

Tabela 2. Warunki przeprowadzonych reakcji (PCR-RFLP, PCR oraz trawienia enzymami restrykcyjnymi) dla polimorfizmów rs3782218 w genie *NOS1*, rs1137933 w genie *NOS2*, a także rs1799983, rs2070744 i rs61722009 w genie *NOS3* [60].

SNP (*Gen*)	**Sekwencje starterów**	**Warunki reakcji**
rs3782218 (*NOS1*)	Forward: 5’ CTG AGA GCA GAA GGT GGG TG 3’ Reverse: 5’ GTC CTG GAT GGG TTT CCC TG 3’	wstępna denaturacja: 95°C przez 15 min denaturacja: 95°C przez 40 s hybrydyzacja: 62,0°C przez 35 s elongacja: 72°C przez 40 s końcowa elongacja: 72°C przez 10 min
	Enzym restrykcyjny	**Warunki trawienia enzymem restrykcyjnym**
	Hpy99I	37°C przez 1 godz.
rs1137933 (*NOS2*)	**Sekwencje starterów**	**Warunki reakcji**
	Forward: 5’ CTC ACC AAA AAG TCT TCA GAC TCA CA 3’ Reverse: 5’ GGC CCC AGT TAA ATT GTG TCT ACC 3’	wstępna denaturacja: 95°C przez 15 min denaturacja: 95°C przez 40 s hybrydyzacja: 59,0°C przez 35 s elongacja: 72°C przez 40 s końcowa elongacja: 72°C przez 10 min
	Enzym restrykcyjny	**Warunki trawienia enzymem restrykcyjnym**
	Hin1I	37°C przez 16 godz.
rs1799983 (*NOS3*)	**Sekwencje starterów**	**Warunki reakcji**
	Forward: 5’ GAC CCT GGA GAT GAA GGC AG 3’ Reverse: 5’ CAT CCC ACC CAG TCA ATC CC 3’	wstępna denaturacja: 95°C przez 5 min denaturacja: 95°C przez 40 s hybrydyzacja: 60,4°C przez 35 s elongacja: 72°C przez 40 s końcowa elongacja: 72°C przez 10 min
	Enzym restrykcyjny	**Warunki trawienia enzymem restrykcyjnym**
	MboI	37°C przez 16 godz.
rs2070744 (*NOS3*)	**Sekwencje starterów**	**Warunki reakcji**
	Forward: 5’ CTA GTG GCC TTT CTC CAG CC 3’ Reverse: 5’ GCC CAG CAA GGA TGT AGT GA 3’	wstępna denaturacja: 95°C przez 15 min denaturacja: 95°C przez 40 s hybrydyzacja: 62,0°C przez 35 s elongacja: 72°C przez 1 min końcowa elongacja: 72°C przez 10 min
	Enzym restrykcyjny	**Warunki trawienia enzymem restrykcyjnym**
	MspI	37°C przez 16 godz.
rs61722009 (*NOS3*)	**Sekwencje starterów**	**Warunki reakcji**
	Forward: 5’ CTA TGG TAG TGC CTT GGC TGG AG 3’ Reverse: 5’ GTC ACA GGC GTT CCA GTA ACT AAG 3’	wstępna denaturacja: 95°C przez 15 min denaturacja: 95°C przez 20 s hybrydyzacja: 58,0°C przez 35 s elongacja: 72°C przez 40 s

końcowa elongacja: 72°C przez 10 min

Uzyskane po restrykcji fragmenty DNA uwidoczniono na 2% lub 3% żelu agarozowym (nr kat.: SY 521011, Syngen Biotech, Wrocław, Polska) z dodatkiem Green DNA Gel Stain (nr kat.: SY 521031, Syngen Biotech, Wrocław, Polska).

Dokowanie molekularne

W ramach badań będących częścią **I artykułu oryginalnego** przeprowadzono analizy *in silico* interakcji między dwiema domenami ACE (domeną N i domeną C) a wybranymi inhibitorami ACE (benazeprylem, enalaprylem, ramiprylem, lizynoprylem). Wyboru leków dokonano na podstawie wywiadu z pacjentami włączonymi do badania. Substancje te występowały w lekach stosowanych przez pacjentów.

W celu wykonania obliczeń dokowania trójwymiarowego (3D) pobrano struktury domeny N i C z bazy Protein Data Bank (PDB). Natomiast trójwymiarowe struktury ligandów (wybranych inhibitorów ACE) pobrano z otwartej bazy PubChem. Przed samą procedurą dokowania modele 3D zostały przygotowane ręcznie z wykorzystaniem oprogramowania UCSF Chimera (wersja 1.15) [56]. Z kolei dokowanie molekularne przeprowadzono za pomocą oprogramowania AutoDock Vina (wersja 1.1.2) [57,58], które zostało także wykorzystane do obliczenia przewidywanego powinowactwa wiązania [kcal/mol]. W celu wizualizacji wyników użyto trzech programów: UCSF Chimera, BIOVIA Discovery Studio Visualizer (wersja 21.1.0.20298) [59] oraz PyMOL (wersja 2.5.2).

Analiza statystyczna

Uzyskane wyniki poddano analizie statystycznej, którą przeprowadzono przy użyciu pakietu SATISTICA 13.3 (Statsoft Polska, Sp. z o. o., Kraków, Polska) z wykorzystaniem licencji Uniwersytetu Medycznego im. Piastów Śląskich we Wrocławiu. Za poziom istotności statystycznej przyjęto $p<0,05$.

Normalność zmiennych zbadano za pomocą testu Shapiro-Wilka, z kolei jednorodność wariancji zbadano z wykorzystaniem testu Levene'a. Jeżeli wartości zmiennych spełniały warunki rozkładu normalnego, to w celu porównania dwóch grup stosowano parametryczny test t-Studenta. W przeciwnym wypadku zastosowano nieparametryczny test U Manna-Whitneya. Z kolei w celu porównania trzech lub więcej grup zastosowano nieparametryczny test Kruskala-Wallisa, ponieważ wszystkie wartości zmiennych nie spełniały warunków rozkładu normalnego.

Częstotliwość genotypów porównano za pomocą testu χ^2 oraz dokładnego testu Fishera. W celu oceny wpływu poszczególnych genotypów na ryzyko wystąpienia cukrzycowej choroby nerek oraz na prawdopodobieństwo konieczności zastosowania terapii nerkozastępczej przeprowadzono także analizę regresji logistycznej.

VII. OMÓWIENIE WYNIKÓW I DYSKUSJA

NO odgrywa istotną rolę w wielu procesach fizjologicznych, jednak procesy prowadzące do zmienionych stężeń lub aktywności NOS mogą przyczyniać się do rozwoju wielu chorób. W komórkach β trzustki są ekspresjonowane wszystkie trzy izoformy. NOS1 można znaleźć głównie w ziarnistościach wydzielniczych insuliny oraz w mitochondriach i jądrze komórkowym [34]. Z kolei ekspresja NOS2 pojawia się dopiero po ekspozycji komórek na wyższe stężenia glukozy [61,62]. Wiadomo jednak, że w stanach insulinooporności NOS2 uczestniczy w rozwoju stanu zapalnego, co prowadzi do zaburzeń homeostazy [35]. NOS3 występuje również w komórkach trzustki, ale wciąż jest zbyt mało danych na temat jej funkcji [63]. Ponadto sugeruje się, że zmiany związane z NO i NOS mogą odgrywać ważną rolę nie tylko w rozwoju insulinooporności, ale też T2D oraz jej powikłań w postaci cukrzycowej choroby nerek [64,65].

Przykładowo polimorfizm rs61722009 w genie *NOS3*, spotykany też często pod nazwą 27-bp VNTR, wielokrotnie był wiązany ze zwiększonym ryzykiem rozwoju cukrzycowej choroby nerek [66]. Jednakże już w innym badaniu – skupiającym się na analizie trzech polimorfizmów w genie *NOS3*: rs1799983, rs2070744 i rs61722009 – zauważono nieco inne zależności [67]. Co prawda allel T (polimorfizm rs1799983) i allel C (polimorfizm rs2070744) występowały częściej u pacjentów z cukrzycową chorobą nerek w porównaniu do pacjentów bez tej choroby, jednakże w przypadku alleli polimorfizmu rs61722009 nie poczyniono już takich obserwacji. Nie stwierdzono też istotnych zmian w stężeniach NO między poszczególnymi grupami pacjentów wydzielonymi ze względu na genotypy [67]. Mimo wszystko uzyskane wyniki implikują, iż polimorfizmy NOS3 mogą stanowić genetyczne determinanty w rozwoju cukrzycowej choroby nerek u pacjentów z T2D.

Polimorfizmy w genach kodujących izoformy NOS nie są związane jednak wyłącznie z występowaniem cukrzycowej choroby nerek czy też T2D. Choć badania nad wpływem polimorfizmów *NOS* na rozwój otyłości pozostają niespójne, to wykazano wpływ polimorfizmu rs2070744 w genie *NOS3* na rozwój zespołu metabolicznego (MetS, ang. *metabolic syndrome*) u otyłych dzieci i młodzieży [68]. Natomiast według badań przeprowadzonych przez Teixeirę i wsp. [69] nosiciele allelu T polimorfizmu rs1799983 w genie *NOS3* mogą być bardziej narażeni na ryzyko chorób sercowo-naczyniowych oraz zaburzenia metaboliczne.

Wykazano również wysoką ekspresję białka NOS2 w wysepkach trzustkowych u pacjentów z T2D [70]. Ciekawa wydaje się być też obserwacja, iż to NO syntetyzowany przy udziale NOS2 najczęściej powoduje dysfunkcję komórek β, upośledzone wydzielanie insuliny,

hiperglikemię i rozwój cukrzycy [71]. Co więcej, zmiany w stężeniach i aktywnościach poszczególnych izoform NOS, ale też polimorfizmy *NOS*, są wiązane z chorobami układu sercowo-naczyniowego, takimi jak nadciśnienie tętnicze, miażdżyca czy choroba wieńcowa [72-74].

W **artykule przeglądowym** podsumowano stwierdzeniem, że zmiany genetyczne związane z izoformami NOS mogą wpływać na kilka narządów lub układów, stanowią zatem ciekawy obszar badań z punktu widzenia współczesnej medycyny. Pozyskane w ramach nich wyniki mogą przyczynić się do lepszego zrozumienia zaburzeń molekularnych występujących w określonych jednostkach chorobowych, co w efekcie może usprawnić ich diagnostykę i/lub przyczynić się do polepszenia terapii.

W ramach **I artykułu oryginalnego** ocenie zostały poddane dwa polimorfizmy w genie *ACE* – rs4343 i rs4646994 – oraz ich znaczenie w rozwoju cukrzycowej choroby nerek i zwiększonego prawdopodobieństwa zastosowania terapii nerkozastępczej. Spoiwem łączącym wszystkie artykuły została jednostka chorobowa – cukrzycowa choroba nerek – a tym samym zebrana grupa badana, spośród której część pacjentów była poddana terapii środkami z grupy ACEi. Została również przeprowadzona analiza *in silico* w celu porównania wiązania leków z grupy ACEi z dwiema domenami ACE – domeną N i domeną C.

Wykazano, że polimorfizmy rs4343 i rs4646994 nie miały wpływu na stężenie ACE. Zauważono natomiast niższą aktywność tego enzymu u pacjentów z cukrzycową chorobą nerek i genotypem A/A polimorfizmu rs4343 w porównaniu do grupy kontrolnej. Jednakże pacjenci z grupy kontrolnej mieli zazwyczaj wyższe stężenie ACE. Niska aktywność ACE u chorych mogła być wynikiem kompensacyjnej adaptacji, która miała na celu zmniejszenie produkcji dodatkowej Ang II [75,76].

W badaniu tym analizowano także zależność łączącą stężenia cynku oraz miedzi z poszczególnymi grupami badanymi. O ile w przypadku miedzi nie zaobserwowano różnic istotnych statystycznie, to stężenia cynku miały związek z występowaniem cukrzycowej choroby nerek. Wykazano niskie stężenie tego pierwiastka u pacjentów z cukrzycową chorobą nerek w porównaniu z osobami z grupy kontrolnej. W literaturze obserwuje się zależność między niską podażą cynku w diecie oraz jego niskimi stężeniami w surowicy krwi a zwiększoną zapadalnością na cukrzycę czy choroby układu sercowo-naczyniowego. Wysoce prawdopodobne jest to, że zarówno choroba może wpływać na metabolizm cynku, jak i to, że jego niskie stężenia skutkują zaburzeniami gospodarki węglowodanowej [77,78]. W dodatku niedobór cynku może być wynikiem stanu zapalnego lub być związany ze stresem oksydacyjnym, a obydwa te zjawiska towarzyszą wielu chorobom przewlekłym, w tym

cukrzycy i jej powikłaniom [77]. Jednak w przypadku pacjentów po przeszczepie nerki zaobserwowano wyższe stężenia cynku w porównaniu z grupą kontrolną. Z kolei w przypadku innych badań osoby po przeszczepie miały zazwyczaj niższe stężenia tego pierwiastka [79,80]. Z kolei po rozkładzie grup ze względu na poszczególne genotypy zaobserwowano sytuację odwrotną – pacjenci po przeszczepie z genotypami A/A polimorfizmu rs4343 i D/D polimorfizmu rs4646994 mieli niższe stężenia cynku w porównaniu z odpowiadającymi im osobami w grupach kontrolnych.

Z kolei na podstawie wyników regresji logistycznej można było zauważyć, iż polimorfizm rs4343 może służyć jako przydatne narzędzie diagnostyczne. Genotypy G/G i G/A oraz sam allel G były związane ze zwiększonym ryzykiem rozwoju cukrzycowej choroby nerek lub zwiększonym prawdopodobieństwem konieczności leczenia nerkozastępczego. Wyniki te zdają się być potwierdzane przez inne badania, które również wskazują na znaczenie allelu G w patogenezie powikłań cukrzycowych [81,82]. Wykazano także wpływ wzrostu BMI oraz wieku na zwiększone ryzyko cukrzycowej choroby nerek, co również dobrze koresponduje z obecnym stanem wiedzy, zgodnie z którym otyłość jest jednym z czynników rozwoju cukrzycy i przyczynia się do rozwoju jej powikłań [83,84]. Nie zaobserwowano podobnych zależności w przypadku drugiego badanego polimorfizmu (rs4646994). Jednakże w celu potwierdzenia tych wyników należałoby przeprowadzić badania kohortowe z udziałem większej populacji.

Jednak najciekawszymi wynikami wydają się być te uzyskane w ramach analizy *in silico*. Należy zaznaczyć, że co prawda podobne badania były już prowadzone, jednakże głównie z wykorzystaniem kaptoprylu oraz lizynoprylu jako ligandów [85,86]. Uwzględniono wszystkie cztery leki z grupy ACEi, które są zarejestrowane w Polsce jako mogące być stosowane w leczeniu cukrzycowej choroby nerek: benazepryl, enalapryl, lizynopryl i ramipryl [87]. Należy zaznaczyć, iż ACE posiada dwa miejsca aktywne – jedno w domenie N i jedno w domenie C [46-48]. Jednakże allel I polimorfizmu rs4646994 powoduje przedwczesną terminację kodonu, w wyniku czego enzym posiada tylko jedno miejsce aktywne w domenie N [45]. Oznacza to, że potencjalne leki są w stanie przyłączyć się tylko do jednego miejsca aktywnego, co może wpływać na ich skuteczność. Ponadto w wielu pracach wykazano, że stosowane leki wiążą się z poszczególnymi domenami, wykazując różne powinowactwo wiązania [45-48]. W niniejszej analizie *in silico* zaobserwowano, że benazepryl i lizynopryl wykazują znaczną preferencję do domeny C, chociaż obliczona wartość dla domeny N wciąż była istotna. Niemniej jednak enalapryl i ramipryl mogą wiązać się z bardzo podobną skutecznością z obiema domenami, dlatego polimorfizm rs4646994 nie powinien mieć

istotnego wpływu na ich działanie w leczeniu cukrzycowej choroby nerek. Może to wskazywać na to, iż w doborze leków z grupy ACEi należałoby kierować się również kryterium genetycznym, a wszystko po to, aby możliwie zmaksymalizować skuteczność stosowanej terapii.

Głównym zamierzeniem **II artykułu oryginalnego** była ocena wybranych polimorfizmów wszystkich trzech izoform NOS – które zostały szeroko opisane w **artykule przeglądowym** – na grupie pacjentów analizowanych również w ramach **I artykułu oryginalnego**. Dotychczasowe badanie uwzględniały bowiem zazwyczaj polimorfizmy tylko jednej izoformy NOS, a kompleksowe podsumowanie roli polimorfizmów *NOS* w patofizjologii powikłań cukrzycowych może przyczynić się do ich lepszego poznania, a tym samym opracowania szybszej metody ich diagnozowania i/lub skuteczniejszego leczenia.

Biorąc pod uwagę zależność między badanymi polimorfizmami a stężeniami poszczególnych izoform, polimorfizmy genów *NOS1* i *NOS2* (odpowiednio rs3782218 i rs1137933) nie wykazały wpływu na stężenia NOS1 i NOS2. Zauważono jednak, że polimorfizmy w genie *NOS3* (rs1799983, rs2070744 i rs61722009) były związane z różnicami w stężeniach NOS3 pomiędzy badanymi grupami. Mogło to być jednak wynikiem tego, że badani z grupy kontrolnej nawet przed podziałem ze względu na genotypy wykazywali wyższe stężenia NOS3 w porównaniu do pozostałych dwóch grup. Rzeczywiście zostało to potwierdzone innymi badaniami, iż polimorfizmy *NOS3* mogą wpływać na stężenie NO, a w dodatku w przypadku cukrzycowej choroby nerek często obserwuje się zmienione stężenia tego parametru [40,88,89].

Podobnie jak w przypadku **I artykułu oryginalnego** zaobserwowano zależność między stężeniami cynku a badanymi polimorfizmami. Jednakże podobnie jak w przypadku stężeń NOS3 – jest to najprawdopodobniej wynik trwającej choroby, a nie wpływ czynników genetycznych. Za każdym razem bowiem różnice te dotyczyły niższych stężeń badanego pierwiastka w grupach pacjentów z cukrzycową chorobą nerek lub pacjentów po przeszczepie nerki w porównaniu z grupą kontrolną. Podobnie jak w przypadku **I artykułu oryginalnego** niedobór cynku jest w literaturze często kojarzony z takimi chorobami jak cukrzyca czy jej powikłania [77-79].

Wykazano także wyższe stężenia glukozy w grupie pacjentów z cukrzycową chorobą nerek oraz w grupie pacjentów po przeszczepie nerki w porównaniu z grupą kontrolną, co było niezależne od analizowanych genotypów. Jednakże należy pamiętać, iż są to pacjenci chorujący na powikłania po cukrzycy, często z długoletnią chorobą, u których stężenie glukozy może być ciężkie do wyrównania mimo przyjmowanych leków [90].

Opierając się na wynikach regresji logistycznej, zauważono, iż polimorfizm rs3782218 w genie *NOS1* może być potencjalnie wykorzystany jako użyteczne narzędzie do przewidywania ryzyka rozwoju cukrzycowej choroby nerek. Wyniki wskazywały, że obecność genotypu C/C jest powiązaną z ponad dziesięciokrotnie większym ryzykiem rozwoju cukrzycowej choroby nerek. Z kolei allel C polimorfizmu rs3782218 w genie *NOS1* był związany ze zwiększonym prawdopodobieństwem konieczności zastosowania terapii nerkozastępczej, natomiast allel G polimorfizmu rs1137933 w genie *NOS2* wiązałby się ze zmniejszonym ryzykiem zastosowania tego rodzaju terapii. Niestety w literaturze brak jest podobnych analiz, co może wiązać się z faktem, iż większość badań o podobnej tematyce skupia się na polimorfizmach w genie *NOS3* [91,92]. Ponadto wykazano, że wiek jest istotnym czynnikiem, który wpływa na zwiększone ryzyko rozwoju cukrzycowej choroby nerek zwiększone prawdopodobieństwo leczenia nerkozastępczego, co znajduje potwierdzenie w innych badaniach [93,94].

VIII. PODSUMOWANIE I WNIOSKI

Cukrzycowa choroba nerek jest istotnym powikłaniem cukrzycy, które może w znaczący sposób obniżać jakoś życia pacjentów, a nawet powodować jego skrócenie. Długotrwała choroba może prowadzić do konieczności zastosowania terapii nerkozastępczej, co niesie ze sobą wysokie koszty, ale też stwarza pewne ryzyko dla chorego. Z tego też powodu poznanie nowych czynników mogących przyczyniać się do zwiększonej progresji cukrzycowej choroby nerek niesie ze sobą szanse na opracowanie nowych parametrów, które mogą posłużyć w usprawnieniu diagnostyki tego schorzenia, a tym samym przyczynić się do szybszego wdrożenia terapii.

W niniejszej rozprawie doktorskiej zaprezentowano wyniki badań potwierdzające tezę, iż polimorfizmy genów kodujących izoformy NOS oraz genu kodującego ACE mogą być istotnymi czynnikami wpływającymi na zwiększone ryzyko rozwoju cukrzycowej choroby nerek i/lub zwiększone prawdopodobieństwo konieczności zastosowania terapii nerkozastępczej. Ponadto zaobserwowano także zmiany w stężeniu NOS3 oraz w aktywności ACE pomiędzy badanymi grupami, uwzględniając podziały ze względu na jednostkę chorobową oraz genotyp w obrębie badanych polimorfizmów. Jednocześnie badania potwierdzają dotychczasowe obserwacje, iż wiek oraz otyłość mają również swój udział w patogenezie cukrzycowej choroby nerek.

Wykazano także zależność między skutecznością wybranych leków z grupy ACEi – benazeprylu oraz lizynoprylu – a wariantem genetycznym polimorfizmu rs4646994 w genie *ACE*. Jest to potwierdzenie dotychczasowych doniesień, iż polimorfizm ten może wpływać na efektywność terapii lekami z grupy ACEi. Jednakże ze względu na stosunkowo niewielką populację badaną przyjmującą te środki terapeutyczne, badania należałoby kontynuować, uwzględniając większą liczbę pacjentów.

Pomimo pewnych ograniczeń na podstawie otrzymanych wyników można wyciągnąć następujące wnioski:

1. Polimorfizmy genów kodujących izoformy NOS, a w szczególności NOS2 oraz NOS3, są wiązane ze zwiększonym ryzykiem lub progresją otyłości, insulinooporności, cukrzycy oraz cukrzycowej choroby nerek.
2. Polimorfizmy: rs4343 w genie *ACE*, rs3782218 w genie *NOS1* oraz rs1137933 w genie *NOS2* mogą być związane ze zmienionym ryzykiem rozwoju cukrzycowej choroby nerek i/lub zmienionym prawdopodobieństwem konieczności zastosowania terapii nerkozastępczej. Genotypy G/G i G/A, a także sam allel G polimorfizmu rs4343 w genie *ACE*, jak również genotyp C/C polimorfizmu rs3782218 w genie *NOS1* były związane ze

zwiększonym ryzykiem rozwoju cukrzycowej choroby nerek. Z kolei genotypy G/G i G/A, oraz allel G polimorfizmu rs4343 w genie ACE, a także allel C polimorfizmu rs3782218 w genie *NOS1* były związane ze zwiększonym prawdopodobieństwem konieczności zastosowania terapii nerkozastępczej. Natomiast ze zmniejszonym ryzykiem zastosowania tej terapii był powiązany allel G polimorfizmu rs1137933 w genie *NOS2*.

3. Benazepryl i lizynopryl wykazują różne powinowactwo wiązania do dwóch domen ACE, co może przekładać się na skuteczność stosowanej terapii. Polimorfizm rs4646994 w genie *ACE* może zatem odgrywać kluczową rolę przy wyborze środka terapeutycznego.

IX. PIŚMIENNICTWO

1. Sulaiman, M.K. Diabetic nephropathy: recent advances in pathophysiology and challenges in dietary management. *Diabetol Metab Syndr.* **2019**, 11, 7. doi: 10.1186/s13098-019-0403-4.
2. Madziarska, K.; Banasik, M. Chorzy na cukrzycę w programach hemodializy i dializy otrzewnowej – zagrożenia, których można uniknąć. *Probl Lek.* **2006**, 45, 240-241.
3. Sagoo, M.K.; Gnudi, L. Diabetic Nephropathy: An Overview. *Methods Mol Biol.* **2020**, 2067, 3-7. doi: 10.1007/978-1-4939-9841-8_1.
4. Misra, A.; Gopalan, H.; Jayawardena, R.; Hills, A.P.; Soares, M.; Reza-Albarrán, A.A.; Ramaiya, K.L. Diabetes in developing countries. *J Diabetes.* **2019**, 11, 7, 522-539. doi: 10.1111/1753-0407.12913.
5. Samsu, N. Diabetic Nephropathy: Challenges in Pathogenesis, Diagnosis, and Treatment. *Biomed Res Int.* **2021**, 2021, 1497449. doi: 10.1155/2021/1497449.
6. Shahbazian, H.; Rezaii, I. Diabetic kidney disease; review of the current knowledge. *J Renal Inj Prev.* **2013**, 2, 2, 73-80. doi: 10.12861/jrip.2013.24.
7. Akhtar, M.; Taha, N.M.; Nauman, A.; Mujeeb, I.B.; Al-Nabet, A.D.M.H. Diabetic Kidney Disease: Past and Present. *Adv Anat Pathol.* **2020**, 27, 2, 87-97. doi: 10.1097/PAP.0000000000000257.
8. Dronavalli, S.; Duka, I.; Bakris, G. The pathogenesis of diabetic nephropathy. *Nat Rev Endocrinol.* **2008**, 4, 444–452. doi: 10.1038/ncpendmet0894
9. Natesan, V.; Kim, S.J. Diabetic Nephropathy - a Review of Risk Factors, Progression, Mechanism, and Dietary Management. *Biomol Ther (Seoul).* **2021**, 29, 4, 365-372. doi: 10.4062/biomolther.2020.204.
10. Kimmelstiel, P.; Wilson, C. Benign and Malignant Hypertension and Nephrosclerosis. A Clinical and Pathological Study. *Am J Pathol.* **1936**, 12, 1, 45-48.
11. Tavafi, M. Complexity of diabetic nephropathy pathogenesis and design of investigations. *J Renal Inj Prev.* **2013**, 2, 2, 59-62. doi: 10.12861/jrip.2013.20.
12. Kriz W, Löwen J, Gröne HJ. The complex pathology of diabetic nephropathy in humans. *Nephrol Dial Transplant.* **2023**, 38, 10, 2109-2119. doi: 10.1093/ndt/gfad052.
13. Arora, M.K.; Singh, U.K. Molecular mechanisms in the pathogenesis of diabetic nephropathy: an update. *Vascul Pharmacol.* **2013**, 58, 4, 259-271. doi: 10.1016/j.vph.2013.01.001.
14. Rayego-Mateos, S.; Morgado-Pascual, J.L.; Opazo-Ríos, L.; Guerrero-Hue, M.; García-Caballero, C.; Vázquez-Carballo, C.; Mas, S.; Sanz, A.B.; Herencia, C.; Mezzano, S.;

Gómez-Guerrero, C.; Moreno, J.A.; Egido, J. Pathogenic Pathways and Therapeutic Approaches Targeting Inflammation in Diabetic Nephropathy. *Int J Mol Sci.* **2020**, 21, 11, 3798. doi: 10.3390/ijms21113798.

15. Rahimi, Z. The Role of Renin Angiotensin Aldosterone System Genes in Diabetic Nephropathy. *Can J Diabetes.* **2016**, 40, 2, 178-183. doi: 10.1016/j.jcjd.2015.08.016.
16. Gastaldi, G.; Pannier, F.; Roztočil, K.; Lugli, M.; Mansilha, A.; Haller, H.; Rabe, E.; VAN Rijn, M.J. Chronic venous disease and diabetic microangiopathy: pathophysiology and commonalities. *Int Angiol.* **2021**, 40, 6, 457-469. doi: 10.23736/S0392-9590.21.04664-2.
17. Premaratne, E.; Verma, S.; Ekinci, E.I.; Theverkalam, G.; Jerums, G.; MacIsaac, R.J. The impact of hyperfiltration on the diabetic kidney. *Diabetes Metab.* **2015**, 41, 1, 5-17. doi: 10.1016/j.diabet.2014.10.003.
18. Elmarakby, A.A.; Sullivan, J.C. Relationship between oxidative stress and inflammatory cytokines in diabetic nephropathy. *Cardiovasc Ther.* **2012**, 30, 1, 49-59. doi: 10.1111/j.1755-5922.2010.00218.x.
19. Lewandowski, Ł.; Urbanowicz, I.; Kepinska, M.; Milnerowicz, H. Concentration/activity of superoxide dismutase isozymes and the pro-/antioxidative status, in context of type 2 diabetes and selected single nucleotide polymorphisms (genes: INS, SOD1, SOD2, SOD3) – Preliminary findings. *Biomed Pharmacother.* **2021**, 137, 111396, doi: 10.1016/j.biopha.2021.111396.
20. Ganten, D.; Ruckpaul, K. Nitrosative Stress. W: Encyclopedic Reference of Genomics and Proteomics in Molecular Medicine; Springer: Berlin/Heidelberg, Niemcy, 2005.
21. Pérez-Torres, I.; Manzano-Pech, L.; Rubio-Ruíz, M.E.; Soto, J.A.; Guarner-Lans, V. Nitrosative stress and its association with cardiometabolic disorders. *Molecules* **2020**, 25, 11, 2555. doi: 10.3390/molecules25112555.
22. Förstermann, U.; Sessa, W.C. Nitric oxide synthases: regulation and function. *Eur Heart J.* **2012**, 33, 829-837, 837a-837d. doi: 10.1093/eurheartj/ehr304.
23. Griffith, O..; Stuehr, D.J. Nitric oxide synthases: properties and catalytic mechanism. *Annu Rev Physiol.* **1995**, 57, 707-736. doi: 10.1146/annurev.ph.57.030195.003423.
24. Knott, A.B.; Bossy-Wetzel, E. Nitric oxide in health and disease of the nervous system. *Antioxid Redox Signal.* **2009**, 11, 3, 541-554. doi: 10.1089/ars.2008.2234.
25. Fish, J.E.; Marsden, P.A. Endothelial nitric oxide synthase: insight into cell-specific gene regulation in the vascular endothelium. *Cell Mol Life Sci.* **2006**, 63, 2, 144-162. doi: 10.1007/s00018-005-5421-8.

26. Förstermann, U.; Münzel, T. Endothelial nitric oxide synthase in vascular disease: from marvel to menace. *Circulation.* **2006**, 113, 13, 1708-1714. doi: 10.1161/CIRCULATIONAHA.105.602532.
27. Karupiah, G.; Xie, Q.W.; Buller, R.M.; Nathan, C.; Duarte, C.; MacMicking, J.D. Inhibition of viral replication by interferon-gamma-induced nitric oxide synthase. *Science.* **1993**, 261, 5127, 1445-1448. doi: 10.1126/science.7690156.
28. Ługowski, M.; Saczko, J.; Kulbacka, J.; Banaś T. Reaktywne formy tlenu i azotu [Reactive oxygen and nitrogen species]. *Pol Merkur Lekarski.* **2011**, 31, 185, 313-317.
29. Lowenstein, C.J.; Padalko, E. iNOS (NOS2) at a glance. *J Cell Sci.* **2004**, 117, Pt 14, 2865-2867. doi: 10.1242/jcs.01166.
30. Maron, B.A.; Tang, S.S.; Loscalzo, J. S-nitrosothiols and the S-nitrosoproteome of the cardiovascular system. *Antioxid Redox Signal.* **2013**, 18, 270-287. doi: 10.1089/ars.2012.4744.
31. Shahani, N.; Sawa, A. Protein S-nitrosylation: role for nitric oxide signaling in neuronal death. *Biochim Biophys Acta.* **2012**, 1820, 6, 736-742. doi: 10.1016/j.bbagen.2011.07.010.
32. Hu, Q.; Jiang, L.; Yan, Q.; Zeng, J.; Ma, X.; Zhao, Y. A natural products solution to diabetic nephropathy therapy. *Pharmacol Ther.* **2023**, 241, 108314. doi: 10.1016/j.pharmthera.2022.108314.
33. Sagoo, M.K.; Gnudi, L. Diabetic nephropathy: Is there a role for oxidative stress? *Free Radic Biol Med.* **2018**, 116, 50–63, doi: 10.1016/j.freeradbiomed.2017.12.040.
34. Mezghenna, K.; Pomiès, P; Chalançon, A.; Castex, F.; Leroy, J.; Niclauss, N.; Nadal, B.; Cambier, L.; Cazevieille, C.; Petit, P.; Gomis, R.; Berney, T.; Gross, R.; Lajoix, A.D. Increased neuronal nitric oxide synthase dimerisation is involved in rat and human pancreatic beta cell hyperactivity in obesity. *Diabetologia.* **2011**, 54, 11, 2856-2866. doi: 10.1007/s00125-011-2264-8.
35. Muniyappa, R.; Sowers, J.R. Role of insulin resistance in endothelial dysfunction. *Rev Endocr Metab Disord.* **2013**, 14, 1, 5-12. doi: 10.1007/s11154-012-9229-1.
36. Gusti, A.M.T.; Qusti, S.Y.; Bahijri, S.M.; Toraih, E.A.; Bokhari, S.; Attallah, S.M.; Alzahrani, A.; Alshehri, W.M.A.; Alotaibi, H.; Fawzy, M.S. Glutathione S-Transferase (GSTT1 rs17856199) and Nitric Oxide Synthase (NOS2 rs2297518) Genotype Combination as Potential Oxidative Stress-Related Molecular Markers for Type 2 Diabetes Mellitus. *Diabetes Metab Syndr Obes.* **2021**, 14, 1385-1403. doi: 10.2147/DMSO.S300525.

37. Dellamea, B.S.; Pinto, L,C.; Leitão, C.B.; Santos, K.G.; Canani, L.H. Endothelial nitric oxide synthase gene polymorphisms and risk of diabetic nephropathy: a systematic review and meta-analysis. *BMC Med Genet.* **2014**, 15, 9. doi: 10.1186/1471-2350-15-9.
38. Nowakowska, J.; Sobkowiak, P.; Bręborowicz, A.; Mrówczyńska, M.; Wojsyk-Banaszak, I.; Szczepankiewicz, A. Nitric Oxide Synthase 2 Promoter Polymorphism Is a Risk Factor for Allergic Asthma in Children. *Medicina (Kaunas).* **2021**, 57, 12, 1341. doi: 10.3390/medicina57121341.
39. Solanki, K.; Rajpoot, S.; Bezsonov, E.E.; Orekhov, A.N.; Saluja, R.; Wary, A.; Axen, C.; Wary, K.; Baig, M.S. The expanding roles of neuronal nitric oxide synthase (NOS1). *PeerJ.* **2022**, 10, e13651. doi: 10.7717/peerj.13651.
40. Dobrijević, Z.; Stevanović, J.; Robajac, D.; Penezić, A.; Četić, D.; Baralić, M.; Nedić, O. Association between nitric oxide synthase (NOS3) gene polymorphisms and diabetic nephropathy: An updated meta-analysis. *Mol Cell Endocrinol.* **2024**, 586, 112197. doi: 10.1016/j.mce.2024.112197.
41. Raina, P.; Sikka, R.; Gupta, H.; Matharoo, K.; Bali, S.K, Singh V, Bhanwer A. Association of eNOS and MCP-1 Genetic Variants with Type 2 Diabetes and Diabetic Nephropathy Susceptibility: A Case-Control and Meta-Analysis Study. *Biochem Genet.* **2021**, 59, 4, 966-996. doi: 10.1007/s10528-021-10041-2.
42. Khurana, V.; Goswami, B. Angiotensin converting enzyme (ACE). *Clin Chim Acta.* **2022**, 524, 113-122. doi: 10.1016/j.cca.2021.10.029.
43. Hou, R.; He, Y.; Yan, G.; Hou, S.; Xie, Z.; Liao, C. Zinc enzymes in medicinal chemistry. *Eur J Med Chem.* **2021**, 226, 113877. doi: 10.1016/j.ejmech.2021.113877.
44. Aggarwal, N.; Kare, P.K.; Varshney, P.; Kalra, O.P.; Madhu, S.V.; Banerjee, B.D.; Yadav, A.; Raizada, A.; Tripathi, A.K. Role of angiotensin converting enzyme and angiotensinogen gene polymorphisms in angiotensin converting enzyme inhibitor-mediated antiproteinuric action in type 2 diabetic nephropathy patients. *World J Diabetes.* **2017**, 8, 3, 112-119. doi: 10.4239/wjd.v8.i3.112.
45. Widodo, W.; Wisnasari, S.; Saifur Rohman, M.; Yunita, L.; Lukitasari, M.; Nuril, M.; Holil, K.; Laila Purwaningroom, D. Alu insertion/deletion of ACE gene polymorphism might not affect significantly the serum bradykinin level in hypertensive patients taking ACE inhibitors. *Egypt J Med Hum Genet.* **2017**, 18, 2, 187-191, doi: 10.1016/j.ejmhg.2016.08.001.
46. Ceconi, C.; Francolini, G.; Olivares, A.; Comini, L.; Bachetti, T.; Ferrari, R. Angiotensin-converting enzyme (ACE) inhibitors have different selectivity for bradykinin binding sites

of human somatic ACE. *Eur J Pharmacol.* **2007**, 577, 1-3, 1-6. doi: 10.1016/j.ejphar.2007.07.061.

47. Fernandez, J.K.; Hayashi, M.A.; Camargo, A.C.; Neshich, G. Structural basis of the lisinopril-binding specificity in N- and C-domains of human somatic ACE. *Biochem Biophys Res Commun.* **2003**, 308, 2, 219-226. doi: 10.1016/s0006- 291x(03)01363-9.
48. Denti, P.; Sharp, S.K.; Kröger, W.L.; Schwager, S.L.; Mahajan, A.; Njoroge, M.; Gibhard, L.; Smit, I.; Chibale, K.; Wiesner, L.; Sturrock, E.D.; Davies, N.H. Pharmacokinetic evaluation of lisinopril-tryptophan, a novel C-domain ACE inhibitor. *Eur J Pharm Sci.* **2014**, 56, 113-119. doi: 10.1016/j.ejps.2014.01.012.
49. Papadopoulou, A.; Fragkou, P.C.; Maratou, E.; Dimopoulou, D.; Kominakis, A.; Kokkinopoulou, I.; Kroupis, C.; Nikolaidou, A.; Antonakos, G.; Papaevangelou, V.; Armaganidis, A.; Tsantes, A.; Polyzogopoulou, E.; Tsiodras, S.; Antoniadou, A.; Moutsatsou, P. Angiotensin-converting-enzyme insertion/deletion polymorphism, ACE activity, and COVID-19: A rather controversial hypothesis. A case-control study. *J Med Virol.* **2022**, 94, 3, 1050-1059. doi: 10.1002/jmv.27417.
50. Cotta Filho, C.K.; Oliveira-Paula, G.H.; Rondon Pereira, V.C. Lacchini, R. Clinically relevant endothelial nitric oxide synthase polymorphisms and their impact on drug response. *Expert Opin Drug Metab Toxicol.* **2020**, 16, 10, 927-951. doi: 10.1080/17425255.2020.1804857.
51. Silva, P.S.; Fontana, V.; Luizon, M.R.; Lacchini, R.; Silva, W.A. Jr; Biagi, C.; Tanus-Santos, J.E. eNOS and BDKRB2 genotypes affect the antihypertensive responses to enalapril. *Eur J Clin Pharmacol.* **2013**, 69, 2, 167-177. doi: 10.1007/s00228-012-1326-2.
52. Rahimi, Z. ACE insertion/deletion (I/D) polymorphism and diabetic nephropathy. *J Nephropathol.* **2012**, 1, 3, 143–151. doi: 10.5812/nephropathol.8109.
53. Yu, Z.Y.; Chen, L.S.; Zhang, L.C.; Zhou, T.B. Meta-analysis of the relationship between ACE I/D gene polymorphism and end-stage renal disease in patients with diabetic nephropathy. *Nephrology (Carlton).* **2012**, 17, 5, 480–487. doi: 10.1111/j.1440-1797.2012.01592.x.
54. Ismail, M.F.; Shaker, O.G.; Ashour, E.; Yousif, H.M.; Afify, M.; Gouda, W. The combined effect of ACE, TCF7L2, and PPARGC1A gene polymorphisms in diabetic nephropathy. *Egypt J Chem* **2017**, 60, 5, 869–881, doi: 10.21608/ejchem.2017.1335.1084.
55. Król-Kulikowska, M.; Abramenko, N.; Jakubek, M.; Banasik, M.; Kepinska, M. The role of angiotensin-converting enzyme (ACE) polymorphisms in the risk of development and treatment of diabetic nephropathy. *J Clin Med.* **2024**, 13, 4, 995. doi: 10.3390/jcm13040995.

56. Pettersen, E.F.; Goddard, T.D.; Huang, C.C.; Couch, G.S.; Greenblatt, D.M.; Meng, E.C.; Ferrin, T.E. UCSF Chimera – a visualization system for exploratory research and analysis. *J Comput Chem.* **2004**, 25, 13, 1605-1612. doi: 10.1002/jcc.20084.
57. Eberhardt, J.; Santos-Martins, D.; Tillack, A.F.; Forli, S. AutoDock Vina 1.2.0: New Docking Methods, Expanded Force Field, and Python Bindings. *J Chem Inf Model.* **2021**, 61, 8, 3891-3898. doi: 10.1021/acs.jcim.1c00203.
58. Trott, O.; Olson, A.J. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem.* **2010**, 31, 2, 455-461. doi: 10.1002/jcc.21334.
59. Biovia, D.S. BIOVIA Discovery Studio Visualizer (2D Diagram and Scheme of the Interactions with Amino Acids); BIOVIA Workbook: San Diego, CA, USA, 2020.
60. Król-Kulikowska, M.; Banasik, M.; Kepinska, M. The effect of selected nitric oxide synthase polymorphism on the risk of developing diabetic nephropathy. *Antioxidants (Basel).* **2024**, 13, 7, 838. doi:10.3390/antiox13070838.
61. Henningsson, R.; Salehi, A.; Lundquist, I. Role of nitric oxide synthase isoforms in glucose-stimulated insulin release. *Am J Physiol Cell Physiol.* **2002**, 283, 1, C296-C304. doi: 10.1152/ajpcell.00537.2001.
62. Jimenez-Feltstrom, J.; Lundquist, I.; Salehi, A. Glucose stimulates the expression and activities of nitric oxide synthases in incubated rat islets: an effect counteracted by GLP-1 through the cyclic AMP/PKA pathway. *Cell Tissue Res.* **2005**, 319, 2, 221-230. doi: 10.1007/s00441-004-1013-4.
63. Kurohane Kaneko, Y.; Ishikawa, T. Dual role of nitric oxide in pancreatic β-cells. *J Pharmacol Sci.* **2013**, 123, 4, 295-300. doi: 10.1254/jphs.13r10cp.
64. Wang, D.; Liu, L.; Zhang, C.; Lu, W.; Wu, F.; He, X. Evaluation of Association Studies and Meta-Analyses of eNOS Polymorphisms in Type 2 Diabetes Mellitus Risk. *Front Genet.* **2022**, 13, 887415. doi: 10.3389/fgene.2022.887415.
65. Varghese, S.; Kumar, S.G. Role of eNOS and TGFβ1 gene polymorphisms in the development of diabetic nephropathy in type 2 diabetic patients in South Indian population. *Egypt J Med Hum Genet.* **2022**, 23, 2. doi: 10.1186/s43042-022-00216-w.
66. Rippin, J.D.; Patel, A.; Belyaev, N.D.; Gill, G.V.; Barnett, A.H.; Bain, S.C. Nitric oxide synthase gene polymorphisms and diabetic nephropathy. *Diabetologia.* **2003**, 46, 3, 426-428. doi: 10.1007/s00125-003-1046-3.
67. Shoukry, A.; Shalaby, S.M.; Abdelazim, S.; Abdelazim, M.; Ramadan, A.; Ismail, M.I.; Fouad, M. Endothelial nitric oxide synthase gene polymorphisms and the risk of diabetic

nephropathy in type 2 diabetes mellitus. *Genet Test Mol Biomarkers.* **2012**, 16, 6, 574-579. doi: 10.1089/gtmb.2011.0218.

68. Miranda, J.A.; Belo, V.A.; Souza-Costa, D.C.; Lanna, C.M.; Tanus-Santos, J.E. eNOS polymorphism associated with metabolic syndrome in children and adolescents. *Mol Cell Biochem.* **2013**, 372, 1-2, 155-160. doi: 10.1007/s11010-012-1456-y.
69. Teixeira, T.G.; Tibana, R.A.; Nascimento, D.D.; de Sousa, N.M.; de Souza, V.C.; Vieira, D.C.; Nóbrega, O.T.; de Almeida, J.A.; Navalta, J.; Prestes, J. Endothelial nitric oxide synthase Glu298Asp gene polymorphism influences body composition and biochemical parameters but not the nitric oxide response to eccentric resistance exercise in elderly obese women. *Clin Physiol Funct Imaging.* **2016**, 36, 6, 482-489. doi: 10.1111/cpf.12255.
70. Muhammed, S.J.; Lundquist, I.; Salehi, A. Pancreatic β-cell dysfunction, expression of iNOS and the effect of phosphodiesterase inhibitors in human pancreatic islets of type 2 diabetes. *Diabetes Obes Metab.* **2012**, 14, 11, 1010-1009. doi: 10.1111/j.1463-1326.2012.01632.x.
71. Bahadoran, Z.; Mirmiran, P.; Ghasemi, A. Role of Nitric Oxide in Insulin Secretion and Glucose Metabolism. *Trends Endocrinol Metab.* **2020**, 31, 2, 118-130. doi: 10.1016/j.tem.2019.10.001.
72. Lundberg, J.O.; Weitzberg, E. Nitric oxide signaling in health and disease. *Cell.* **2022**, 185, 16, 2853-2878. doi: 10.1016/j.cell.2022.06.010.
73. Tran, N.; Garcia, T.; Aniqa, M.; Ali, S.; Ally, A.; Nauli, S.M. Endothelial Nitric Oxide Synthase (eNOS) and the Cardiovascular System: in Physiology and in Disease States. *Am J Biomed Sci Res.* **2022**, 15, 2, 153-177.
74. Sydorchuk, A.; Sydorchuk, L.; Gutnitska, A.F.; Margaryan, A.; Valentina; Dzhuryak; Sydorchuk, R.; Iftoda, O. Linkage of Metabolic Disorders, Endothelial Dysfunction and NOS3 (rs2070744) and GNB3 (rs5443) Genes Polymorphisms in Hypertensive Patients. *Biointerface Research in Applied Chemistry* **2023**, 13, 2, 123. doi: 10.33263/briac132.123.
75. Bertoncello, N.; Moreira, R.P.; Arita, D.Y.; Aragão, D.S.; Watanabe, I.K.; Dantas, P.S.; Santos, R.; Mattar-Rosa, R.; Yokota, R.; Cunha, T.S.; Casarini, D.E. Diabetic Nephropathy Induced by Increased Ace Gene Dosage Is Associated with High Renal Levels of Angiotensin (1-7) and Bradykinin. *J Diabetes Res.* **2015**, 2015, 674047. doi: 10.1155/2015/674047.
76. de Alcantara Santos, R.; Guzzoni, V.; Silva, K.A.S.; Aragão, D.S.; de Paula Vieira, R.; Bertoncello, N.; Schor, N.; Aimbire, F.; Casarini, D.E.; Cunha, T.S. Resistance exercise shifts the balance of renin-angiotensin system toward ACE2/Ang 1-7 axis and reduces

inflammation in the kidney of diabetic rats. *Life Sci.* **2021**, 287, 120058. doi: 10.1016/j.lfs.2021.120058.

77. Al-Timimi, D.J.; Sulieman, D.M.; Hussen, K.R. Zinc status in type 2 diabetic patients: relation to the progression of diabetic nephropathy. *J Clin Diagn Res.* **2014**, 8, 11, CC04-CC08. doi: 10.7860/JCDR/2014/10090.5082.
78. Nie, P.; Lou, Y.; Bai, X.; Zhu, Y.; Guo, Q.; Luo, P.; Zhang, W.; Li, B. Influence of zinc levels and Nrf2 expression in the clinical and pathological changes in patients with diabetic nephropathy. *Nutr Diabetes.* **2022**, 12, 1, 37. doi: 10.1038/s41387-022-00212-4.
79. Nikoobakht, M.R.; Pourmand, G.; Allameh, F.; Dialameh, H.; Sharifi, A.; Hashemiaghdam, A. Serum trace elements before and 3 months after renal transplantation in kidney recipients: An Iranian study. *Indian J. Transplant.* **2014**, 8, 8–11. doi: 10.1016/j.ijt.2013.12.002.
80. Muralidhara, K.; Kannan, S.; Ahamed, I.; Kishore, K.; Vincent, L.; Hegde, N. A prospective observational study on the traditional and novel risk factors associated with post-transplant diabetes mellitus in renal transplant recipients. *Biomedicine.* **2021**, 41, 482–488. doi: 10.51248/.v41i2.1063.
81. Schüler, R.; Osterhoff, M.A.; Frahnow, T.; Möhlig, M.; Spranger, J.; Stefanovski, D.; Bergman, R.N.; Xu, L.; Seltmann, A.C.; Kabisch, S.; Hornemann, S.; Kruse, M.; Pfeiffer, A.F.H. Dietary Fat Intake Modulates Effects of a Frequent ACE Gene Variant on Glucose Tolerance with association to Type 2 Diabetes. *Sci Rep.* **2017**, 7, 1, 9234. doi: 10.1038/s41598-017-08300-7.
82. ALkafaji, M.J.M.; Shehab, A.F.; Omair, H.A. ACE Polymorphisms Rs (4343) and Its Association with Ace Enzyme in Some of Patients with Diabetic Nephropathy in Some Population of Salah Al-Din Governorate in Iraq. *Pak Heart J.* **2023**, 56, 193–199.
83. Duan, J.; Wang, C.; Liu, D.; Qiao, Y.; Pan, S.; Jiang, D.; Zhao, Z.; Liang, L.; Tian, F.; Yu, P.; Zhang, Y.; Zhao, H.; Liu, Z. Prevalence and risk factors of chronic kidney disease and diabetic kidney disease in Chinese rural residents: a cross-sectional survey. *Sci Rep.* **2019**, 9, 1, 10408. doi: 10.1038/s41598-019-46857-7.
84. Hussain, S.; Jamali, M.C.; Habib, A.; Hussain, M.S.; Akhtar, M.; Najmi, A.K. Diabetic kidney disease: An overview of prevalence, risk factors, and biomarkers. *Clin Epidemiol. Glob Health.* **2021**, 9, 2–6. doi: 10.1016/j.cegh.2020.05.016.
85. Selmi, A.; Aydi, R.; Kammoun, O.; Bougatef, H.; Bougatef, A.; Miled, N.; Alghamdi, O.A.; Kammoun, M. Synthesis, crystal structure, molecular docking studies and biological

evaluation of aryl substituted dihydroisoquinoline imines as a potent angiotensin converting enzyme inhibitor. *J Mol Struct.* **2021**, 1235, 130230. doi: 10.1016/j.molstruc.2021.130230.

86. Dey, T.K.; Chatterjee, R.; Mandal, R.S.; Roychoudhury, A.; Paul, D.; Roy, S.; Pateiro, M.; Das, A.K.; Lorenzo, J.M.; Dhar, P. ACE Inhibitory Peptides from Bellamya bengalensis Protein Hydrolysates: In Vitro and In Silico Molecular Assessment. *Processes.* **2021**, 9, 1316. doi: 10.3390/pr9081316.
87. Januszewicz, A.; Januszewicz, W.; Szczepańska-Sadowska, E.; Sznajderman, M. Nadciśnienie Tętnicze; Wydawnictwo Medycyna Praktyczna: Kraków, Poland, 2007; 1079–1080.
88. Varghese, S.; Kumar, S.G. Association between genetic variants of NOS3, TGF-β and susceptibility of diabetic nephropathy: A meta-analysis. *Meta Gene.* **2019**, 21, 100573. doi: 10.1016/j.mgene.2019.100573.
89. Oliveira-Paula, G.H.; Lacchini, R.; Tanus-Santos, J.E. Endothelial nitric oxide synthase: From biochemistry and gene structure to clinical implications of NOS3 polymorphisms. *Gene.* **2016**, 575, 2 Pt 3, 584-99. doi: 10.1016/j.gene.2015.09.061.
90. Shahid, S.M.; Nawab, S.N.; Shaikh, R.; Mahboob, T. Glycemic control, dyslipidemia and endothelial dysfunction in coexisted diabetes, hypertension and nephropathy. *Pak J Pharm Sci.* **2012**, 25, 1, 123-129.
91. Mohammadi, M.; Yaghooti, H.; Adibmanesh, A.; Mohammadtaghvaie, N.; Akhormeh, A.K.; Eslami, M. Endothelial Nitric Oxide Synthase 4a/B Polymorphism and Its Interaction with Enos G894T Variants in Type 2 Diabetic Patients: Modifying the Risk of Diabetic Nephropathy. *Iran J Public Health.* **2022**, 51, 1, 219-220. doi: 10.18502/ijph.v51i1.8318.
92. Li, H.; Shu, G.; Gao, H. Epithelial nitric oxide synthases (eNOS) 894 G < T polymorphism and diabetic nephropathy susceptibility: A meta-Analysis. *Pteridines.* **2022**, 33, 49–57. doi: 10.1515/pteridines-2022-0042.
93. Duan, J.-Y.; Duan, G.-C.; Wang, C.-J.; Liu, D.-W.; Qiao, Y.-J.; Pan, S.-K.; Jiang, D.-K.; Liu, Y.; Zhao, Z.-H.; Liang, L.-L.; Tian, F.; Liu, Z.-S. Prevalence and risk factors of chronic kidney disease and diabetic kidney disease in a central Chinese urban population: A cross-sectional survey. *BMC Nephrol.* **2020**, 21, 1–13. doi: 10.1186/s12882-020-01761-5.
94. Natesan, V.; Kim, S.J. Diabetic Nephropathy – a Review of Risk Factors, Progression, Mechanism, and Dietary Management. *Biomol Ther (Seoul).* **2021**, 29, 4, 365-372. doi: 10.4062/biomolther.2020.204.

X. WYKAZ SKRÓTÓW

ACE – (ang. *angiotensin-converting enzyme*), enzym konwertujący angiotensynę, konwertaza angiotensyny

ACEi – inhibitory ACE

Ang I – angiotensyna I

Ang II – angiotensyna II

AGEs – (ang. *advanced glycation end-products*), zaawansowane produkty glikacji

BH_4 – tetrahydrobiopteryna

CRP – (ang. *C-reactive protein*), białko C-reaktywne

EDTA – (ang. *ethylenediaminetetraacetic acid*), kwas wersenowy

eGFR – (ang. estimated glomerular filtration rate), estymowany wskaźnik filtracji kłębuszkowej

ESRD – (ang. *end-stage renal disease*), schyłkowa niewydolność nerek

FAAS – (ang. *Flame Atomic Absorption Spectrometry*), atomowa spektrometria absorpcyjna z atomizacją w płomieniu

FAD – dinukleotyd flawinoadeninowy

FMN – mononukleotyd flawinowy

GFR – (ang. *glomerular filtration rate*), wskaźnik filtracji kłębuszkowej

MDRD – (ang. *Modification of Diet in Renal Disease*)

MetS – (ang. *metabolic syndrome*), zespół metaboliczny

NADPH – fosforan dinukleotydu nikotynoamidoadeninowego

NO – (ang. *nitric oxide*), tlenek azotu

NOS – (ang. *nitric oxide synthase*), syntaza tlenku azotu

PCR-RFLP – (ang. *polymerase chain reaction – restriction fragment length polymorphism*), łańcuchowa reakcja polimerazy – polimorfizm długości fragmentów restrykcyjnych

PDB – Protein Data Bank

RAAS – (ang. *renin-angiotensin-aldosterone system*), układ renina-angiotensyna-aldosteron

RNS – (ang. *reactive nitrogen species*), reaktywne formy azotu

ROS – (ang. *reactive nitrogen species*), reaktywne formy tlenu

SNP – (ang. *single nucleotide polymorphism*), polimorfizm pojedynczego nukleotydu

T1D – (ang. type 1 diabetes), cukrzyca typu 1

T2D – (ang. type 2 diabetes), cukrzyca typu 2

VNTR – (ang. *variable number tandem repeat*), zmienna liczba powtórzeń tandemowych

XI. SPIS TABEL I RYCIN

1. Spis tabel

2. Spis rycin

XII. ZAŁĄCZNIKI

1. **Załącznik 1. Publikacje wchodzące w skład rozprawy doktorskiej.**
2. **Załącznik 2. Oświadczenie autorki rozprawy doktorskiej.**
3. **Załącznik 3. Oświadczenia współautorów publikacji.**
4. **Załącznik 4. Życiorys naukowy autorki rozprawy doktorskiej.**
5. **Załącznik 5. Wykaz publikacji i abstraktów potwierdzony przez bibliotekę.**

1. Załącznik 1. Publikacje wchodzące w skład rozprawy doktorskiej.

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Review

Human Nitric Oxide Synthase—Its Functions, Polymorphisms, and Inhibitors in the Context of Inflammation, Diabetes and Cardiovascular Diseases

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Abstract: In various diseases, there is an increased production of the free radicals needed to carry out certain physiological processes but their excessive amounts can cause oxidative stress and cell damage. Enzymes play a major role in the transformations associated with free radicals. One of them is nitric oxide synthase (NOS), which catalyzes the formation of nitric oxide (NO). This enzyme exists in three forms (NOS1, NOS2, NOS3), each encoded by a different gene. The following work presents the most important information on the NOS isoforms and their role in the human body, including NO synthesis in various tissues and cells, intercellular signaling and activities supporting the immune system and regulating blood vessel functions. The role of NOS in pathological conditions such as obesity, diabetes and heart disease is considered. Attention is also paid to the influence of the polymorphisms of these genes, encoding particular isoforms, on the development of these pathologies and the role of NOS inhibitors in the treatment of patients.

Keywords: nitric oxide synthase; oxidative stress; single nucleotide polymorphism; obesity; type 2 diabetes; heart diseases

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1. Introduction

During various processes taking place in the human body, free radicals and reactive oxygen species (ROS) are formed as natural metabolism products. Some of them act as signaling molecules that control physiological processes. Unfortunately, their excess can cause tissue damage [1]. For this reason, cells are forced to maintain a balance in the production of ROS to maintain homeostasis. To this end, compounds called antioxidants, which include a number of enzymes, including superoxide dismutase (SOD), catalase and glutathione peroxidase, play a role. However, maintaining control over these processes is not always possible. A condition in which too much ROS is produced and/or not effectively neutralized is referred to as oxidative stress. The imbalance between the excess of ROS and the biological ability to detoxify reactive products may accompany many pathological conditions, such as atherosclerosis or diabetes, but may also play a significant role in preventing aging as a result of mitohormesis [2].

In addition to ROS, reactive nitrogen species (RNS), a group of molecules derived from nitric oxide (NO), are characterized by high chemical reactivity due to having unpaired electrons [3]. They can, together with ROS, damage cellular structures. Their excessive production causes a phenomenon analogous to oxidative stress, referred to as nitrosative stress [4]. It is a state of imbalance between the amount of RNS formed and the biological capacity to render the reactive species harmless. It often damages proteins, lipids and even DNA, which can induce apoptosis. In turn, stronger nitrosative stress can cause necrosis. A high level of nitrosative stress reduces the adenosine triphosphate (ATP) pool, which prevents a cell from entering the path of controlled, apoptotic death, causing its necrosis [5].

NO is produced by a reaction catalyzed by nitric oxide synthase (NOS). Three isoforms of this enzyme, each associated with a different place of expression and action in the body, were distinguished [6]. Recently, there have been many reports on the impact of individual NOS isoforms and disturbances in their activity on the risk of various diseases, including metabolic and cardiovascular diseases. NO deficiency is one of the leading causes of endothelial dysfunction [7]. It is related to improper regulation of vasorelaxation, i.e., lowering the tone of blood vessels. In turn, such disorders are part of the pathogenesis of such diseases as atherosclerosis, hypotension, diabetes and hypercholesterolemia [8]. Attention can also be drawn to the importance of particular genotypes of selected polymorphisms of *NOS* genes. Nevertheless, there are still quite a few issues to ponder over or delve into more closely. This review was intended to inspire researchers to conduct further research, which can be translated into clinical significance. The aim of this study was to summarize the current knowledge of NO and NOS and their impact on selected disease states.

2. Characteristics of NO and Its Derivatives

The relevant compounds belonging to the RNS group are NO and its derivatives—nitrosyl cation (NO^+), nitrosyl anion (NO^-) and peroxynitrite ($ONOO^-$). The most known NO derivatives are shown in Table 1. NO is formed from arginine in the reaction catalyzed by NOS. Although the half-life of NO is only a few seconds [9], it is a highly reactive molecule that, along with other free radicals, can cause the formation of new RNS, which, in turn, react with cell proteins and may impair their function due to the oxidation or nitrosylation of amino acid residues. NO acts as a cellular signaling molecule; it modulates muscle tone, regulates insulin secretion and modulates airway tone and intestinal peristalsis. In addition, it plays an essential role in angiogenesis and nerve development [10]. Dysregulated production of NO can lead to many pathological conditions, such as stroke, inflammation and hypertension. Therefore, NOS activity control using isoform-selective NOS inhibitors brings with it great hopes for treating diseases associated with NO production [11].

Table 1. The most well-known nitric oxide (NO) derivatives [12–21].

Molecule Name	Summary Formula	Reactivity	Characteristics
nitrosyl cation	NO^+	a strong oxidizing agent	Intermediate in the amine diazotization reaction
nitrosyl anion	NO^-	a strong oxidizing agent	Participates in the nitrosylation reaction of metals, forming metal nitrosyl complexes
nitrogen dioxide	$\bullet NO_2$	a strong oxidizing agent	A good oxidizer; it combusts, sometimes explosively, with many compounds, such as hydrocarbons
dinitrogen trioxide	N_2O_3	a strong oxidizing agent	Partially dissociates into NO and NO_2; vapors very toxic by inhalation; reactivity likely to resemble that of nitrogen dioxide
peroxynitrite	$ONOO^-$	highly reactive; a very strong oxidant and nitrating agent	Essentially stable but its protonated form (ONOOH) decomposes rapidly via homolysis of the O-O bond to form about 28% free NO_2 and OH radicals
nitrite	NO_2^-	very reactive; a member of reactive nitrogen species	A nitrogen oxoanion formed by loss of a proton from nitrous acid; used for NO measurement
nitrate	NO_3^-	very reactive; a member of reactive nitrogen species	A nitrogen oxoanion formed by loss of a proton from nitric acid; used for NO measurement
nitroxyl	HNO	very reactive towards nucleophiles, including thiols	A weak acid; can be formed as a short-lived intermediate in the solution phase

NO has long been known to be present in bacteria but for years there was no evidence for its biological functions in mammals [22]. Evidence since that time has established a significant role for NO as a messenger molecule in at least three systems: white blood cells, where NO mediates tumoricidal and bactericidal effects; blood vessels, where it represents endothelium-derived relaxing factor activity; and as a neuronal constituent with functions very much like those of a neurotransmitter [23].

In recent years, the study of the role of NO in cellular signaling has become one of the most rapidly growing biology areas. In many instances, NO mediates its biological effects by activating guanylyl cyclase and increasing cyclic guanosine monophosphate (cGMP) synthesis from guanosine triphosphate (GTP) [24]. However, the list of NO effects that are independent of cyclic GMP is also growing at a rapid rate; for example, NO can interact with transition metals such as iron, thiol groups, other free radicals, oxygen, superoxide anion, unsaturated fatty acids and other molecules [25]. Some of these reactions result in the oxidation of NO to nitrite and nitrate and terminate its effect, while different reactions can lead to altered protein structure, function or catalytic capacity. These diverse effects of NO, which are either cyclic GMP-dependent or independent, can change and regulate important physiological and biochemical events in cell regulation and function. NO can function as an intracellular messenger, autacoid, paracrine substance, neurotransmitter or as a hormone that can be carried to distant sites for effects [25]. Thus, it is a unique simple molecule with an array of signaling functions. However, as with any messenger molecule, there can be excess or deficiency of the substance resulting in pathological events.

In addition to NO, its derivatives are known to play a role in the pathophysiology of various diseases. It is worth paying attention here to the formation of RNS. Considered in terms of strict chemical criteria, RNS encompasses such a diverse range of compounds, with such contrasting and distinct properties, that their only unifying characteristic is that they can be derived from NO. Recent advances give essential insights into the biology of specific RNS, their effects on physiological functions and their potential participation in the development of diseases [26]. In biological systems, the primary source of all RNS is NO. The rapid reactions of NO with free radicals have proved to be significant routes to the formation of RNS and, at present, the best known of them is the reaction with peroxide (O^{-2}) to produce $ONOO^-$ [26–28]. Peroxynitrite is chemically unstable under physiological conditions resulting in the formation of nitrate through isomerization. Since nitrate is essentially biochemically inert in mammalian cells, this reaction has been shown to be an excellent method to scavenge and neutralize O^{-2} [29]. As studies on this reaction progressed, a new perspective emerged when researchers realized that $ONOO^-$ is reactive with all the major classes of biomolecules and, therefore, has the potential to mediate cytotoxicity independently of NO or O^{-2} [30].

$ONOO^-$ is a highly reactive species which can directly react with various biological targets and components of a cell, including lipids, thiols, amino acid residues, DNA bases and low-molecular-weight antioxidants [31]. However, these reactions happen at a relatively slow rate. This slow reaction rate allows it to react more selectively throughout the cell. Furthermore, $ONOO^-$ can react with other molecules to form additional types of RNS, including nitrogen dioxide ($\bullet NO_2$) and dinitrogen trioxide (N_2O_3), as well as different types of chemically reactive free radicals [32]. $ONOO^-$ can react with proteins that contain transition metal centers and, through this, modify proteins such as hemoglobin, myoglobin and cytochrome c. This molecule can change protein structure through reactions with various amino acids through cysteine oxidation or tyrosine nitration. However, $ONOO^-$ does not react directly with tyrosine. Tyrosine reacts with other RNS produced by peroxynitrite. These reactions affect protein structure and function and can potentially cause changes in the catalytic activity of enzymes, alter cytoskeletal organization and impair cell signal transduction [33]. Such changes at the molecular level can underlie many diseases, such as cardiovascular diseases, diabetes, chronic inflammatory diseases and cancer, and neurodegenerative disorders.

3. The Structure of NOS and Its Isoforms

To fully understand the role and function of NO, it is necessary to become familiar with the enzyme responsible for its synthesis and the structural differences in the enzyme isoforms. NO synthases (NOS) are a group of enzymes that catalyze the synthesis of NO from the nitrogen residue of the amino acid L-arginine in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen. NOS is an enzyme that binds to flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, tetrahydrobiopterin (BH_4) and calmodulin. To date, three different types of this enzyme have been found in mammals. We can distinguish two constitutive NOS isoforms, the activity of which depends on the Ca^{2+}/calmodulin complex—NOS associated with signal transduction in central and peripheral neurons (NOS1, ncNOS, bNOS) and endothelial NOS (NOS3, eNOS, ecNOS) related to NO synthesis in blood vessels [34]. A separate gene encodes both isoforms. NOS1 is located on the longer arm of chromosome 12 at position 24.22 and encodes a protein of 1434 amino acids [35]. In turn, NOS3 is located on the longer arm of chromosome 7 at position 36.1 and encodes a protein of 1153 amino acids [36]. NOS1 is especially important for the brain and peripheral nervous system, where NO performs functions as a neurotransmitter, and has been implicated in neurotoxicity associated with stroke and neurodegenerative diseases, neural regulation of smooth muscle, including peristalsis, and penile erection [37]. In turn, NOS3 is responsible for the production of NO in the vascular endothelium [38], a monolayer of flat cells lining the interior surface of blood vessels [39]. NO produced by NOS3 in the vascular endothelium plays critical roles in regulating vascular tone, cellular proliferation, leukocyte adhesion and platelet aggregation [40]. Therefore, a functional NOS3 is essential for a healthy cardiovascular system. The third form of NOS was originally isolated and sequenced from mouse macrophages [41]; its activity depends on the Ca^{2+}/calmodulin complex but does not require high Ca^{2+} levels. It is completely active at normal intracellular Ca^{2+} levels [34]. Inducible NOS (NOS2, iNOS, mNOS, macNOS) is involved in the immune response and synthesizes NO, which is an essential pro-inflammatory cytotoxic agent, as a defense mechanism; for example, NO is responsible for inhibiting the production of IL-12 and macrophages. Furthermore, its expression occurs due to various inflammatory cytokines, including IL-1, IL-2, TNFα and lipopolysaccharide (LPS). NOS2 can be produced in most nucleated cells of the body and plays a vital role in eliminating or suppressing intracellular pathogens, including viruses [42]. The gene encoding NOS2 is located on the longer arm of chromosome 17 at position 11.2 and encodes a protein of 1203 amino acids [43]. The dual role of NOS2 in cancer development is known in the literature. It depends on the local concentration of NOS2 in the tumor microenvironment or disease state. This NOS isoform modulates key issues such as malignant transformation, angiogenesis and metastasis. However, NO used by macrophages has a cytotoxic or cytostatic effect on cancer cells [44]. In fact, the role of NOS2 in the cancer process is very complex; hence we can talk about it as a tumor promoter and suppressor at the same time [44,45].

The structures of the three known isoforms are similar to each other; they are all dimers made of two identical subunits [46–48]. Each of the monomers has three domains: the reductase domain, the oxygenase domain and the calmodulin-binding domain, which is shown in Figure 1. The reductase domain consists of binding sites for FMN, FAD and NADPH, while the oxygenase domain is responsible for binding tetrahydrobiopterin (BH_4). The task of the reductase domain, within which FMN and FAD play the role of functional groups, is to transport electrons from NADPH to the oxygenase domain of the opposite subunit. In turn, calmodulin binding is necessary to maintain the activity of each of the NOS isoforms [12,49].

All three isoforms catalyze the same reaction. In the first stage, the enzyme catalyzes the oxidation of L-arginine, thanks to which an intermediate compound—N-hydroxy-L-arginine—is formed, which is then oxidized to L-citrulline and NO is created [12,50].

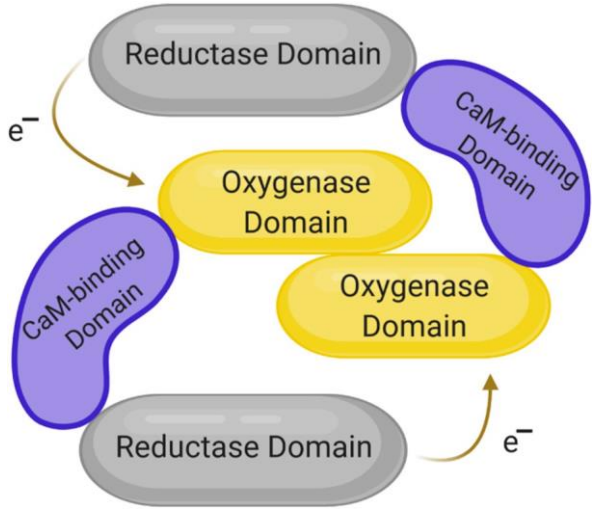

Figure 1. Schematic structure of nitric oxide synthase (NOS). Modified based on [49,50]. Created with BioRender.com. Legend: CaM—calmodulin; e^-—free electron.

NO, being a product of the NOS-catalyzed reaction, is also responsible for regulating this enzyme's expression and activity. By reacting with the amino acid residues of the molecule and forming the S-nitroso group, NO can reversibly inhibit NOS activity [51]. Negative feedback of NO has been shown through a process called S-nitrosylation [52]. NOS1 and NOS2 can also undergo S-nitrosylation, although dynamic regulation of their function through such a route has not been proven. In addition, these two isoenzymes can form ferrous-nitrosylcomplexes in their heme groups, which causes their partial inactivation [53]. The factor limiting NO synthesis is the L-arginine substrate's availability, which may be particularly important for cells in which the NOS2 isoenzyme is available [54]. NO synthesis catalyzed by NOS is shown in Figure 2.

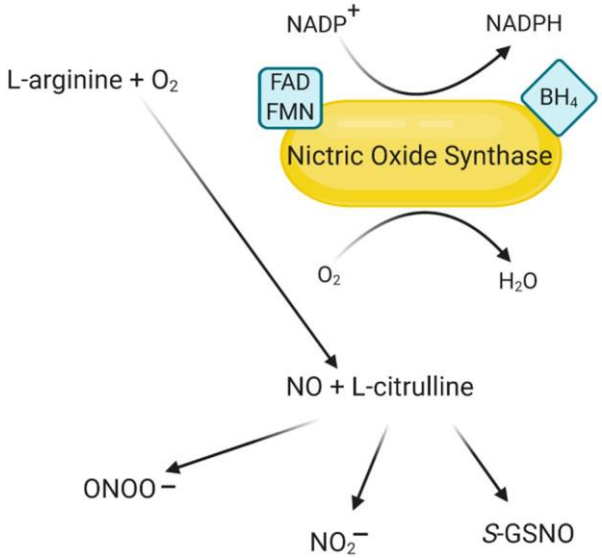

Figure 2. Synthesis of NO and related products. Modified based on [55–57]. Created with BioRender.com. Legend: FAD—flavin adenine dinucleotide; FMN—flavin mononucleotide; BH_4—tetrahydrobiopterin; H_2O—water; $NADP^+$—glutamate dehydrogenase; NADPH—reduced nicotinamide adenine dinucleotide phosphate; O_2—oxygen; S-GSNO—S-nitrosoglutathione.

4. The Role of NO and NOS in the Context of Inflammation, Diabetes and Cardiovascular Diseases

Although NO is a ubiquitous intercellular transmitter in all vertebrates, responsible for modulating blood flow and nervous activity, its excessive production can lead to nitrosative stress, leading to many pathological conditions. NO itself is not very toxic since the body is able to minimize the means that cause its accumulation. This action involves a group of scavenging enzymes, SOD or catalase, thanks to which NO is quickly removed by diffusion through tissues into erythrocytes. It is then transformed into nitrate by reaction with oxyhemoglobin [58,59].

NO derivatives, such as peroxynitrite ($ONOO^-$), are much more potent oxidants. The $ONOO^-$-forming reaction occurs very quickly and no enzyme is required. NO is the only known biological molecule that reacts very quickly with superoxide. At the same time, it is produced in such high concentrations that it can overcome endogenous levels of SOD and react with the superoxide before SOD removes it. It was formerly thought that NO alone directly attacks and damages cell DNA. It is currently believed that this effect depends precisely on the conversion of NO into higher nitrogen oxides. However, NO can reversibly inhibit transition metal enzymes or free radical intermediates in the catalytic cycle. It also demonstrates the ability to inhibit catalase and cytochrome P-450 reversibly. It can also inhibit ribonucleotide reductases, the enzymes responsible for DNA synthesis [50,58–60].

4.1. The Role of NO and NOS in Inflammation

In 1994, the relationship between the increase in NOS activity and inflammation development was shown [61]. The study involved NOS2 and cyclooxygenase (COX), which converts arachidonic acid into prostaglandin H2 that is then further metabolized to prostanoids. The parameters given were measured in the acute, chronic and receding stages of a mouse model of the granulomatosis air sac. COX and NOS2 activity were measured in acute phase skin samples for up to 24 h. Activities in granulomatous tissue were measured after 3, 5, 7, 14 and 21 days for chronic and resolving inflammation. The activity of tested NOS2 increased over 24 h on the skin and there was also a significant increase in granulomatous tissue between day 3 and day 7, followed by a decrease on day 14 and a further increase on day 21. However, in the chronic receding phase, decreased activity of both tested enzymes could be observed. This may indicate their diverse regulation, which may result from the changing cytokine pattern during the inflammatory response [61].

A few years later, other researchers also considered the role of NO and NOS in the immune response and inflammation [62]. It was already known that NO is synthesized by many cell types that are involved in immunity and inflammatory reactions. Ultimately, NO is an important molecule that participates in the body's defense reactions against pathogenic microorganisms. The main enzyme involved in its production during inflammation is NOS2, which causes long-term NO synthesis at a high level. However, the role of NO in immune diseases and inflammation is still unclear. At high concentrations generated by NOS2, NO is rapidly oxidized to RNS, which mediate most of the effects of NO on the immune system. RNS can modify key signaling molecules, such as kinases and transcription factors, e.g., phosphoinositide 3-kinases (PI3K) [63]. They also inhibit several critical enzymes in mitochondrial respiration, for example nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) or monoamine oxidases (MAO), which leads to depletion of ATP and cellular energy [64,65].

The latest studies also focus on the role of NO in accompanying inflammation, including inflammatory joint disease and the role of this molecule in endothelial function. Endothelial dysfunction is attributed to a reduction in the biological activity of NO in rheumatoid arthritis (RA). However, the relationship between NO and endothelial inflammation and dysfunction in RA has not yet been thoroughly investigated and explained [66]. Research conducted by Garg et al. [67] showed that serum nitrite levels in RA patients were significantly higher compared to the control group. A positive correlation between

the concentrations of nitrate, C-reactive protein (CRP) and TNF-α was also observed. These studies show that inflammatory disease activity and endothelial dysfunction in RA are associated with increased levels of pro-inflammatory cytokines and NO. The release of cytokines induced the production of NO, which mediates endothelial dysfunction. Therefore, it should be noted that NO plays an essential role in inflammation-induced endothelial dysfunction in RA [67].

4.2. The Role of NOS in Obesity

At the beginning of the 21st century, it was shown that NO is produced in adipose tissue and that lipolysis can be inhibited by this molecule. One of the studies included obese men who had NOS expression analyzed in the subcutaneous fat [68]. The results showed that NOS2 and NOS3 mRNA expression was detected in isolated fat cells and pieces of adipose tissue. The mRNA of NOS1, however, was not detected. Hormone-sensitive lipase (HSL), the enzyme responsible for regulating lipolysis, showed reduced activity in obese people. The expression of HSL in the subcutaneous fat was also examined in the same subgroup of patients. According to the results of this study, HSL levels were reduced in obese patients. The study showed that NOS2 and NOS3, but not NOS1, were present in human subcutaneous fat. In addition, NOS3 expression and NOS3 protein levels were increased in obese patients, while HSL protein levels were reduced. Increased NO production and reduced HSL levels may be able to induce reduced lipolysis of subcutaneous fat in obesity [68].

A few years later, intensive work began on the inhibition of NOS2 that would contribute to the treatment of obesity. The study involved obese mice that had reduced scatter sensitivity to satiety signals [69]. The nodose ganglia and jejunum were analyzed by immunoblotting for NOS2 expression. NOS2 expression and NO production were found to be increased in the nodose ganglia and the small intestine in obese mice. It was also observed that NOS2 pretreatment with inhibitors—L-NIL (hydrochloride) and N^{ω}-propyl-L-arginine—in obese mice increased the excitability of the nodal neuron and thus restored afferent sensitivity to satiety signals and reduced short-term energy intake. In obese mice given NOS2 inhibitors daily for three weeks, reduced energy intake and reduced weight gain in the first week and less epididymal fat at the end of three weeks were seen compared to saline mice. The results of these studies show that inhibiting NOS2 or blocking the action of NO on afferent pathways can be used to treat obesity.

In 2020, Udi et al. [70] investigated the effect of a new hybrid inhibitor—dual cannabinoid receptor type 1 (CB_1 receptor)/NOS2 antagonist—on the relief of obesity-induced chronic kidney disease (CKD). To this end, said formulation was orally administered to mice for 28 days. The inhibitor was shown to reduce morphological and functional changes in the kidneys caused by obesity by reducing kidney inflammation, fibrosis, oxidative stress and kidney damage. This study shows that blocking CB_1 receptors and NOS2 may be of great therapeutic importance in alleviating obesity-related CKD.

Research on the relationship between gene polymorphisms encoding NOS isoforms and the development of obesity is inconsistent. However, the polymorphism of the NOS3 (rs2070744) seems to be of significant interest [71]. The influence of this polymorphism on metabolic syndrome (MetS) in obese children and adolescents has been demonstrated. The distribution of NOS3 genotypes in the studied groups was compared. It has also been shown that the CC genotype of the rs2070744 polymorphism is associated with MetS in obese children and adolescents [72]. However, more research is certainly needed on the influence of NOS3 polymorphisms and other genetic markers on the risk of developing metabolic diseases.

Finally, Teixeira et al. [73] investigated the kinetic response of NO after a session of acute eccentric resistance exercise (ERE) and the possible effect of the rs1799983 polymorphism in the NOS3 in elderly obese women. To this end, 87 women completed seven sets of ten eccentric repetitions at 110% of the ten maximum repetitions. The group with the GG genotype was characterized by higher body weight, obesity, higher BMI and relatively

higher muscle strength, with significantly lower concentrations of triglycerides, VLDL and urea compared to the groups with the TT and TG genotypes. Hence, carriers of T should pay more attention to cardiovascular risk factors and metabolic disorders.

4.3. The Role of NOS in Insulin Resistance and Diabetes

Expression of all three NOS isoforms can be detected in pancreatic β cells. NOS1 is located mainly in the secretory granules of insulin and in the mitochondria and the cell nucleus [74]. In contrast, NOS2 is not detectable in β-cells at basal glucose levels; its expression occurs only after exposure to higher glucose concentrations [75,76]. However, it is known that in the states of insulin resistance, NOS2 participates in the deregulation of metabolic processes of tissues by disturbing the balance in glucose and lipid homeostasis and endothelial dysfunction through the creation of local and systemic inflammatory environments [77]. This is due to increased nitrosation stress, which affects the action of proteins involved in the maintenance of metabolism and vascular homeostasis through cysteine S-glutathionylation and the nitration of tyrosine residues of other vital proteins. The presence of NOS3 in pancreatic cells has also been confirmed but there is still too little data on its function [78].

An important issue is also the role of mitochondria in NO generation. It is well known that NO can act as an inducer of mitochondrial permeability transition (MPT) through its direct effect on MPT pores [79]. Additionally, NO may induce indirect effects secondary to the inhibition of oxidative phosphorylation, which may trigger apoptosis by inducing mitochondrial membrane permeabilization [80]. These activities may contribute to the development of diabetes mellitus, as diabetes is closely associated with changes in the structure and function of the mitochondria at the cellular level [81,82]. Disruption of glucose uptake, which is the primary source of energy, disrupts the metabolism of cellular energy and thus the functioning of the mitochondria. Thus, the mutual connection between the functioning of mitochondria and the pathophysiology of diabetes is visible. Moreover, the development of oxidative stress accompanies both diabetes and the induction of MPT pores. On this basis, it can be concluded that the MPT pores are directly involved in the pathology of diabetes [83]. However, additional studies are needed to clearly confirm or rule out this thesis.

NO from NOS1 and NOS3 can act as a mediator or inhibitor in the negative feedback associated with glucose-stimulated insulin secretion (GSIS) [84]. In addition, NOS1-derived NO increases glucokinase (GK) activity through S-nitrosylation of cysteine residues, a critical process that mediates the dissociation of GK from and enhances insulin secretion [85]. In isolated islets of Langerhans, increasing the glucose dose increases the activity of all three NOS isoforms. However, the activity of NOS1 and NOS3 is more quickly adapted to increasing glucose concentration [86]. Inhibition of the activity of these two isoforms in islets of Langerhans enhances GSIS. This negative feedback effect inhibits excessive insulin secretion in response to high glucose levels and protects pancreatic β cells. One possible mechanism of NO negative feedback on GSIS is the inhibition of phospho-fructokinase and glucose metabolism in pancreatic β-cells [87]. Apart from the production of NO, NOS1 also exhibits cytochrome C reductase activity [88]. NOS1 inhibits GSIS by increasing NO production and stimulating GSIS through its nonoxidative activity; a balance between these two activities is essential for proper insulin secretion in response to glucose [89].

NOS2 protein expression is high in pancreatic islets of patients with type 2 diabetes (T2D) and inhibition of NOS2 expression restores disturbed GSIS [90]. NO derived from NOS2 most often causes β-cell dysfunction, impaired insulin secretion, hyperglycemia and the development of diabetes [91]. NOS2, through the cGMP-independent mechanism, inhibits insulin secretion [87] as a result of inhibition of the mitochondrial electron transport chain (complexes I and II) and the activity of mitochondrial aconitase [92], S-nitrosylation of critical thiol groups involved in the secretory process [93] and also tyrosine nitration and subsequent GK regulation [94].

The hypothalamic NOS–NO system is involved in the central regulation of glucose homeostasis and NOS1 is the main isoform involved in this process [95]. The central NOS–NO system regulates insulin secretion and its peripheral action and the acute blockage of NOS in the CNS causes hyperglycemia, peripheral insulin resistance and decreased insulin secretion [96]. Elevated NO concentrations in the hypothalamus lead to liver insulin resistance and increased GSIS [97]. However, the mechanisms by which the central NOS–NO system regulates insulin secretion and mediates the effects of insulin on peripheral tissues are not yet well understood.

Endothelial dysfunction, such as impaired NO production, is considered an early stage in the development of insulin resistance, atherosclerosis and T2D [98]. Studies assessing the relationship between ischemic heart disease (CHD) and endothelial dysfunction have clearly shown that reduced NO-dependent endothelial vasodilatation is an early functional disorder in the development of atherosclerotic lesions [99]. Moreover, as previously mentioned, among the many effects of NO, its ability to modulate peripheral and hepatic glucose metabolism and insulin secretion has also been demonstrated. It has also been suggested that changes in NO play an important role in the development of insulin resistance and T2D [100].

In T2D, polymorphisms in the NOS3 seem to attract particular attention, such as the tandem repeat polymorphism (VNTR) of the NOS3, which has been associated with the development of diabetic nephropathy. A study involving Japanese patients showed that the VNTR of the NOS3 might be associated with the progression of diabetic nephropathy in people diagnosed with T2D [101].

Another study focused on assessing *NOS3* polymorphisms in the context of the risk of diabetic nephropathy concerned the analysis of polymorphisms: rs1799983, rs2070744 and 27-bp VNTR [102]. A total of 400 patients with T2D were enrolled in this study. The group with diabetic nephropathy consisted of 200 patients; the group with diabetes without nephropathy also consisted of 200 patients. Genetic analysis of the *NOS3* polymorphisms was carried out for all subjects. The T allele of the rs1799983 polymorphism and the C allele of the rs2070744 polymorphism were significantly more frequent in diabetic nephropathy patients than in patients without nephropathy. However, in the case of the 27-bp VNTR polymorphism, there was no significant change in NO concentrations in the different genotyping groups of patients with diabetic nephropathy and without it. The obtained results suggest that the *NOS3* polymorphisms could indeed constitute genetic determinants of the development of diabetic nephropathy in patients with T2D in the studied population

4.4. The Role of NOS in Heart Disease

NOS1 is the primary endogenous source of NO for the heart, facilitating myocardial relaxation and modulating contractions. Therefore, this isoform plays a key role in protecting the myocardium against the effects of increased oxidative stress, systolic/diastolic dysfunction, adverse structural remodeling and heart failure disorders. In a healthy heart, NOS1-derived NO attenuates the underlying inotropy of the heart by modulating L-type calcium channel (LTCC) activity in the plasma membrane to reduce the amplitude of intracellular Ca^{2+} transition states [103] through S-nitrosylation or cGMP-dependent mechanisms. In the sarcoplasmic reticulum, NO facilitates myocyte relaxation by promoting intracellular Ca^{2+} reuptake [104]. NO derived from NOS1 can be activated directly by S-nitrosylation [105] or indirectly by peroxynitrite-dependent S-glutathionylation [106]. In addition, NOS1 may affect myocardial function by regulating mitochondrial proteins. It has been reported that NO derived from NOS1 inhibits the mitochondrial respiratory chain, including complexes I, III and IV [107–109], and reduces mitochondrial oxygen consumption, thus affecting heart metabolism.

In states like ischemia-reperfusion injury [110], infarction [111], hypertrophy and heart failure [112,113], the expression and activity of NOS1 are increased. Various studies indicate that NO from NOS1 prevents diastolic dysfunction, increases the β-adrenergic reserve, reduces left ventricular hypertrophy and protects the heart against arrhythmogen-

esis [114]. Zhang et al. [114] suggest that the increase in NOS1 concentration and activity is an early event after pathogenic trauma and during disease progression and that NOS1 has a protective function in the heart. It has been shown that acute in vitro treatment with angiotensin II of isolated left ventricular (LV) myocytes significantly increases the expression and activity of NOS1. In turn, NOS1-derived NO decreased NADPH oxidase superoxide production and facilitated the relaxation of LV myocytes through cGMP/PKG-dependent PLN-Ser16 phosphorylation. Similarly, NOS1 expression and activity were increased in the LV myocytes of rats with Ang II-induced early hypertension. NOS1 activity was equal to the ratio of phosphorylated NOS1 levels to total NOS1 levels [115].

Nishijima et al. [116] investigated the effect of NOS2 on atrial oxidative stress and electrophysiological changes in the heart in dogs. To this end, the animals were divided into two groups—one received a placebo while the other received active treatment (NOS cofactor, BH_4 and NOS substrate). Heart failure increased atrial NOS2 and decreased atrial BH_4, while L-arginine remained unchanged. Heart failure resulted in left atrial oxidative stress that was weakened by treatment with BH_4 and L-arginine. This indicates that chronic ischemic heart failure leads to atrial oxidative stress and electrophysiological abnormalities through BH_4 depletion and NOS2 decoupling. Thus, modulation of NOS2 activity by supplementing BH_4 may be an effective way to reduce the frequency of atrial arrhythmias.

Research confirms that the other two NOS isoforms may also be associated with the pathophysiology of heart disease. Liu et al. [117] evaluated the role of NOS3 in the pathogenesis of hypoplastic coronary arteries. For this purpose, they used three groups of mice—wild-type (WT), NOS3-deficient and mice with heart-specific NOS3 overexpression. NOS3 deficiency resulted in coronary artery hypoplasia in fetal mice and spontaneous myocardial infarction in postpartum hearts. In NOS3-deficient mice at birth, significant reductions in coronary artery diameter, vessel density and volume were found. In addition, NOS3-deficient mice showed substantial increases in ventricular wall thickness, myocardial volume and cardiomyocyte size compared to WT mice. Therefore, it should be assumed that NOS3 is essential for the development of coronary arteries and that its deficiency leads to hypoplastic coronary arteries.

It is known that single nucleotide polymorphisms (SNPs) in NOS are associated with the cardiovascular system's pathophysiology. Levinsson et al. [118] investigated NOS variants' association with CHD and hypertension. For this purpose, 560 people diagnosed with CHD were genotyped at 58 selected SNPs in the NOS that were most strongly associated with the aforementioned ailments. It turned out that the SNP of NOS1, rs3782218, showed the most consistent association with both phenotypes. In turn, the association with CHD was observed with two other SNPs—those of NOS1 (rs2682826) and NOS3 (rs1549758). In the case of arterial hypertension, additional SNPs were observed, including the SNP of NOS3, rs3918226. It was thereby confirmed that NOS1 is the most important risk gene for NOS-dependent coronary artery disease.

However, another study showed an association between NOS polymorphisms and increased susceptibility to the development of atherosclerosis and coronary artery disease (CAD). For this purpose, the researchers tested for the 27-bp tandem repeat polymorphism (VNTR) in intron 4 of the *NOS3* in 141 unrelated CAD patients with positive coronary angiograms and 159 age-matched controls with no symptomatic history of CAD [119]. Although the frequency of different genotypes of this polymorphism differed significantly between patients with CAD and the control group, it cannot be determined whether this polymorphism was an independent factor in developing the studied disease. For this purpose, more detailed studies should be conducted on a larger number of subjects.

Selected polymorphisms of the various NOS isoforms, along with their associated diseases, are presented in Table 2.

Table 2. Selected polymorphisms of NOS and their characteristics [71–73,101,102,118–124].

Polymorphism	Isoform	Location	Disease	References
rs3782218	NOS1	C2637T	ischemic heart disease, hypertension	[118]
rs2682826	NOS1	C276T	ischemic heart disease	[118]
rs2779248	NOS2	T278C	type 2 diabetes	[120,121]
rs1137933	NOS2	C231T	type 2 diabetes	[120,121]
Tandem repeat polymorphism (VNTR)	NOS3	27-bp VNTR	diabetic nephropathy, acute eccentric resistance exercise, metabolic syndrome, atherosclerosis, coronary artery disease	[101,102,119]
rs1799983	NOS3	G894T	diabetic nephropathy	[73,102,122]
rs2070744	NOS3	T786C	diabetic nephropathy	[71,72,102]
rs1549758	NOS3	C774T	ischemic heart disease, coronary artery disease	[118,123]
rs3918226	NOS3	C665T	hypertension	[118,124]

5. Selected NOS Inhibitors and Their Role in Therapy

As already explained, NO plays a vital role in the homeostasis of various physiological systems, including micro- and macrovascularization, inhibition of platelet aggregation and regulation of neurotransmission in the central nervous system and gastrointestinal, respiratory and genitourinary systems. However, its overproduction is associated with many diseases, such as arthritis, asthma, cerebral ischemia, Parkinson's disease, neurodegeneration and seizures [125]. For this reason, greater interest should be directed to the design of NOS inhibitors with therapeutic purposes.

The first designed NOS inhibitors appeared in the 1980s and 1990s and were based on L-arginine, an enzyme substrate. This approach led to strong compounds but, unfortunately, with a poor level of selectivity among the isoforms. At the end of the 1990s, the first crystal structures of NOS2 and NOS3 were revealed, showing a high degree of similarity, especially in their active site. One of the most critical moments in the history of NOS inhibitors was the description of highly selective NOS2 inhibitors by Garvey et al. in 1994 [126]. The compounds were isothiourea derivatives designed as reversible inhibitors that competed with the L-arginine of human NOS2, with 190-fold selectivity compared to NOS3 but only about 5-fold compared to NOS1 [126,127]. However, further studies of this group led to the design of a highly selective compound for both NOS2 and NOS3 that could penetrate cells and tissues [127,128]. The crystal structure of NOS1 was described in 2002 and this achievement made it possible to design selective inhibitors [129,130]. NOS isoforms were approved as targets for new drugs shortly after their X-ray crystallography became available. Since then, the design of effective and selective inhibitors has become an important approach in developing modern drugs that cover the biochemical pathways of NO associated with many dysfunctions of the human body [131–133].

A number of NOS inhibitors have already been evaluated in clinical trials. Some of them are presented in Table 3. One of them was so-called tilarginine, a nonselective L-NMMA compound that has been assessed in North America and Europe. The administration of a 1 mg/kg bolus and a 5-h infusion did not reduce mortality in patients with refractory cardiogenic shock complicating myocardial infarction despite an open infarction artery. Although good results were shown in phase II, it was not successful in phase III [134,135]. In another study, L-NMMA showed no differences in mean arterial pressure (MAP) after 2 h compared to the placebo group [136].

Table 3. NOS inhibitors and research conducted on them [134–146].

Inhibitor Name	Country/Continent	Application in Clinical Test/Research	References
Tilarginine (L-NMMA)	North America, Europe	Patients with cardiogenic shock; patients with breast cancer	[134–136]
N(G)-nitro-L-arginine methyl ester (L-NAME)	North America, Asia	Patients with cardiogenic shock; patients with septic shock	[137,138]
Asymmetric dimethylarginine (ADMA)	Europe	Possible use as a cardiovascular risk factor	[139,140]
N(G)-methyl-l-arginine hydrochloride	Europe	Used to restore the balance of vasomotor tone in patients with septic shock	[141,142]
7-nitroindazole (7-NI)	Europe, North America	Anticonvulsive properties in seizure models in rodents	[143,144]
Aminoguanidine	Asia	Alleviation of graft-versus-host disease in mice; alleviation of the susceptibility of mice to bacterial infections	[145,146]

However, when evaluating another inhibitor, N(G)-nitro-L-arginine methyl ester, in the treatment of refractory cardiogenic shock, it was shown that death after one month was 27% in the study group compared to 67% in the control group [137]. Further studies have been conducted to verify this; however, it was found that nonselective NOS inhibitors were of no clinical interest [147].

It should certainly be emphasized that the research on NOS inhibitors clearly moved forward, all thanks to X-ray crystallographic studies of this enzyme. This helps, in structure-based design approaches, in the search for selective inhibitors and in understand their mechanisms of action. Regardless, efforts have been made to give them a drug-like profile.

6. Conclusions

It is well recognized that NO has an essential role in many physiological processes. However, disturbances in their production, caused, e.g., by altered concentration or activity of NOSs, may drive the development of many pathologies. The relationship between the occurrence of specific polymorphisms of genes encoding isoforms of NOS and the development of insulin resistance has been demonstrated. Several genetic variants in the *NOS3* locus are associated with the development of type 2 diabetes and susceptibility to other metabolic complications. In turn, polymorphisms in *NOS2* are associated with a higher plasma glucose concentration and variants in the promoter sequence of this gene also correlate with type 2 diabetes. However, polymorphisms of the genes encoding NOSs are not only associated with metabolic disorders. Certain changes in *NOS1* appear to be related to high blood pressure or heart diseases such as CHD.

As can be seen from the examples above, the genetic changes associated with NOS can affect several organs or systems. Therefore, research on the polymorphisms of genes encoding NOS isoforms seems interesting and justified. Such studies may contribute to a better understanding of the molecular disorders that occur in specific disease entities. In addition, although many of these diseases are very common and many studies are carried out on them, many processes in the human body have still not been fully explained yet. Focusing on these topics can help confirm the role of genetic predisposition in the populations with these conditions. Furthermore, studies on the effects of NOS inhibitors in treating various diseases also seem to be necessary; however, to confirm their effectiveness, additional or more extended clinical trials are required.

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References

1. Togliatto, G.; Lombardo, G.; Brizzi, M.F. The future challenge of reactive oxygen species (ros) in hypertension: From bench to bed side. *Int. J. Mol. Sci.* **2017**, *18*, 1988. [CrossRef]
2. Ristow, M. Unraveling the truth about antioxidants: Mitohormesis explains ros-induced health benefits. *Nat. Med.* **2014**, *20*, 709–711. [CrossRef]
3. Ferreira, C.A.; Ni, D.; Rosenkrans, Z.T.; Cai, W. Scavenging of reactive oxygen and nitrogen species with nanomaterials. *Nano Res.* **2018**, *10*, 4955–4984. [CrossRef]
4. Ganten, D.; Ruckpaul, K. Nitrosative Stress. In *Encyclopedic Reference of Genomics and Proteomics in Molecular Medicine*; Springer: Berlin/Heidelberg, Germany, 2005. [CrossRef]
5. Pérez-Torres, I.; Manzano-Pech, L.; Rubio-Ruíz, M.E.; Soto, M.E.; Guarner-Lans, V. Nitrosative stress and its association with cardiometabolic disorders. *Molecules* **2020**, *25*, 2555. [CrossRef] [PubMed]
6. Förstermann, U.; Sessa, W.C. Nitric oxide synthases: Regulation and function. *Eur. Heart J.* **2012**, *33*, 829–837. [CrossRef] [PubMed]
7. Feng, Q.; Hedner, T. Endothelium-Derived relaxing factor (EDRF) and nitric oxide (NO). II. Physiology, pharmacology and pathophysiological implications. *Clin. Physiol.* **1990**, *10*, 503–526. [CrossRef] [PubMed]
8. Naseem, K.M. The role of nitric oxide in cardiovascular diseases. *Mol. Asp. Med.* **2005**, *26*, 33–65. [CrossRef]
9. Thomas, D.D.; Liu, X.; Kantrow, S.P.; Lancaster, J.R., Jr. The biological lifetime of nitric oxide: Implications for the perivascular dynamics of NO and O2. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 355–360. [CrossRef]
10. Ziche, M.; Morbidelli, L. Nitric oxide and angiogenesis. *J. Neurooncol.* **2000**, *50*, 139–148. [CrossRef]
11. Ji, H.; Tan, S.; Igarashi, J.; Li, H.; Derrick, M.; Martásek, P.; Roman, L.J.; Vásquez-Vivar, J.; Poulos, T.L.; Silverman, R.B. Selective neuronal nitric oxide synthase inhibitors and the prevention of cerebral palsy. *Ann. Neurol.* **2009**, *65*, 209–217. [CrossRef]
12. Ługowski, M.; Saczko, J.; Kulbacka, J.; Banas´, T. Reaktywne formy tlenu i azotu. *Pol. Merk. Lek.* **2011**, *185*, 313–317. [PubMed]
13. Bailey, S.M.; Landar, A.; Darley-Usmar, V. Mitochondrial proteomics in free radical research. *Free Radic. Biol. Med.* **2005**, *38*, 175–188. [CrossRef] [PubMed]
14. PubChem Substance and Compound Databases—NCBI—NIH. Available online: https://pubchem.ncbi.nlm.nih.gov/compound/Nitrilooxonium (accessed on 18 December 2020).
15. Hayton, T.W.; Legzdins, P.; Sharp, W.B. Coordination and organometallic chemistry of metal-NO complexes. *Chem. Rev.* **2002**, *102*, 935–992. [CrossRef] [PubMed]
16. PubChem Substance and Compound Databases—NCBI—NIH. Available online: https://pubchem.ncbi.nlm.nih.gov/compound/Nitrogen-dioxide (accessed on 18 December 2020).
17. PubChem Substance and Compound Databases—NCBI—NIH. Available online: https://pubchem.ncbi.nlm.nih.gov/compound/Dinitrogen-trioxide (accessed on 18 December 2020).
18. Goldstein, S.; Merényi, G. The chemistry of peroxynitrite: Implications for biological activity. *Methods Enzymol.* **2008**, *436*, 49–61. [CrossRef] [PubMed]
19. PubChem Substance and Compound Databases—NCBI—NIH. Available online: https://pubchem.ncbi.nlm.nih.gov/compound/Nitrite (accessed on 18 December 2020).
20. PubChem Substance and Compound Databases—NCBI—NIH. Available online: https://pubchem.ncbi.nlm.nih.gov/compound/Nitrate (accessed on 18 December 2020).
21. Bianco, C.L.; Toscano, J.P.; Bartberger, M.D.; Fukuto, J.M. The chemical biology of HNO signaling. *Arch. Biochem. Biophys.* **2017**, *617*, 129–136. [CrossRef] [PubMed]
22. Lancaster, J.R., Jr. Historical origins of the discovery of mammalian nitric oxide (nitrogen monoxide) production/physiology/pathophysiology. *Biochem. Pharmacol.* **2020**, *176*, 113793. [CrossRef] [PubMed]
23. Tuteja, N.; Chandra, M.; Tuteja, R.; Misra, M.K. Nitric oxide as a unique bioactive signaling messenger in physiology and pathophysiology. *J. Biomed. Biotechnol.* **2004**, *4*, 227–237. [CrossRef]

24. Murad, F. Discovery of some of the biological effects of nitric oxide and its role in cell signaling. *Biosci. Rep.* **2004**, *24*, 452–474. [CrossRef]
25. Murad, F. Nitric oxide signaling: Would you believe that a simple free radical could be a second messenger, autacoid, paracrine substance, neurotransmitter, and hormone? *Recent Prog. Horm. Res.* **1998**, *53*, 43–59. [PubMed]
26. Patel, R.P.; McAndrew, J.; Sellak, H.; White, C.R.; Jo, H.; Freeman, B.A.; Darley-Usmar, V.M. Biological aspects of reactive nitrogen species. *Biochim. Biophys. Acta* **1999**, *1411*, 385–400. [CrossRef]
27. Crow, J.P.; Beckman, J.S. The importance of superoxide in nitric oxide-dependent toxicity: Evidence for peroxynitrite-mediated injury. *Adv. Exp. Med. Biol.* **1996**, *387*, 147–161. [CrossRef] [PubMed]
28. Kurutas, E.B. The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: Current state. *Nutr J.* **2016**, *15*, 71. [CrossRef] [PubMed]
29. Rubanyi, G.M.; Ho, E.H.; Cantor, E.H.; Lumma, W.C.; Botelho, L.H. Cytoprotective function of nitric oxide: Inactivation of superoxide radicals produced by human leukocytes. *Biochem. Biophys. Res. Commun.* **1991**, *181*, 1392–1397. [CrossRef]
30. Beckman, J.S.; Beckman, T.W.; Chen, J.; Marshall, P.A.; Freeman, B.A. Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 1620–1624. [CrossRef]
31. O'Donnell, V.B.; Eiserich, J.P.; Chumley, P.H.; Jablonsky, M.J.; Krishna, N.R.; Kirk, M.; Barnes, S.; Darley-Usmar, V.M.; Freeman, B.A. Nitration of unsaturated fatty acids by nitric oxide-derived reactive nitrogen species peroxynitrite, nitrous acid, nitrogen dioxide, and nitronium ion. *Chem. Res. Toxicol.* **1999**, *12*, 83–92. [CrossRef]
32. Wink, D.A.; Darbyshire, J.F.; Nims, R.W.; Saavedra, J.E.; Ford, P.C. Reactions of the bioregulatory agent nitric oxide in oxygenated aqueous media: Determination of the kinetics for oxidation and nitrosation by intermediates generated in the NO/O2 reaction. *Chem. Res. Toxicol.* **1993**, *6*, 23–27. [CrossRef]
33. Pacher, P.; Beckman, J.S.; Liaudet, L. Nitric oxide and peroxynitrite in health and disease. *Psychol. Rev.* **2007**, *87*, 315–424. [CrossRef]
34. Griffith, O.W.; Stuehr, D.J. Nitric oxide synthases: Properties and catalytic mechanism. *Annu. Rev. Physiol.* **1995**, *57*, 707–736. [CrossRef]
35. GeneCards—Human Genes|Gene Database|Gene Search. Available online: https://www.genecards.org/cgi-bin/carddisp.pl?gene=NOS1 (accessed on 4 December 2020).
36. GeneCards—Human Genes|Gene Database|Gene Search. Available online: https://www.genecards.org/cgi-bin/carddisp.pl?gene=NOS3 (accessed on 4 December 2020).
37. Knott, A.B.; Bossy-Wetzel, E. Nitric oxide in health and disease of the nervous system. *Antioxid. Redox Signal.* **2009**, *11*, 541–553. [CrossRef]
38. Fish, J.E.; Marsden, P.A. Endothelial nitric oxide synthase: Insight into cell-specific gene regulation in the vascular endothelium. *Cell Mol. Life Sci.* **2006**, *63*, 144–162. [CrossRef]
39. Sumpio, B.E.; Riley, J.T.; Dardik, A. Cells in focus: Endothelial cell. *Int. J. Biochem. Cell Biol.* **2002**, *34*, 1508–1512. [CrossRef]
40. Förstermann, U.; Münzel, T. Endothelial nitric oxide synthase in vascular disease: From marvel to menace. *Circulation* **2006**, *113*, 1708–1714. [CrossRef] [PubMed]
41. Hevel, J.M.; White, K.A.; Marietta, M.A. Purification of the inducible murine macrophage nitric oxide synthase. Identification as a flavoprotein. *J. Biol. Cherm.* **1991**, *266*, 22789–22791. [PubMed]
42. Karupiah, G.; Xie, Q.W.; Buller, M.L.; Nathan, C.; Duarte, C.; MacMicking, J.D. Inhibition of viral replication by interferon-y-induced nitric oxide synthase. *Science* **1993**, *261*, 1445–1448. [CrossRef] [PubMed]
43. GeneCards—Human Genes|Gene Database|Gene Search. Available online: https://www.genecards.org/cgi-bin/carddisp.pl?gene=NOS2 (accessed on 4 December 2020).
44. Lechner, M.; Lirk, P.; Rieder, J. Inducible nitric oxide synthase (iNOS) in tumor biology: The two sides of the same coin. *Semin. Cancer Biol.* **2005**, *15*, 277–289. [CrossRef] [PubMed]
45. Vanninia, F.; Kashfia, K.; Nath, N. The dual role of iNOS in cancer. *Redox Biol.* **2015**, *6*, 334–343. [CrossRef]
46. Fischmann, T.O.; Hruza, A.; Niu, X.D.; Fossetta, J.D.; Lunn, C.A.; Dolphin, E.; Prongay, A.J.; Reichert, P.; Lundell, D.J.; Narula, S.K.; et al. Structural characterization of nitric oxide synthase isoforms reveals striking active-site conservation. *Nat. Struct. Biol.* **1999**, *6*, 233–242. [CrossRef]
47. Mehl, M.; Daiber, A.; Herold, S.; Shoun, H.; Ullrich, V. Peroxynitrite reaction with heme proteins. *Nitric Oxide* **1999**, *3*, 142–152. [CrossRef]
48. Roman, L.J.; Martasek, P.; Masters, B.S. Intrinsic and extrinsic modulation of nitric oxide synthase activity. *Chem. Rev.* **2002**, *102*, 1179–1190. [CrossRef]
49. Garcin, E.D.; Bruns, C.M.; Lloyd, S.J.; Hosfield, D.J.; Tiso, M.; Gachhui, R.; Stuehr, D.J.; Tainer, J.A.; Getzoff, E.D. Structural basis for isozyme-specific regulation of electron transfer in nitric-oxide synthase. *J. Biol. Chem.* **2004**, *279*, 37918–37927. [CrossRef]
50. Lowenstein, C.J.; Padalko, E. iNOS (NOS2) at a glance. *J. Cell Sci.* **2004**, *117*, 2865–3286. [CrossRef] [PubMed]
51. Maron, B.A.; Tang, S.S.; Loscalzo, J. S-Nitrosothiols and the S-nitrosoproteome of the cardiovascular system. *Antioxid. Redox Signal.* **2013**, *18*, 270–287. [CrossRef] [PubMed]
52. Shahani, N.; Sawa, A. Protein S-nitrosylation: Role for nitric oxide signaling in neuronal death. *Biochim. Biophys. Acta* **2012**, *1820*, 736–742. [CrossRef] [PubMed]

53. Abu-Soud, H.M.; Wang, J.; Rousseau, D.L.; Fukuto, J.M.; Ignarro, L.J.; Stuehr, D.J. Neuronal nitric oxide synthase self-inactivates by forming a ferrous-nitrosyl complex during aerobic catalysis. *J. Biol. Chem.* **1995**, *270*, 22997–23006. [CrossRef]
54. Morris, S.M., Jr. Enzymes of arginine metabolism. *J. Nutr.* **2004**, *134*, 2743S–2747S. [CrossRef] [PubMed]
55. Maréchal, A.; Mattioli, T.A.; Stuehr, D.J.; Santolini, J. Activation of peroxynitrite by inducible nitric-oxide synthase. A direct source of nitrative stress. *J. Biol. Chem.* **2007**, *282*, 14101–14112. [CrossRef]
56. Negrerie, M.; Berka, V.; Vos, M.H.; Liebl, U.; Lambry, J.-C.; Tsai, A.-L.; Martin, J.-L. Geminate recombination of nitric oxide to endothelial nitric oxide-synthase and mechanistic implications. *J. Biol. Chem.* **1999**, *274*, 24694–24702. [CrossRef]
57. Centers for Disease Control and Prevention (US); National Center for Chronic Disease Prevention and Health Promotion (US); Office on Smoking and Health (US). *How Tobacco Smoke Causes Disease: The Biology and Behavioral Basis for Smoking-Attributable Disease: A Report of the Surgeon General*; Centers for Disease Control and Prevention (US): Atlanta, GA, USA, 2010.
58. Roy, B.; Lepoivre, M.; Henry, Y.; Fontecave, M. Inhibition of ribonucleotide reductase by nitric oxide derived from thionitrites: Reversible modifications of both subunits. *Biochemistry* **1995**, *34*, 5411–5418. [CrossRef]
59. Kröncke, K.D.; Fehsel, K.; Kolb-Bachofen, V. Inducible nitric oxide synthase in human diseases. *Clin. Exp. Immunol.* **1998**, *113*, 147–156. [CrossRef]
60. Zhou, L.; Zhu, D.Y. Neuronal nitric oxide synthase: Structure, subcellular localization, regulation, and clinical implications. *Nitric. Oxide* **2009**, *20*, 223–230. [CrossRef]
61. Vane, J.R.; Mitchell, J.A.; Appleton, I.; Tomlinson, A.; Bishop-Bailey, D.; Croxtall, J.; Willoughby, D.A. Inducible isoforms of cyclooxygenase and nitric-oxide synthase in inflammation. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 2046–2050. [CrossRef] [PubMed]
62. Coleman, J.W. Nitric oxide in immunity and inflammation. *Int. Immunopharmacol.* **2001**, *1*, 1397–1406. [CrossRef]
63. Koundouros, N.; Poulogiannis, G. Phosphoinositide 3-Kinase/Akt signaling and redox metabolism in cancer. *Front. Oncol.* **2018**, *8*, 160. [CrossRef] [PubMed]
64. Montezano, A.C.; Touyz, R.M. Reactive oxygen species and endothelial function–role of nitric oxide synthase uncoupling and Nox family nicotinamide adenine dinucleotide phosphate oxidases. *Basic Clin. Pharmacol. Toxicol.* **2012**, *110*, 87–94. [CrossRef]
65. Muriel, P.; Pérez-Rojas, J.M. Nitric oxide inhibits mitochondrial monoamine oxidase activity and decreases outer mitochondria membrane fluidity. *Comp. Biochem. Physiol. Toxicol. Pharmacol.* **2003**, *136*, 191–197. [CrossRef]
66. van't Hof, R.J.; Hocking, L.; Wright, P.K.; Ralston, S.H. Nitric oxide is a mediator of apoptosis in the rheumatoid joint. *Rheumatology* **2000**, *39*, 1004–1008. [CrossRef]
67. Garg, N.; Syngle, A.; Krishan, P. Nitric oxide: Link between inflammation and endothelial dysfunction in rheumatoid arthritis. *Int. J. Angiol.* **2017**, *26*, 165–169. [CrossRef]
68. Elizalde, M.; Rydén, M.; van Harmelen, V.; Eneroth, P.; Gyllenhammar, H.; Holm, C.; Ramel, S.; Ölund, A.; Arner, P.; Andersson, K. Expression of nitric oxide synthases in subcutaneous adipose tissue of nonobese and obese humans. *J. Lipid Res.* **2000**, *41*, 1244–1251. [PubMed]
69. Yu, Y.; Park, S.J.; Beyak, M.J. Inducible nitric oxide synthase-derived nitric oxide reduces vagal satiety signalling in obese mice. *J. Physiol.* **2019**, *597*, 1487–1502. [CrossRef]
70. Udi, S.; Hinden, L.; Ahmad, M.; Drori, A.; Iyer, M.R.; Cinar, R.; Herman-Edelstein, M.; Tam, J. Dual inhibition of cannabinoid CB1 receptor and inducible NOS attenuates obesity-induced chronic kidney disease. *Br. J. Pharmacol.* **2020**, *177*, 110–127. [CrossRef]
71. Pawlik, A.; Błaszczyk, H.; Rac´, M.; Maciejewska-Skrendo, A.; Safranow, K.; Dziedziejko, V. NOS3 gene rs1799983 and rs2070744 polymorphisms in patients with unstable angina. *J. Vasc. Res.* **2020**, *57*, 136–142. [CrossRef] [PubMed]
72. Miranda, J.A.; Belo, V.A.; Souza-Costa, D.C.; Lanna, C.M.; Tanus-Santos, J.E. eNOS polymorphism associated with metabolic syndrome in children and adolescents. *Mol. Cell Biochem.* **2013**, *372*, 155–160. [CrossRef] [PubMed]
73. Teixeira, T.G.; Tibana, R.A.; Nascimento, D.D.; de Sousa, N.M.; de Souza, V.C.; Vieira, D.C.; Nóbrega, O.T.; de Almeida, J.A.; Navalta, J.; Prestes, J. Endothelial nitric oxide synthase Glu298Asp gene polymorphism influences body composition and biochemical parameters but not the nitric oxide response to eccentric resistance exercise in elderly obese women. *Clin. Physiol. Funct. Imaging.* **2016**, *36*, 482–489. [CrossRef] [PubMed]
74. Mezghenna, K.; Pomiès, P.; Chalançon, A.; Castex, F.; Leroy, J.; Niclauss, N.; Nadal, B.; Cambier, L.; Cazevieille, C.; Petit, P.; et al. Increased neuronal nitric oxide synthase dimerisation is involved in rat and human pancreatic beta cell hyperactivity in obesity. *Diabetologia* **2011**, *54*, 2856–2866. [CrossRef]
75. Henningsson, R.; Salehi, A.; Lundquist, I. Role of nitric oxide synthase isoforms in glucose-stimulated insulin release. *Am. J. Physiol. Cell Physiol.* **2002**, *283*, C296–C304. [CrossRef]
76. Jimenez-Feltstrom, J.; Lundquist, I.; Salehi, A. Glucose stimulates the expression and activities of nitric oxide synthases in incubated rat islets: An effect counteracted by GLP-1 through the cyclic AMP/PKA pathway. *Cell Tissue Res.* **2005**, *319*, 221–230. [CrossRef]
77. Muniyappa, R.; Sowers, J.R. Role of insulin resistance in endothelial dysfunction. *Rev. Endocr. Metab. Disord.* **2013**, *14*, 5–12. [CrossRef]
78. Kaneko, Y.K.; Ishikawa, T. Dual role of nitric oxide in pancreatic β-cells. *J. Pharmacol. Sci.* **2013**, *123*, 295–300. [CrossRef]
79. Balakirev, M.Y.; Khramtsov, V.V.; Zimmer, G. Modulation of the mitochondrial permeability transition by nitric oxide. *Eur. J. Biochem.* **1997**, *246*, 710–718. [CrossRef]
80. Vieira, H.; Kroemer, G. Mitochondria as targets of apoptosis regulation by nitric oxide. *IUBMB Life* **2003**, *55*, 613–616. [CrossRef]

81. Montgomery, M.K.; Turner, N. Mitochondrial dysfunction and insulin resistance: An update. *Endocr. Connect.* **2015**, *4*, R1–R15. [CrossRef] [PubMed]
82. Prasun, P. Mitochondrial dysfunction in metabolic syndrome. *Biochim. Biophys. Acta Mol. Basis Dis.* **2020**, *1866*, 165838. [CrossRef] [PubMed]
83. Belosludtsev, K.N.; Belosludtseva, N.V.; Dubinin, M.V. Diabetes mellitus, mitochondrial dysfunction and Ca^{2+}-dependent permeability transition pore. *Int. J. Mol. Sci.* **2020**, *21*, 6559. [CrossRef] [PubMed]
84. Mosén, H.; Ostenson, C.G.; Lundquist, I.; Alm, P.; Henningsson, R.; Jimenez-Feltström, J.; Guenifi, A.; Efendic, S.; Salehi, A. Impaired glucose-stimulated insulin secretion in the GK rat is associated with abnormalities in islet nitric oxide production. *Regul. Pept.* **2008**, *151*, 139–146. [CrossRef] [PubMed]
85. Ding, S.Y.; Tribble, N.D.; Kraft, C.A.; Markwardt, M.; Gloyn, A.L.; Rizzo, M.A. Naturally occurring glucokinase mutations are associated with defects in posttranslational S-nitrosylation. *Mol. Endocrinol.* **2010**, *24*, 171–177. [CrossRef] [PubMed]
86. Nakada, S.; Ishikawa, T.; Yamamoto, Y.; Kaneko, Y.; Nakayama, K. Constitutive nitric oxide synthases in rat pancreatic islets: Direct imaging of glucose-induced nitric oxide production in beta-cells. *Pflugers Arch.* **2003**, *447*, 305–311. [CrossRef] [PubMed]
87. Tsuura, Y.; Ishida, H.; Hayashi, S.; Sakamoto, K.; Horie, M.; Seino, Y. Nitric oxide opens ATP-sensitive K+ channels through suppression of phosphofructokinase activity and inhibits glucose-induced insulin release in pancreatic beta cells. *J. Gen. Physiol.* **1994**, *104*, 1079–1098. [CrossRef]
88. Klatt, P.; Heinzel, B.; John, M.; Kastner, M.; Böhme, E.; Mayer, B. Ca2+/calmodulin-dependent cytochrome c reductase activity of brain nitric oxide synthase. *J. Biol. Chem.* **1992**, *267*, 11374–11378. [PubMed]
89. Gheibi, S.; Ghasemi, A. Insulin secretion: The nitric oxide controversy. *EXCLI J.* **2020**, *19*, 1227–1245. [CrossRef]
90. Muhammed, S.J.; Lundquist, I.; Salehi, A. Pancreatic β-cell dysfunction, expression of iNOS and the effect of phosphodiesterase inhibitors in human pancreatic islets of type 2 diabetes. *Diabetes Obes. Metab.* **2012**, *14*, 1010–1019. [CrossRef]
91. Bahadoran, Z.; Mirmiran, P.; Ghasemi, A. Role of nitric oxide in insulin secretion and glucose metabolism. *Trends Endocrinol. Metab.* **2020**, *31*, 118–130. [CrossRef] [PubMed]
92. Corbett, J.A.; Sweetland, M.A.; Wang, J.L.; Lancaster, J.R., Jr.; McDaniel, M.L. Nitric oxide mediatescytokine-induced inhibition of insulin secretion byhuman islets of Langerhans. *Proc. Natl Acad. Sci. USA* **1993**, *90*, 1731–1735. [CrossRef] [PubMed]
93. Panagiotidis, G.; Akesson, B.; Rydell, E.L.; Lundquist, I. Influence of nitric oxide synthase inhibition, nitric oxide and hydroperoxide on insulin release induced by various secretagogues. *Br. J. Pharmacol.* **1995**, *114*, 289–296. [CrossRef] [PubMed]
94. Kim, J.Y.; Song, E.H.; Lee, H.J.; Oh, Y.K.; Park, Y.S.; Park, J.-W.; Kim, B.J.; Kim, D.J.; Lee, I.; Song, J.; et al. Chronic ethanol consumption-induced pancreaticb-cell dysfunction and apoptosisthrough glucokinase nitration and its down-regulation. *J. Biol. Chem.* **2010**, *285*, 37251–37262. [CrossRef]
95. Fioramonti, X.; Marsollier, N.; Song, Z.; Fakira, K.A.; Patel, R.M.; Brown, S.; Duparc, T.; Pica-Mendez, A.; Sanders, N.M.; Knauf, C.; et al. Ventromedial hypothalamic nitric oxide production is necessary for hypoglycemia detection and counterregulation. *Diabetes* **2010**, *59*, 519–528. [CrossRef]
96. Shankar, R.; Zhu, J.S.; Ladd, B.; Henry, D.; Shen, H.Q.; Baron, A.D. Central nervous system nitric oxide synthase activity regulates insulin secretion and insulin action. *J. Clin. Investig.* **1998**, *102*, 1403–1412. [CrossRef]
97. Chen, W.; Balland, E.; Cowley, M.A. Hypothalamic insulin resistance in obesity: Effects on glucose homeostasis. *Neuroendocrinology* **2017**, *104*, 364–381. [CrossRef]
98. Dhananjayan, R.; Koundinya, K.S.; Malati, T.; Kutala, V.K. Endothelial dysfunction in type 2 diabetes mellitus. *Indian. J. Clin. Biochem.* **2016**, *31*, 372–379. [CrossRef]
99. Matsuzawa, Y.; Lerman, A. Endothelial dysfunction and coronary artery disease: Assessment, prognosis, and treatment. *Coron. Artery Dis.* **2014**, *25*, 713–724. [CrossRef]
100. Sansbury, B.E.; Hill, B.G. Regulation of obesity and insulin resistance by nitric oxide. *Free Radic. Biol. Med.* **2014**, *73*, 383–399. [CrossRef]
101. Rippin, J.D.; Patel, A.; Belyaev, N.D.; Gill, G.V.; Barnett, A.H.; Bain, S.C. Nitric oxide synthase gene polymorphisms and diabetic nephropathy. *Diabetologia* **2003**, *46*, 426–428. [CrossRef] [PubMed]
102. Shoukry, A.; Shalaby, S.M.; Abdelazim, S.; Abdelazim, M.; Ramadan, A.; Ismail, M.I.; Fouad, M. Endothelial nitric oxide synthase gene polymorphisms and the risk of diabetic nephropathy in type 2 diabetes mellitus. *Genet. Test Mol. Biomark.* **2012**, *16*, 574–579. [CrossRef] [PubMed]
103. Sears, C.E.; Bryant, S.M.; Ashley, E.A.; Lygate, C.A.; Rakovic, S.; Wallis, H.L.; Neubauer, S.; Terrar, D.A.; Casadei, B. Cardiac neuronal nitric oxide synthase isoform regulates myocardial contraction and calcium handling. *Circ. Res.* **2003**, *92*, e52–e59. [CrossRef] [PubMed]
104. Zhang, Y.H.; Zhang, M.H.; Sears, C.E.; Emanuel, K.; Redwood, C.; El-Armouche, A.; Kranias, E.G.; Casadei, B. Reduced phospholamban phosphorylation is associated with impaired relaxation in left ventricular myocytes from neuronal NO synthase-deficient mice. *Circ. Res.* **2008**, *102*, 242–249. [CrossRef] [PubMed]
105. Burger, D.E.; Lu, X.; Lei, M.; Xiang, F.L.; Hammoud, L.; Jiang, M.; Wang, H.; Jones, D.L.; Sims, S.M.; Feng, Q. Neuronal nitric oxide synthase protects against myocardial infarction-induced ventricular arrhythmia and mortality in mice. *Circulation* **2009**, *120*, 1345–1354. [CrossRef] [PubMed]
106. Adachi, T.; Weisbrod, R.M.; Pimentel, D.R.; Ying, J.; Sharov, V.S.; Schöneich, C.; Cohen, R.A. S-Glutathiolation by peroxynitrite activates SERCA during arterial relaxation by nitric oxide. *Nat. Med.* **2004**, *10*, 1200–1207. [CrossRef]

107. Torres, J.; Darley-Usmar, V.; Wilson, M.T. Inhibition of cytochrome c oxidase in turnover by nitric oxide: Mechanism and implications for control of respiration. *Biochem. J.* **1995**, *312*, 169–173. [CrossRef]
108. Welter, R.; Yu, L.; Yu, C.A. The effects of nitric oxide on electron transport complexes. *Arch. Biochem. Biophys.* **1996**, *331*, 9–14. [CrossRef]
109. Chouchani, E.T.; Methner, C.; Nadtochiy, S.M.; Logan, A.; Pell, V.R.; Ding, S.; James, A.M.; Cochemé, H.M.; Reinhold, J.; Lilley, K.S.; et al. Cardioprotection by S-nitrosation of a cysteine switch on mitochondrial complex I. *Nat. Med.* **2013**, *19*, 753–759. [CrossRef]
110. Strasen, J.; Ritter, O. Role of nNOS in cardiac ischemia-reperfusion injury. *Trends Cardiovasc. Med.* **2011**, *21*, 58–63. [CrossRef]
111. Saraiva, R.M.; Minhas, K.M.; Raju, S.V.; Barouch, L.A.; Pitz, E.; Schuleri, K.H.; Vandegaer, K.; Li, D.; Hare, J.M. Deficiency of neuronal nitric oxide synthase increases mortality and cardiac remodeling after myocardial infarction: Role of nitroso-redox equilibrium. *Circulation* **2005**, *112*, 3415–3422. [CrossRef] [PubMed]
112. Damy, T.; Ratajczak, P.; Shah, A.M.; Camors, E.; Marty, I.; Hasenfuss, G.; Marotte, F.; Samuel, J.L.; Heymes, C. Increased neuronal nitric oxide synthase-derived NO production in the failing human heart. *Lancet* **2004**, *363*, 1365–1367. [CrossRef]
113. Carnicer, R.; Crabtree, M.J.; Sivakumaran, V.; Casadei, B.; Kass, D.A. Nitric oxide synthases in heart failure. *Antioxid. Redox Signal.* **2013**, *18*, 1078–1099. [CrossRef] [PubMed]
114. Zhang, Y.H.; Jin, C.Z.; Jang, J.H.; Wang, Y. Molecular mechanisms of neuronal nitric oxide synthase in cardiac function and pathophysiology. *J. Physiol.* **2014**, *592*, 3189–3200. [CrossRef]
115. Kar, R.; Kellogg, D.L., 3rd; Roman, L.J. Oxidative stress induces phosphorylation of neuronal NOS in cardiomyocytes through AMP-activated protein kinase (AMPK). *Biochem. Biophys. Res. Commun.* **2015**, *459*, 393–397. [CrossRef]
116. Nishijima, Y.; Sridhar, A.; Bonilla, I.; Velayutham, M.; Khan, M.; Terentyeva, R.; Li, C.; Kuppusamy, P.; Elton, T.S.; Terentyev, D.; et al. Tetrahydrobiopterin depletion and NOS2 uncoupling contribute to heart failure-induced alterations in atrial electrophysiology. *Cardiovasc. Res.* **2011**, *91*, 71–79. [CrossRef]
117. Liu, Y.; Lu, X.; Xiang, F.L.; Poelmann, R.E.; Gittenberger-de Groot, A.C.; Robbins, J.; Feng, Q. Nitric oxide synthase-3 deficiency results in hypoplastic coronary arteries and postnatal myocardial infarction. *Eur. Heart J.* **2014**, *35*, 920–931. [CrossRef]
118. Levinsson, A.; Olin, A.C.; Björck, L.; Rosengren, A.; Nyberg, F. Nitric oxide synthase (NOS) single nucleotide polymorphisms are associated with coronary heart disease and hypertension in the INTERGENE study. *Nitric. Oxide* **2014**, *39*, 1–7. [CrossRef]
119. Salimi, S.; Firoozrai, M.; Nourmohammadi, I.; Shabani, M.; Shafiee, S.M.; Mohebbi, A.; Tavilani, H. Lack of evidence for contribution of intron4a/b polymorphism of endothelial nitric oxide synthase (NOS3) gene to plasma nitric oxide levels. *Acta Cardiol.* **2008**, *63*, 229–234. [CrossRef]
120. Chen, F.; Li, Y.M.; Yang, L.Q.; Zhong, C.G.; Zhuang, Z.X. Association of NOS2 and NOS3 gene polymorphisms with susceptibility to type 2 diabetes mellitus and diabetic nephropathy in the Chinese Han population. *IUBMB Life* **2016**, *68*, 516–525. [CrossRef]
121. Garme, Y.; Moudi, M.; Saravani, R.; Galavi, H. Nitric oxide synthase 2 polymorphisms (rs2779248T/C and rs1137933C/T) and the risk of type 2 diabetes in zahedan, southeastern iran. *Iran J. Public Health* **2018**, *47*, 1734–1741. [PubMed]
122. Möllsten, A.; Wessman, M.; Svensson, M.; Forsblom, C.; Parkkonen, M.; Brismar, K.; Groop, P.H.; Dahlquist, G. Glu298Asp and NOS4ab polymorphisms in diabetic nephropathy. *Ann. Med.* **2006**, *38*, 522–528. [CrossRef] [PubMed]
123. Li, X.; Lin, Y.; Zhang, R. Associations between endothelial nitric oxide synthase gene polymorphisms and the risk of coronary artery disease: A systematic review and meta-analysis of 132 case-control studies. *Eur. J. Prev. Cardiol.* **2019**, *26*, 160–170. [CrossRef] [PubMed]
124. Zhang, X.; Lynch, A.I.; Davis, B.R.; Ford, C.E.; Boerwinkle, E.; Eckfeldt, J.H.; Leiendecker-Foster, C.; Arnett, D.K. Pharmacogenetic association of NOS3 variants with cardiovascular disease in patients with hypertension: The GenHAT study. *PLoS ONE* **2012**, *7*, e34217. [CrossRef] [PubMed]
125. Uttara, B.; Singh, A.V.; Zamboni, P.; Mahajan, R.T. Oxidative stress and neurodegenerative diseases: A review of upstream and downstream antioxidant therapeutic options. *Curr. Neuropharmacol.* **2009**, *7*, 65–74. [CrossRef] [PubMed]
126. Garvey, E.P.; Oplinger, J.A.; Tanoury, G.J.; Sherman, P.A.; Fowler, M.; Marshall, S.; Harmon, M.F.; Paith, J.E.; Furfine, E.S. Potent and selective inhibition of human nitric oxide synthases. Inhibition by non-amino acid isothioureas. *J. Biol. Chem.* **1994**, *269*, 26669–26676. [PubMed]
127. Alderton, W.K.; Cooper, C.E.; Knowles, R.G. Nitric oxide synthases: Structure, function and inhibition. *Biochem. J.* **2001**, *357*, 593–615. [CrossRef]
128. Garvey, E.P.; Oplinger, J.A.; Furfine, E.S.; Kiff, R.J.; Laszlo, F.; Whittle, B.J.; Knowles, R.G. 1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase in vitro and in vivo. *J. Biol. Chem.* **1997**, *272*, 4959–4963. [CrossRef]
129. Silverman, R.B. Design of selective neuronal nitric oxide synthase inhibitors for the prevention and treatment of neurodegenerative diseases. *Acc. Chem. Res.* **2009**, *42*, 439–451. [CrossRef]
130. Annedi, S.C. Cell-Permeable inhibitors of neuronal nitric oxide synthase open new prospects for the treatment of neurological disorders. *J. Med. Chem.* **2015**, *58*, 1064–1066. [CrossRef]
131. Paige, J.S.; Jaffrey, S.R. Pharmacologic manipulation of nitric oxide signaling: Targeting NOS dimerization and protein-protein interactions. *Curr. Top. Med. Chem.* **2007**, *7*, 97–114. [CrossRef]
132. Poulos, T.L.; Li, H. Structural basis for isoform-selective inhibition in nitric oxide synthase. *Acc. Chem. Res.* **2013**, *46*, 390–398. [CrossRef] [PubMed]
133. Vallance, P.; Leiper, J. Blocking NO synthesis: How, where and why? *Nat. Rev. Drug Discov.* **2002**, *1*, 939–950. [CrossRef] [PubMed]

134. Alexander, J.H.; Reynolds, H.R.; Stebbins, A.L.; Dzavik, V.; Harrington, R.A.; Van der Werf, F.; Hechman, J.S. Effect of tilarginine acetate in patients with acute myocardial infarction and cardiogenic shock: The TRIUMPH randomized controlled trial. *J. Am. Med Assoc.* **2007**, *297*, 1657–1666. [CrossRef]
135. Wong, V.W.; Lerner, E. Nitric oxide inhibition strategies. *Future Sci. OA* **2015**, *1*, FSO35. [CrossRef]
136. Dzavík, V.; Cotter, G.; Reynolds, H.R.; Alexander, J.H.; Ramanathan, K.; Stebbins, A.L.; Hathaway, D.; Farkouh, M.E.; Ohman, E.M.; Baran, D.A.; et al. Should we inhibit nitric Oxide synthase in Cardiogenic shocK 2 (SHOCK-2) investigators. Effect of nitric oxide synthase inhibition on haemodynamics and outcome of patients with persistent cardiogenic shock complicating acute myocardial infarction: A phase II dose-ranging study. *Eur. Heart J.* **2007**, *28*, 1109–1116. [CrossRef]
137. Cottera, G.; Kaluskia, E.; Miloa, O.; Blatta, A.; Salaha, A.; Hendlera, A.; Krakovera, R.; Golickb, A.; Vereda, Z. LINCS: L-NAME (a NO synthase inhibitor) in the treatment of refractory cardiogenic shock: A prospective randomized study. *Eur. Heart J.* **2003**, *24*, 1287–1295. [CrossRef]
138. Yargiçoglu, P.; Yaras¸, N.; Agar, A.; Gümüs¸lü, S.; Abidin, I.; Bilmen, S. Effects of N-nitro l-arginine methyl ester (l-NAME), a potent nitric oxide synthase inhibitor, on visual evoked potentials of rats exposed to different experimental stress models. *Acta Physiol. Scand.* **2004**, *180*, 307–316. [CrossRef]
139. Sibal, L.; Agarwal, S.C.; Home, P.D.; Boger, R.H. The role of asymmetric dimethylarginine (ADMA) in endothelial dysfunction and cardiovascular disease. *Curr. Cardiol. Rev.* **2010**, *6*, 82–90. [CrossRef]
140. Böger, R.H. Asymmetric dimethylarginine (ADMA) and cardiovascular disease: Insights from prospective clinical trials. *Vasc. Med.* **2005**, *10*, S19–S25. [CrossRef]
141. Grover, R.; Zaccardelli, D.; Colice, G.; Guntupalli, K.; Watson, D.; Vincent, J.L. An open-label dose escalation study of the nitric oxide synthase inhibitor, N(G)-methyl-L-arginine hydrochloride (546C88), in patients with septic shock. Glaxo Wellcome International Septic Shock Study Group. *Crit. Care Med.* **1999**, *27*, 913–922. [CrossRef]
142. Hauser, B.; Bracht, H.; Matejovic, M.; Radermacher, P.; Venkatesh, B. Nitric oxide synthase inhibition in sepsis? Lessons learned from large-animal studies. *Anesth. Analg.* **2005**, *101*, 488–498. [CrossRef] [PubMed]
143. Alabadí, J.A.; Thibault, J.L.; Pinard, E.; Seylaz, J.; Lasbennes, F. 7-Nitroindazole, a selective inhibitor of nNOS, increases hippocampal extracellular glutamate concentration in status epilepticus induced by kainic acid in rats. *Brain Res.* **1999**, *839*, 305–312. [CrossRef]
144. Bush, M.A.; Pollack, G.M. Pharmacokinetics and pharmacodynamics of 7-nitroindazole, a selective nitric oxide synthase inhibitor, in the rat hippocampus. *Pharm. Res.* **2001**, *18*, 1607–1612. [CrossRef] [PubMed]
145. Soliman, M.M. Effects of aminoguanidine, a potent nitric oxide synthase inhibitor, on myocardial and organ structure in a rat model of hemorrhagic shock. *J. Emerg. Trauma. Shock.* **2014**, *7*, 190–195. [CrossRef] [PubMed]
146. Sil, S.; Ghosh, T.; Ghosh, R.; Gupta, P. Nitric oxide synthase inhibitor, aminoguanidine reduces intracerebroventricular colchicine induced neurodegeneration, memory impairments and changes of systemic immune responses in rats. *J. Neuroimmunol.* **2017**, *303*, 51–61. [CrossRef] [PubMed]
147. Salem, R.; Mebazaa, A. Nitric oxide inhibition rapidly increases blood pressure with no change in outcome in cardiogenic shock: The TRIUMPH trial. *Crit. Care* **2007**, *11*, 136. [CrossRef]

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The Role of Angiotensin-Converting Enzyme (ACE) Polymorphisms in the Risk of Development and Treatment of Diabetic Nephropathy

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Abstract: Background: Angiotensin-converting enzyme (ACE) is responsible for the production of angiotensin II, and increased production of angiotensin II is observed in diabetes. What is more, *ACE* polymorphisms may play a role in the development of diabetic nephropathy. The aim of this study was to assess the role of selected *ACE* polymorphisms (rs4343 and rs4646994) in the risk of development of diabetic nephropathy and in the likelihood of renal replacement therapy. Methods: *ACE* polymorphisms were analyzed in a group of 225 patients who were divided into three subgroups. The rs4343 polymorphism was determined using the PCR-RFLP, and the rs4646994 polymorphism was determined using the PCR. Molecular docking between domains of ACE and its ligands was performed by using AutoDock Vina. Results: The G/G genotype of rs4343 polymorphism is associated with increased odds of developing diabetic nephropathy. The G allele is also associated with a higher risk of this disease. Similar results were obtained in patients who had already had a kidney transplant as a result of diabetic nephropathy. Conclusions: The presence of G/G and G/A genotypes, and the G allele increases the likelihood of developing diabetic nephropathy. This may also be a risk factor for renal replacement therapy.

Keywords: diabetes nephropathy; kidney transplant; single nucleotide polymorphisms; ACE inhibitors; molecular docking

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1. Introduction

Diabetic nephropathy is one of the most common complications of type 1 and type 2 diabetes, often necessitating kidney transplantation [1,2]. Its pathogenesis is complex, with increasing attention focusing on the role of genetic polymorphisms in its development. In this context, the angiotensin-converting enzyme gene (*ACE*) polymorphisms have garnered particular interest [3–5]. ACE is responsible for the production of angiotensin II, a key component of the renin-angiotensin system that plays a crucial role in blood pressure homeostasis by constricting blood vessels [6]. In diabetic individuals, there is a continuous growth in angiotensin II production, leading to elevated oxidative stress, glomerular hyperfiltration, endothelial damage, thrombosis, inflammation, and vascular remodeling [7]. Some of the *ACE* polymorphisms that may be associated with the development of diabetic nephropathy are rs4343 and rs4646994. The rs4343 polymorphism is located in exon 17 of *ACE*. It belongs to single nucleotide polymorphisms (SNPs) and consists of replacing guanine with adenine [8]. However, this does not affect the change in the

amino acid sequence. In turn, the rs4646994 polymorphism is located in intron 16 of *ACE*. It is due to the presence of the insertion allele or absence allele of a 287 bp Alu repeat sequence [9]. Ismail et al. [10] observed a more frequent occurrence of the DD genotype within the *ACE* polymorphism (rs4646994) and the D allele within the I/D polymorphism in patients with diabetic nephropathy compared to those with diabetes mellitus but without nephropathy [10].

ACE inhibitors (ACEi) are widely used as standard therapy in patients with diabetic nephropathy due to their reported renal protective effects [11]. However, the response to ACEi treatment varies among patients, often being unpredictable, partly due to genetic factors. The contribution of genetics to treatment response differences is primarily associated with the presence of polymorphisms, including single nucleotide polymorphisms (SNPs), insertions/deletions, and variable numbers of tandem repeats (VNTRs) [12,13]. Among the commonly used antihypertensive drugs for diabetic nephropathy treatment are ACEi, such as captopril, lisinopril, or ramipril [14].

The primary mechanism of ACEi action is to inhibit the conversion of angiotensin I to angiotensin II. ACE contains two homologous catalytic domains, the N and C domains, which are capable of cleaving angiotensin I and bradykinin [15,16]. The C domain of ACE is more effective in cleaving angiotensin I to vasoactive angiotensin II [17]. The rs4646994 polymorphism in *ACE* causes premature codon termination, resulting in the enzyme having only one active site in the N domain, thereby limiting drug binding to a single site. In silico analysis is used to visualize and analyze the binding of individual ACEi drugs to these domains [12]. In patients with COVID-19, proteolytic enzymes may selectively affect ACE domains, leading to variable enzyme activity based on the rs4646994 genotype [13]. This is because the genotype within this polymorphism determines how many domains an ACE molecule will have.

The aim of this study was to assess the frequency of two selected *ACE* polymorphisms (rs4343 and rs4646994) in patients with diabetic nephropathy, both with and without kidney transplantation. Additionally, the study aimed to investigate the relationship between specific genotypes, ACE activity, and the concentrations of ACE, creatinine, and C-reactive protein (CRP) in blood serum, as well as glucose in blood plasma. Furthermore, the concentrations of zinc and copper in the serum were determined due to potential disturbances in the metabolism of trace elements like zinc or copper that may occur during the development of diabetic complications [18,19]. The study also explored in silico analysis of interactions between the two ACE domains (N-domain and C-domain) and selected ACE inhibitors (lisinopril, ramipril, enalapril, benazepril).

2. Materials and Methods

2.1. Study Groups

A total of 225 individuals participated in this study, comprising three groups: the diabetic nephropathy group (N = 81), the kidney transplant diabetic nephropathy group (N = 94) and the control group (N = 50). Biological samples were collected from the participants, including blood samples obtained from Łukasiewicz PORT—Polish Center for Technology Development (control group), and blood samples obtained from the Department and Clinic of Nephrology and Transplantation Medicine of the Wroclaw Medical University (diabetic nephropathy group and kidney transplant diabetic nephropathy group). Blood was collected into two tubes: one tube with clotting activators (to obtain serum; cat. No.: BD 368815, Becton Dickinson, Franklin Lakes, NJ, USA) and the other tube with EDTA (to obtain plasma and buffy coat; cat. No.: BD 367864, Becton Dickinson, USA). DNA was isolated from the buffy coat using a ready-made isolation kit (Syngen Blood/Cell DNA Mini Kit, cat. No.: SY221012, Syngen Biotech, Wrocław, Poland).

The control group consisted of individuals with excluded cardiovascular diseases, liver function disorders (measured by GGT activity, ALT, and ASP), atherosclerosis, diabetes (based on insulin and fasting glucose measurements), hypertension (blood pressure measurements), inflammation (C-reactive protein concentration) and tumors. Potential par-

ticipants using medications or dietary supplements within the last 6 months were excluded from the study.

The selection of patients was made on the basis of medical history, laboratory tests, and imaging tests (e.g., USG) to exclude other causes of kidney damage. The following parameters were measured in patients: creatinine, blood morphology, urine general examination (including the presence of protein), albuminuria, sodium/potassium, glucose, and GFR (calculated according to the abbreviated formula MDRD). Qualification for the study required the presence of diabetes, albuminuria, proteinuria, or increased creatinine levels. Patients with other causes of kidney damage were excluded. In addition, patients completed a questionnaire providing information such as age, gender, anthropometric data (weight, height), other chronic diseases, stimulant usage (smoking, alcohol consumption), or medications (Questionnaire S1, Supplementary Materials). All participants were informed about the research objectives and provided written consent for the collection of biological material. The Bioethics Committee at Wroclaw Medical University approved the use of collected biological material for research purposes (No. KB 835/2021). The sample size was determined by power analysis using preliminary data from previous studies, with assumptions of $\alpha = 0.05$ and a power of 80%.

The characteristics of the three studied groups are presented in Table 1. In order to characterize the groups, the following parameters were used: age, sex, BMI values, glucose and creatinine concentrations, GFR values, and CRP concentrations.

Table 1. Values and concentrations of selected parameters characterizing the studied groups.

Parameter	Control Group (N = 50)	Diabetic Nephropathy Group (N = 81)	Kidney Transplant Diabetic Nephropathy Group (N = 94)	p
Age (years)	{25; 34; 47}	{65; 71; 78} *	{55; 62; 69} *,**	<0.001
Sex	Men: 21 Women: 29	Men: 42 Women: 39	Men: 47 Women: 47	0.540
BMI (kg/m^2)	23.83 ± 3.37	30.02 ± 5.33 *	27.05 ± 4.76 *,**	<0.001
Glucose (mg/dL)	{81.00; 85.50; 88.92}	{106.00; 139.50; 178.00} *	{113.00; 139.50; 173.00} *	<0.001
Creatinine (mg/dL)	-	{1.14; 1.36; 1.72}	{1.14; 1.30; 1.70}	0.405
eGFR ($mL/min/1.73\ m^2$)	-	{35.00; 48.00; 58.00}	{42.00; 53.50; 63.00}	0.086
CRP (mg/L)	{0.33; 0.65; 1.10}	{0.72; 1.91; 4.88} *	{0.97; 2.66; 4.11} *	<0.001

Values are shown as mean value ± standard deviation or {1st quartile; median; 3rd quartile}. * $p < 0.05$—compared to control group; ** $p < 0.05$—compared to diabetic nephropathy group.

2.2. Methods

2.2.1. Determination of ACE Activity, and ACE, Glucose, Creatinine, eGFR, and CRP Concentrations

Serum ACE activity was measured using the ACE1 Activity Assay Kit (Colorimetric) (cat. No.: ab273308, Abcam, Cambridge, UK). Serum ACE concentration was measured using the Human ACE (Angiotensin I Converting Enzyme) ELISA Kit (cat. No.: EH0026, Fine Biotech Co., Ltd., Wuhan, China). Glucose, creatinine, and CRP concentrations were measured in the hospital laboratory during routine patient visits. eGFR values were calculated according to the abbreviated MDRD formula.

2.2.2. Determination of Metal Concentrations

Zinc (Zn) and copper (Cu) concentrations in the blood serum were determined using the SOLAAR M6 atomic absorption spectrophotometer (Thermo Elemental Solaar House, Cambridge, UK) at the Laboratory of Atomic Absorption Spectrometry, Department and Clinic of Internal Diseases, Vocational, Hypertension and Clinical Oncology, Wroclaw Medical University. The Flame Atomic Absorption Spectrometry (FAAS) method in an air-acetylene flame was used to measure the concentrations of these metals.

2.2.3. Genotyping Analysis

DNA was isolated from the buffy coat using the Syngen Blood/Cell DNA Mini Kit (cat. No.: SY221012, Syngen Biotech, Wrocław, Poland). The rs4343 polymorphism was determined using the polymerase chain reaction and restriction fragment length polymorphism analysis (PCR-RFLP). In turn, the rs4646994 polymorphism, due to the fact that it is an insertion/deletion polymorphism, was determined using the polymerase chain reaction (PCR). Primers were designed with the Primer-BLAST program based on gene sequences from GenBank (National Center for Biotechnology Information). The sequences of the primers, reaction conditions, and the restriction enzyme used are presented in Table 2.

Table 2. The conditions for PCR and restriction enzyme digestion.

SNP	Primers	PCR-RFLP Conditions
rs4343	Forward primer—5′ CTG ACG AAT GTG ATG GCC GC 3′ Reverse primer—5′ TGA TGA GTT CCA CGT ATT TCG 3′	the initial denaturation—95 °C for 5 min denaturation—95 °C for 40 s annealing—58.4 °C for 35 s elongation—72 °C for 40 s the final elongation—72 °C for 10 min
	Restriction enzyme	**Restriction enzyme digestion conditions**
	BstUI	37 °C for 16 h
rs4646994	Forward primer—5′ CTG GAG ACC ACT CCC ATC CTT TCT 3′ Reverse primer—5′ GAT GTG GCC ATC ACA TTC GTC AGA T 3′	the initial denaturation—95 °C for 5 min denaturation—95 °C for 40 s annealing—60 °C for 35 s elongation—72 °C for 40 s the final elongation—72 °C for 10 min

The digested DNA fragments were visualized using a 2% agarose gel with Green DNA Gel Stain (both from Syngen Biotech, Wrocław, Poland, with cat. no SY 521011 and cat. no SY 521031, respectively). Electropherograms showing restriction digest products are provided in Figures S1 and S2 (Supplementary Materials).

2.2.4. Molecular Docking

For the docking calculations, three-dimensional (3D) crystallographic structures of the N domain and the C domain of the ACE molecule were obtained from the Protein Data Bank (PDB) with PDB entries 5AMB and 6H5W for N-domain and C-domain, respectively [20]. Prior to the docking procedure, the 3D models were manually prepared to ensure accuracy by removing crystallographic waters, ligands, and other unfavorable components. UCSF Chimera software (version 1.15) was utilized for this purpose [21]. Atoms with double conformations were checked and repaired with a self-written script in Python programming language (version Python 3.8).

Three-dimensional structures of ligands (benazepril, enalapril, lisinopril, and ramipril) were retrieved from the PubChem open chemistry database using UCSF Chimera software for its downloading. Molecular docking calculations between two domains of ACE and its ligands were performed with AutoDock Vina software (version 1.1.2) [22,23]. The calculations were carried out using the parameters recommended in the user manual.

AutoDockTools (ADT, version 1.5.7) was employed to find and determine the center and size of the grid box for the docking calculations.

The predicted binding affinity (kcal/mol) was calculated by Auto-Dock Vina. To visualize molecules and analyze the docking results, three pieces of software were employed. To generate overall views of the docking outcomes, UCSF Chimera was used [21], while BIOVIA Discovery Studio Visualizer (version 21.1.0.20298) was utilized to create two-dimensional diagrams and illustrate the interactions of ligands with amino acids [24]. PyMOL (version 2.5.2) was employed to verify the positioning of the ligand on the receptor surface. The choice of drugs was made based on an interview with patients who were included in this study. These substances appeared in the drugs used by the respondents.

2.2.5. Statistical Analysis

Statistical analyses were performed using the STATISTICA 13.3 package (Statsoft Polska, Sp. z o.o., Kraków, Poland) under the Wroclaw Medical University license. The normality of variable distributions was assessed using the Shapiro–Wilk test and the homogeneity of variance was examined using Levene's test.

For testing statistically significant differences between the two groups, the parametric Student's *t*-test was applied to variables with a normal distribution. If the variable did not meet the conditions of a normal distribution, the non-parametric Mann–Whitney U test was used.

To test statistically significant differences among three or more groups, the non-parametric Kruskal–Wallis test was employed in case the variables did not follow a normal distribution.

The frequencies of genotypes were compared using the χ^2 test and Fisher's exact test.

Logistic regression analysis was performed to assess the significance of the effect of polymorphism genotypes on the risk of diabetic nephropathy and the likelihood of renal replacement therapy, expressed as odds ratios (OR) with a 95% confidence interval (CI). Statistical significance was considered for $p < 0.05$.

3. Results

3.1. Concentrations of the Selected Parameters and ACE Activity in the Studied Groups

Higher ACE concentrations were observed in patients with diabetic nephropathy ($p = 0.012$) and in patients with diabetic nephropathy after kidney transplantation ($p = 0.005$) compared to the control group. In turn, in the case of ACE activity, an inverse relationship was observed ($p < 0.001$ and $p = 0.003$, respectively).

For zinc, the group of patients with diabetic nephropathy showed a lower concentration of this element compared to the control group ($p < 0.001$), while the group of patients after kidney transplantation exhibited a higher concentration of zinc compared to the control group ($p < 0.001$). No significant differences in copper concentrations were found when compared to patients with diabetic nephropathy and patients with diabetic nephropathy after kidney transplantation. The results are presented in Table 3.

3.2. The Influence of the rs4343 and the rs4646994 Polymorphisms in ACE on the Concentrations of the Selected Parameters and on ACE Activity

Significant differences in genotypic distribution between the study groups were observed for the rs4343 polymorphism ($p < 0.001$). The G/A genotype appeared least frequently in the control group (8.16%), whereas in the other groups, it was the dominant genotype (50.00% and 46.24%, respectively). Although no similar relationship was observed for the rs4646994 polymorphism, the differences in genotypic distribution were on the verge of statistical significance ($p = 0.056$). The results are presented in Table 4.

Table 3. Concentration of ACE, activity of ACE, and concentrations of zinc and copper in the studied groups.

Parameter	Control Group (*N* = 50)	Diabetic Nephropathy Group (*N* = 81)	Kidney Transplant Diabetic Nephropathy Group (*N* = 94)	*p*
ACE (ng/mL)	{42.64; 71.97; 99.30}	{79.06; 89.52; 101.72} *	{81.25; 89.64; 100.38} *	0.005
ACE (mU/mL)	{0.066; 0.079; 0.092}	{0.026; 0.052; 0.071} *	{0.045; 0.063; 0.076} *	<0.001
Zn (µg/L)	{755.00; 830.00; 913.50}	{720.00; 804.50; 880.00} *	{854.21; 946.55; 1031.87} *	<0.001
Cu (µg/L)	{880.00; 1019.00; 1151.50}	{909.00; 1084.00; 1200.00}	{920.91; 1012.89; 1181.65}	0.293

Values are shown as {1st quartile; median; 3rd quartile}. * $p < 0.05$—compared to the control group.

Table 4. The genotypic distribution of the rs4343 and the rs4646994 polymorphisms of *ACE* in the studied groups.

SNP	Groups (N)	Genotype Frequencies (%)		
		G/G	G/A	A/A
rs4343	Control (*N* = 49)	*N* = 11 (22.45%)	*N* = 4 (8.16%)	*N* = 34 (69.39%)
	Diabetic Nephropathy (*N* = 84)	*N* = 16 (19.05%)	*N* = 42 (50.00%)	*N* = 26 (30.95%)
	Kidney Transplant Diabetic Nephropathy (*N* = 93)	*N* = 26 (27.96%)	*N* = 43 (46.24%)	*N* = 24 (25.80%)
SNP	**Groups (N)**	**Genotype Frequencies (%)**		
		I/I	**I/D**	**D/D**
rs4646994	Control (*N* = 49)	*N* = 0 (0.00%)	*N* = 42 (84.00%)	*N* = 8 (16.00%)
	Diabetic Nephropathy (*N* = 81)	*N* = 11 (13.58%)	*N* = 51 (62.96%)	*N* = 19 (23.46%)
	Kidney Transplant Diabetic Nephropathy (*N* = 93)	*N* = 13 (13.98%)	*N* = 59 (63.44%)	*N* = 21 (22.58)

3.2.1. The Influence of the rs4343 Polymorphism on the Concentrations of the Selected Parameters and on ACE Activity

After subgrouping the population by genotype (rs4343 polymorphism), no differences in ACE concentration were observed ($p = 0.118$). However, statistically lower ACE activity was observed in the group of patients with diabetic nephropathy and the A/A genotype compared to the control group with the same genotype ($p = 0.004$).

No significant differences were observed between creatinine levels ($p = 1.000$), CRP levels ($p = 1.000$), and copper levels ($p = 0.485$), as well as eGFR values ($p = 1.000$) (Table 5). However, statistically significant differences in glucose concentration were noted. Patients with diabetic nephropathy and the A/A genotype had significantly higher glucose concentrations compared to controls with the A/A genotype ($p < 0.001$). In the case of kidney transplant diabetic nephropathy patients, statistically higher glucose concentrations were observed in the following groups: with the G/G genotype compared to controls with G/G ($p = 0.009$).

Table 5. Concentrations and activity of the selected parameters in the studied groups in terms of the rs4343 polymorphism in *ACE*.

Parameter	Control Group (*N* = 49)			Diabetic Nephropathy Group (*N* = 84)			Kidney Transplant Diabetic Nephropathy Group (*N* = 93)		
	G/G (*N* = 11)	G/A (*N* = 4)	A/A (*N* = 34)	G/G (*N* = 16)	G/A (*N* = 42)	A/A (*N* = 26)	G/G (*N* = 26)	G/A (*N* = 43)	A/A (*N* = 24)
ACE (ng/mL)	{67.86; 83.77; 88.92}	{51.04; 60.85; 90.94}	{41.62; 78.13; 101.64}	{81.66; 88.36; 104.25}	{79.39; 90.71; 100.17}	{77.10; 86.63; 101.97}	{79.55; 84.74; 97.05}	{81.02; 90.20; 101.21}	{85.90; 94.63; 100.64}
ACE (mU/mL)	{0.073; 0.078; 0.106}	{0.079; 0.085; 0.088}	{0.063; 0.079; 0.093}	{0.050; 0.063; 0.077}	{0.022; 0.049; 0.074}	{0.025; 0.051; 0.066} **	{0.054; 0.063; 0.073]	{0.040; 0.063; 0.076}	{0.037; 0.064; 0.078}
Glucose (mg/dL)	{81.00; 82.98; 88.92}	{85.50; 90.00; 94.50}	{79.92; 84.47; 88.92}	{102.00; 124.50; 154.00}	{106.00; 139.00; 183.00}	{105.00; 141.00; 163.00} **	{114.00; 126.00; 143.00} *	{111.00; 149.00; 184.00}	{113.00; 155.00; 183.00}
Creatinine (mg/dL)	-	-	-	{1.06; 1.35; 1.46}	{1.21; 1.42; 2.00}	{1.05; 1.24; 1.60}	{1.08; 1.26; 1.41}	{1.14; 1.31; 1.80}	{1.17; 1.30; 1.70}
eGFR (mL/min/1.73 m^2)	-	-	-	{48.50; 52.00; 59.50}	{29.00; 44.00; 54.00}	{44.00; 56.00; 64.00}	{43.00; 56.00; 61.00}	{36.00; 53.00; 63.00}	{45.00; 54.00; 66.00}
CRP (mg/L)	{0.27; 0.64; 1.01}	{0.37; 0.99; 3.12}	{0.33; 0.65; 1.10}	{0.60; 2.55; 4.49}	{1.29; 2.38; 5.35}	{0.23; 0.72; 1.24}	{1.21; 2.79; 4.07}	{1.49; 2.26; 4.33}	{0.70; 0.96; 4.10}
Zn (µg/L)	{845.49; 895.37; 1019.99}	{823.33; 933.47; 1075.62}	{901.82; 972.73; 1043.34}	{817.00; 855.00; 922.00}	{752.00; 800.50; 884.00}	{729.00; 855.50; 937.00} **	{749.00; 825.00; 940.00}	{721.00; 807.00; 887.00}	{672.00; 753.50; 833.00} **
Cu (µg/L)	{986.90; 1046.20; 1184.39}	{1006.96; 1251.14; 2039.81}	{868.53; 998.18; 1155.12}	{896.00; 1009.00; 1205.00}	{884.00; 1002.00; 1168.00}	{859.00; 1058.00; 1129.00}	{904.00; 1047.00; 1158.00}	{911.00; 1092.00; 1254.00}	{900.00; 1074.00; 1225.00}

Values are shown as {1st quartile; median; 3rd quartile}. * $p < 0.05$—compared to the control group with the G/G genotype; ** $p < 0.05$—compared to the control group with the A/A genotype.

Moreover, differences in zinc concentrations were also observed. Patients with diabetic nephropathy and the A/A genotype had lower zinc concentrations compared to the control group with the A/A genotype ($p = 0.021$). On the other hand, kidney transplant diabetic nephropathy patients with the A/A genotype had lower zinc concentrations compared to the control group with the A/A genotype ($p < 0.001$).

The results described above are presented in Table 5.

3.2.2. The Influence of the rs4646994 Polymorphism on the Concentrations of the Selected Parameter and on ACE Activity

After subgrouping the population by genotype of rs4646994 polymorphism, no significant differences were observed between creatinine levels ($p = 1.000$), eGFR values ($p = 1.000$), or copper levels ($p = 0.645$). However, in the case of glucose concentration, the following observations were noted: patients with diabetic nephropathy and patients with the I/D genotype after kidney transplantation had higher glucose concentrations than the control group with the same genotype.

For CRP levels, patients with the I/D genotype ($p = 0.003$) after kidney transplantation had statistically higher CRP levels compared to the control group with the I/D genotype.

Regarding zinc concentrations, statistically significant lower concentrations of this element were observed in the following groups: patients with diabetic nephropathy and the I/D compared to the control group with the same genotype ($p < 0.001$), as well as patients after kidney transplantation and the I/D genotype compared to the control group with the same genotype ($p < 0.001$).

The results described above are presented in Table 6.

3.2.3. The Influence of the rs4646994 Polymorphism on the Concentration of Selected Parameters and ACE Activity in a Group of Patients Using Ramipril

Some of the patients were treated with ACEi. They most often took ramipril (31 patients); they also used perindopril (9 patients), lisinopril (3 patients), and quinapril (1 patient). Due to the small groups, a full statistical analysis of the results was not performed. However, Table 7 shows the relationship between the rs4646994 polymorphism and the values of selected parameters in the group of patients using ramipril. Statistically higher glucose levels were observed in patients with the I/D genotype compared to patients with the I/I genotype ($p = 0.027$).

3.3. The Influence of ACE Polymorphisms on the Risk of Occurrence of Diabetic Nephropathy or the Likelihood of Renal Replacement Therapy

In this study, logistic regression was used to assess the risk of developing diabetic nephropathy or the likelihood of renal replacement therapy based on *ACE* polymorphisms. The results indicate that the G/G genotype (rs4343 polymorphism) is associated with an over 2.68-fold increased odds of developing diabetic nephropathy ($p = 0.014$). Another genotype within this polymorphism, G/A, also seems to be associated with a significantly increased risk of developing this complication of diabetes. However, the wide confidence interval suggests low accuracy in estimating this parameter. Nevertheless, the occurrence of the G allele is associated with a 2.53-fold higher risk of developing nephropathy ($p < 0.001$). Additionally, each subsequent year increases the risk of developing diabetic nephropathy by 17.30% ($p < 0.001$), while an increase in BMI by one unit increases this risk by 26.60% ($p < 0.001$).

Table 6. Concentrations of the selected parameters in the studied groups in terms of the rs46464994 polymorphism in *ACE*.

Parameter	Control Group (*N* = 50)			Diabetic Nephropathy Group (*N* = 81)			Kidney Transplant Diabetic Nephropathy Group (*N* = 93)		
	I/I (*N* = 0)	I/D (*N* = 42)	D/D (*N* = 8)	I/I (*N* = 11)	I/D (*N* = 51)	D/D (*N* = 19)	I/I (*N* = 13)	I/D (*N* = 59)	D/D (*N* = 21)
ACE (ng/mL)	-	{42.64; 71.51; 89.32}	{60.50; 88.52; 99.30}	{80.05; 96.22; 101.98}	{76.48; 87.19; 100.17}	{83.22; 88.32; 101.72}	{86.33; 93.61; 96.98}	{80.05; 89.64; 101.02}	{76.15; 86.66; 100.49}
ACE (mU/mL)	-	{0.063; 0.079; 0.092}	{0.074; 0.077; 0.086}	{0.016; 0.036; 0.070}	{0.022; 0.051; 0.074} *	{0.045; 0.057; 0.073}	{0.027; 0.064; 0.069}	{0.040; 0.062; 0.076}	{0.054; 0.063; 0.073}
Glucose (mg/dL)	-	{81.00; 85.50; 88.92}	{81.99; 85.95; 92.52}	{103.00; 110.00; 146.00}	{107.00; 141.00; 183.00} *	{94.50; 152.00; 202.50}	{130.50; 161.00; 178.00}	{113.00; 139.00; 170.00} *	{112.00; 122.00; 146.00}
Creatinine (mg/dL)	-	-	-	{1.12; 1.24; 1.34}	{1.18; 1.39; 1.80}	{1.14; 1.41; 1.48}	{1.12; 1.27; 1.54}	{1.14; 1.41; 1.80}	{1.06; 1.22; 1.31}
eGFR (mL/min/1.73 m^2)	-	-	-	{54.00; 56.00; 61.00}	{34.00; 45.00; 55.50}	{36.00; 50.00; 61.00}	{45.50; 54.00; 67.00}	{36.00; 51.00; 59.00}	{52.00; 59.00; 64.00}
CRP (mg/L)	-	{0.37; 0.72; 1.15}	{0.15; 0.36; 1.03}	{0.71; 0.98; 1.24}	{0.72; 2.13; 5.42}	{0.60; 1.94; 4.49}	{0.77; 0.93; 4.11}	{0.99; 2.86; 4.33} *	{1.21; 2.43; 3.37}
Zn (µg/L)	-	{890.78; 972.73; 1043.34}	{824.51; 877.31; 995.86}	{762.00; 879.00; 945.00}	{741.00; 807.00; 898.00} *	{759.00; 855.00; 922.00}	{704.00; 744.00; 798.00}	{718.00; 805.00; 887.00} *	{780.00; 825.00; 880.00}
Cu (µg/L)	-	{920.91; 1002.02; 1181.65}	{930.06; 1032.31; 1233.67}	{835.00; 941.00; 1144.00}	{896.00; 1045.00; 1150.00}	{822.00; 962.00; 1205.00}	{820.00; 1097.00; 1225.00}	{911.00; 1087.00; 1248.00}	{934.00; 1088.00; 1139.00}

Values are shown as {1st quartile; median; 3rd quartile}. * $p < 0.05$—compared to the control group with the I/D genotype.

Table 7. Concentrations of the selected parameters in the groups of patients using ramipril in terms of the rs46464994 polymorphism in *ACE*.

Parameter	I/I (*N* = 6)	I/D (*N* = 17)	D/D (*N* = 8)	*p*
BMI (kg/m²)	{22.54; 32.57; 38.81}	{26.29; 27.92; 33.27}	{29.28; 31.99; 40.39}	0.186
ACE (ng/mL)	{86.48; 92.68; 97.97}	{83.10; 89.61; 104.70}	{81.20; 92.97; 97.35}	0.983
ACE (mU/mL)	{0.027; 0.051; 0.066}	{0.018; 0.062; 0.077}	{0.035; 0.069; 0.073}	0.886
Glucose (mg/dL)	{103.00; 106.00; 110.00}	{133.00; 157.00; 216.00} *	{128.00; 146.50; 224.00}	0.027
Creatinine (mg/dL)	1.23 ± 0.21	1.26 ± 0.34	1.30 ± 0.29	0.919
eGFR (mL/min/1.73 m²)	53.67 ± 9.33	61.13 ± 21.36	54.88 ± 17.72	0.620
CRP (mg/L)	{1.31; 2.72; 5.02}	{0.45; 0.72; 1.54}	-	0.395
Zn (µg/L)	778.67 ± 105.56	810.47 ± 150.56	797.50 ± 60.63	0.947
Cu (µg/L)	1007.83 ± 225.69	1067.88 ± 216.30	940.25 ± 188.94	0.386

Values are shown as mean value ± standard deviation or {1st quartile; median; 3rd quartile}. * $p < 0.05$—compared to the I/I group.

Similar results were obtained in the kidney transplant diabetic nephropathy group. The G/G and G/A genotypes within the rs4343 polymorphism were associated with an increased likelihood of renal replacement therapy (approximately 3.35-fold and 15.23-fold, respectively). Likewise, the G allele was associated with a 2.89-fold increased likelihood of renal replacement therapy. Additionally, each subsequent year was associated with a 1.18-fold decreased likelihood of renal replacement therapy ($p < 0.001$), while an increase in BMI by one unit was associated with a 1.21-fold decreased likelihood ($p < 0.001$).

The results described above are presented in Tables 8 and 9.

3.4. Interaction of ACE with Selected Drugs (Benazepril, Enalapril, Lisinopril and Ramipril)

The molecular docking analysis was performed to calculate the binding affinity between the N and C domains of ACE and their ligands—drugs from the ACEi group, which are used in the treatment of diabetic nephropathy. The obtained results indicate that enalapril and ramipril have similar binding affinities for both domains. This means that the two drugs effectively bind to both the N and the C domains. In turn, benazepril was noted to have a lower binding affinity to the C domain compared to the N domain. This indicates that benazepril binds more efficiently with the C domain. A similar relationship was observed with lisinopril, which also showed a lower binding affinity to the C domain compared to the second domain. The discussed results are shown in Table 10. The interaction of enalapril between the N and C domains of ACE is shown in Figure 1. The visualization of interactions with ramipril, benazepril and lisinopril are presented in the Supplementary Materials (Figures S3–S5).

Table 8. The relationship between the selected parameters and the risk of developing diabetic nephropathy.

SNP (*Gene*)	Genotype	Diabetic Nephropathy Group	Control Group	*p*	OR	95% CI OR
rs4343 (*ACE*)	G/G	42	11	0.014	2.675	1.216–5.884
	G/A	85	4	<0.001	13.894	4.662–41.408
	A/A	50	34	-	1.000	-
	G allele	169	26	<0.001	2.530	1.543–4.148
	A allele	185	72	-	1.000	-
rs4646994 (*ACE*)	I/I	24	0	-	-	-
	I/D	110	42	0.131	0.524	0.227–1.211
	D/D	40	8	-	1.000	-
	I allele	168	42	0.382	1.221	0.780–1.911
	D allele	190	58	-	1.000	-
Other Variables	**Category**	**Diabetic Nephropathy Group**	**Control Group**	***p***	**OR**	**95% CI OR**
Age	-	-	-	<0.001	1.173	1.123–1.226
BMI	-	-	-	<0.001	1.266	1.157–1.386
Sex	Men	89	21	-	1.000	-
	Women	85	29	0.270	0.701	0.372–1.318

Table 9. The relationship between the selected parameters and the likelihood of renal replacement therapy.

SNP (*Gene*)	Genotype	Kidney Transplant Diabetic Nephropathy Group	Control Group	*p*	OR	95% CI OR
rs4343 (*ACE*)	G/G	26	11	0.007	3.348	1.392–8.053
	G/A	43	4	<0.001	15.229	4.821–48.103
	A/A	24	34	-	1.000	-
	G allele	95	26	<0.001	2.891	1.697–4.925
	A allele	91	72	-	1.000	-
rs4646994 (*ACE*)	I/I	13	0	-	-	-
	I/D	59	42	0.176	0.535	0.216–1.323
	D/D	21	8	-	1.000	-
	I allele	85	42	0.548	1.162	0.711–1.899
	D allele	101	58	-	1.000	-
Other Variables	**Category**	**Kidney Transplant Diabetic Nephropathy Group**	**Control Group**	***p***	**OR**	**95% CI OR**
Age	-	-	-	<0.001	0.848	0.805–0.894
BMI	-	-	-	<0.001	0.826	0.748–0.911
Sex	Men	47	21	-	1.000	-
	Women	46	29	0.327	1.409	0.710–2.798

Table 10. The binding affinity between two domains of ACE and enalapril, ramipril, benazepril, and lisinopril.

Ligands	The Binding Affinity (kcal/mol)	
	N Domain	C Domain
Enalapril	−7.4	−7.9
Ramipril	−8.0	−8.2
Benazepril	−6.6	−8.3
Lisinopril	−5.8	−6.8

(A)
N domain of ACE with enalapril

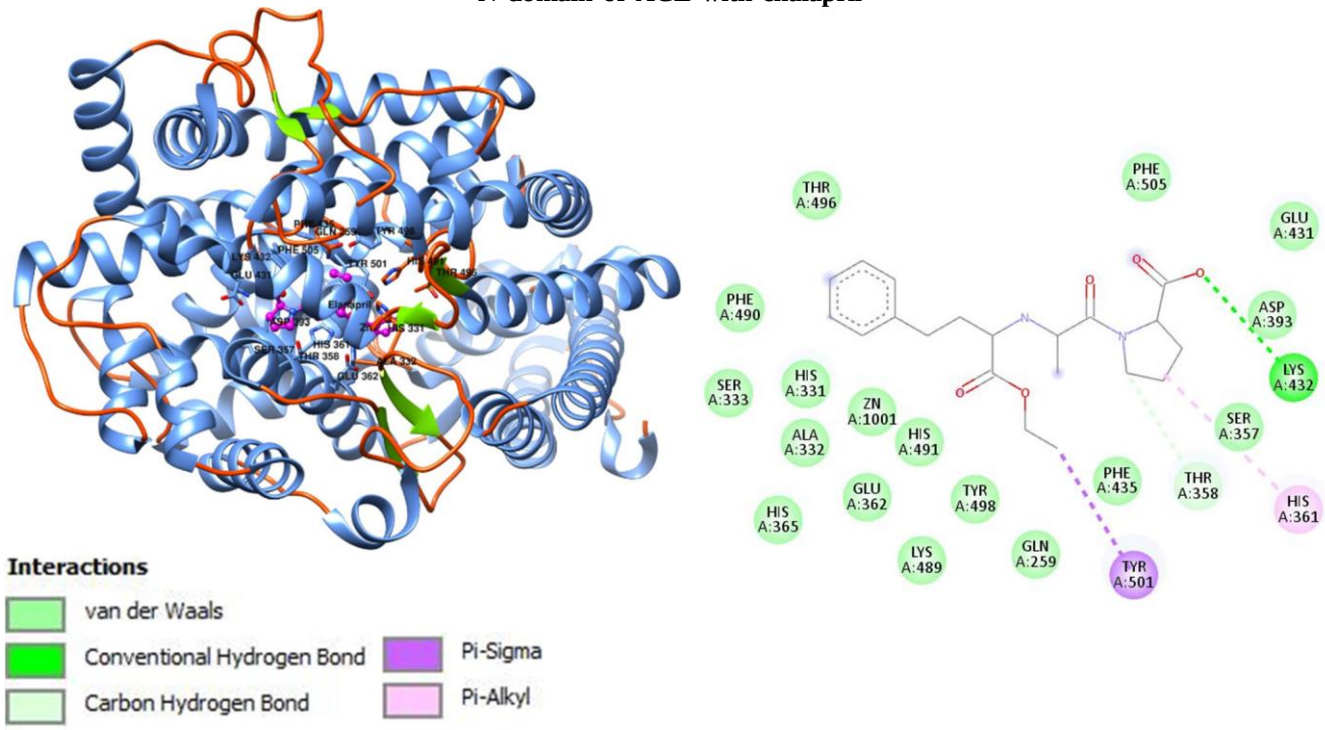

Figure 1. *Cont.*

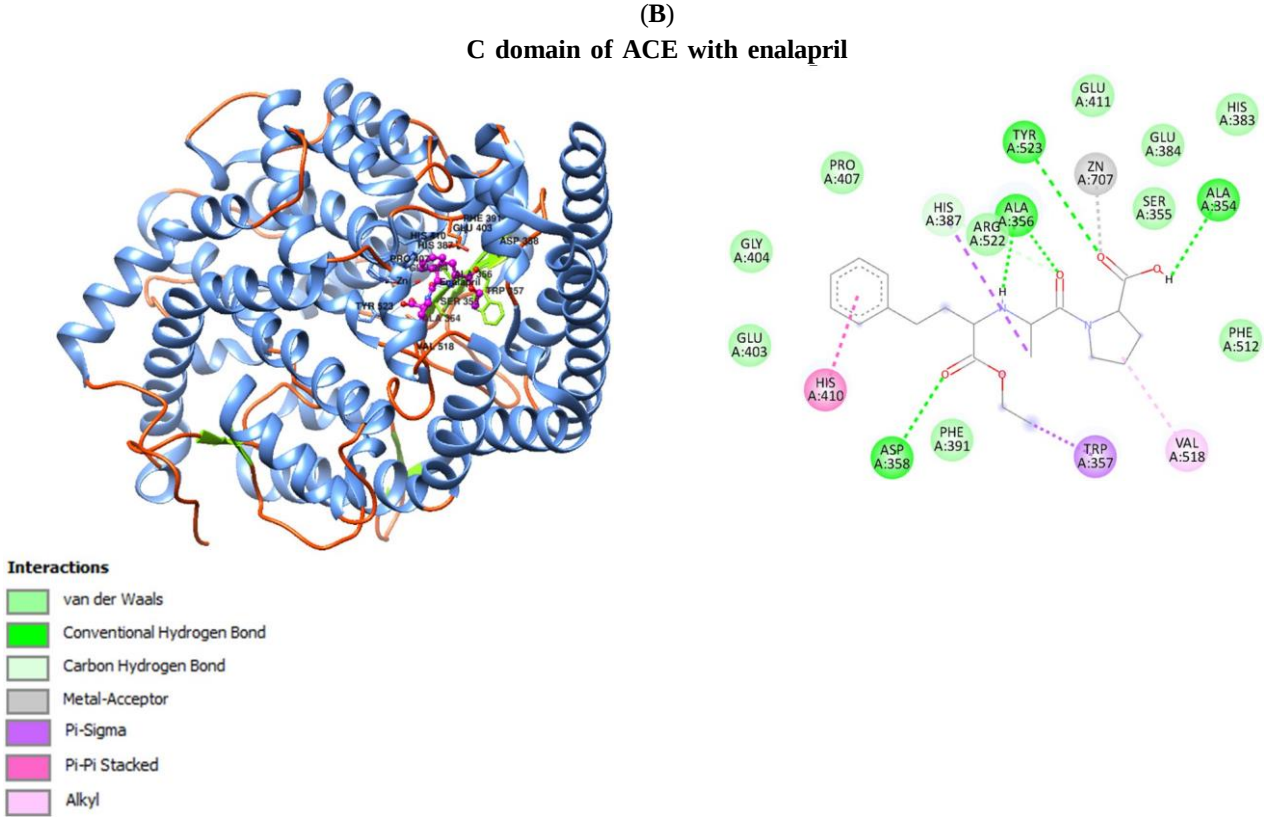

Figure 1. The interaction between N and C domains of ACE and enalapril.

4. Discussion

Diabetic nephropathy is the leading cause of mortality in diabetic patients [25]. Emerging evidence points to the importance of the role played by the ACE molecule in the pathogenesis of diabetic nephropathy [26,27]. Although there are indications that identify *ACE* polymorphisms as one of the risk factors in kidney transplant rejection [28,29], there are no studies that would confirm their role in the development of diabetic nephropathy that leads to transplantation. The current study was undertaken to investigate polymorphisms in the *ACE* gene (rs4343 and rs4646994) in patients with diabetic nephropathy (without and after transplantation), as well as to compare the binding of ACEi drugs used in nephrology with two ACE domains.

The studied polymorphisms (rs4343 and rs4646994) had no effect on ACE concentrations. However, it was noticed that ACE activity in the group of patients with diabetic nephropathy and the A/A genotype was significantly lower compared to the control group. This is interesting because the situation was different in the case of the concentrations of this enzyme—patients from the control group had higher concentrations of this parameter. Low ACE activity in patients with diabetic nephropathy may result from compensatory adaptation aimed at reducing the production of additional angiotensin II (Ang II) [30,31].

This study also demonstrated a relationship between zinc concentrations and the occurrence of diabetic nephropathy. Lower concentrations of this element were observed in patients with diabetic nephropathy compared to the control group, which corresponds to the previously available data [32,33]. A low supply of zinc in the diet and its low concentrations in blood serum are associated with an increased incidence of diabetes and cardiovascular diseases. Unfortunately, it is not always known whether the disease affects zinc metabolism or whether its low concentrations result in carbohydrate metabolism disorders. It is highly probable that these two phenomena coexist [32]. What is interesting is that patients after kidney transplantation had higher zinc concentrations compared to the control group, which would be contrary to other studies [34,35]. However, after a more detailed analysis of the groups, it turned out that these groups of patients with the

A/A (rs4343) and D/D (rs4646994) genotypes had lower zinc concentrations compared to the corresponding control groups. Perhaps this discrepancy is caused by the fact that the results of the statistical test comparing the three groups (the control group, the diabetic nephropathy group, and the kidney transplant diabetic nephropathy group) were influenced by both very low and very high zinc concentrations in the kidney transplant diabetic nephropathy group. In turn, after additional division of this group due to different genotypes, the concentrations of the tested element were distributed slightly differently between the individual groups. However, in the case of copper, no statistical significance was observed.

The influence of genetic factors, including two *ACE* polymorphisms, on an increased risk of developing diabetic nephropathy or an increased likelihood of renal replacement therapy due to ongoing diabetic nephropathy has been investigated. Based on the logistic regression results, it can be seen that the rs4343 polymorphism could be a useful tool in predicting this risk in both cases. Both genotypes containing the G allele (G/G and G/A) and the G allele alone were associated with an increased risk of developing diabetic nephropathy or an increased likelihood of renal replacement therapy. These results are confirmed by other studies, which also indicate the importance of the G allele (rs4343 polymorphism) in the pathogenesis of diabetic complications [36,37]. In order to use the obtained results in clinical terms, cohort studies should be conducted, taking into account the interaction of the studied polymorphism with other gene variants. Similar dependencies were not obtained in the case of the rs4646994 polymorphism, the influence of which was confirmed in many research reports [38–40]. This may be due to the fact that studies were carried out on groups not exceeding 100 patients, so in order to confirm these results, a much larger number of patients with diabetic nephropathy should be examined. Sex also does not seem to be a significant risk factor in the development of the studied disease, which would be consistent with the results obtained by other researchers [41]. However, older age and higher BMI values have been found to increase the risk of developing diabetic nephropathy. This corresponds to the current knowledge, according to which obesity is one of the factors of diabetes development and contributes to its complications [42,43].

Although in silico studies in the context of the impact of the rs4646994 polymorphism on treatment with ACEi drugs have already been conducted, their main target was usually captopril or lisinopril [44,45]. In this study, the interactions between two ACE domains and four drugs that are registered in Poland as those that can be used in the treatment of diabetic nephropathy [46] were taken into account. It should be remembered here that the I allele of the rs4646994 polymorphism causes premature codon termination, resulting in the enzyme having only one active site in the N domain [12]. This means that potential drugs are able to attach to only one active site, which may affect their effectiveness. In addition, it has been proven that different drugs bind to particular domains, showing different binding affinities [12,15–17]. In the present study, it was observed that benazepril and lisinopril display a significant preference for the C domain of the ACE, although the calculated value for the N domain was still relevant. Nevertheless, enalapril and ramipril can bind with very similar efficiency to both domains; therefore, the rs4646994 polymorphism should not have a significant impact on their action in the treatment of diabetic nephropathy. Moreover, after comparing the concentrations of selected parameters in groups based on ramipril use and genotypes, no significant differences were observed. The exception was the glucose concentration, which was higher in the group of patients with the I/D genotype compared to patients with the I/I genotype. However, it should be taken into account that a relatively small group of patients took ramipril; therefore, a larger study group should be used to examine the relationship between the drugs used and the selected genotypes. Therefore, in the case of the other two drugs—benazepril and lisinopril—the genotype within the rs4646994 polymorphism may translate into the effectiveness of these agents. Both drugs bind more effectively to the C domain, so in patients with the I/D and I/I genotypes, the effectiveness of benazepril and lisinopril may be lower compared to patients with the D/D genotype. This could translate into the clinical effectiveness of these drugs, but in

order to confirm the obtained results, direct tests of the effectiveness of individual ACEi should be carried out in groups of patients divided according to the genotype within the rs4646994 polymorphism.

5. Conclusions

In conclusion, the study reveals significant associations between one of the *ACE* polymorphisms (rs4343) and the risk of diabetic nephropathy or the likelihood of renal replacement therapy. The presence of specific genotypes (G/G and G/A) and G alleles increases the likelihood of developing these complications. Additionally, age and BMI were identified as factors influencing the risk of diabetic nephropathy, while sex did not show a significant association. Similar results were obtained to investigate the likelihood of renal replacement therapy.

However, it is important to remember that not only the above-mentioned factors will contribute to the increased risk of renal replacement therapy. Patients who develop diabetic nephropathy and have it for a long time will most likely require this type of therapy in the future. Therefore, it would be appropriate to investigate the impact of *ACE* polymorphisms on the risk of kidney transplant rejection. In addition, extended studies could take into account the impact of selected *ACE* polymorphisms on the development of diabetic nephropathy by assessing the progression of renal failure using the speed of the decline of eGFR. Additional tests should also be performed, such as the assessment of proteinuria as an important factor in the progression of renal failure.

Moreover, the results of in silico analysis seem to be interesting. They indicate the dependence of the effectiveness of treatment with ACEi on the rs4646994 polymorphism. However, it should be noted that these are only preliminary studies. To confirm these results, clinical trials should be conducted on a larger group of patients. Obtaining similar results could contribute to the individualization of therapy and, thus, more effective treatment.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/jcm13040995/s1. Questionnaire S1: Sample of a questionnaire conducted among people suffering from diabetic nephropathy; Figure S1: Example of electropherogram for rs4343 (*ACE*); Figure S2: Example of electropherogram for rs4646994 (*ACE*); Figure S3: The interaction between N and C domains of ACE and ramipril; Figure S4: The interaction between N and C domains of ACE and benazepril; Figure S5: The interaction between N and C domains of ACE and lisinopril.

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References

1. Madziarska, K.; Banasik, M. Chorzy na cukrzyce˛ w programach hemodializy i dializy otrzewnowej—Zagroz˙enia, których moz˙na unikna˛c´ [Diabetes patients in hemodialysis and peritoneal dialysis programs—risks that can be avoided]. *Probl. Lek.* **2006**, *45*, 240–241.
2. Sulaiman, M.K. Diabetic nephropathy: Recent advances in pathophysiology and challenges in dietary management. *Diabetol. Metab. Syndr.* **2019**, *11*, 7. [CrossRef] [PubMed]
3. Rahimi, Z. ACE insertion/deletion (I/D) polymorphism and diabetic nephropathy. *J. Nephropathol.* **2012**, *1*, 143–151. [CrossRef] [PubMed]
4. Yu, Z.Y.; Chen, L.S.; Zhang, L.C.; Zhou, T.B. Meta-analysis of the relationship between ACE I/D gene polymorphism and end-stage renal disease in patients with diabetic nephropathy. *Nephrology* **2012**, *17*, 480–487. [CrossRef] [PubMed]
5. Huang, Z.; Wu, B.; Tao, J.; Han, Z.; Yang, X.; Zhang, L.; Liu, X.; Wang, Z.; Tan, R.; Gu, M.; et al. Association between Angiotensin I-Converting Enzyme Insertion/Deletion Polymorphism and Prognosis of Kidney Transplantation: A Meta-Analysis. *PLoS ONE* **2015**, *10*, e0127320. [CrossRef] [PubMed]
6. Rahimi, Z.; Hasanvand, A.; Felehgari, V. Interaction of MTHFR 1298C with ACE D allele augments the risk of diabetic nephropathy in Western Iran. *DNA Cell Biol.* **2012**, *31*, 553–559. [CrossRef] [PubMed]
7. Ruggenenti, P.; Bettinaglio, P.; Pinares, F.; Remuzzi, G. Angiotensin converting enzyme insertion/deletion polymorphism and renoprotection in diabetic and nondiabetic nephropathies. *Clin. J. Am. Soc. Nephrol.* **2008**, *3*, 1511–1525. [CrossRef] [PubMed]
8. Alimoradi, N.; Sharqi, M.; Firouzabadi, D.; Sadeghi, M.M.; Moezzi, M.I.; Firouzabadi, N. SNPs of ACE1 (rs4343) and ACE2 (rs2285666) genes are linked to SARS-CoV-2 infection but not with the severity of disease. *Virol. J.* **2022**, *19*, 48. [CrossRef]
9. Moradzadegan, A.; Vaisi-Raygani, A.; Nikzamir, A.; Rahimi, Z. Angiotensin converting enzyme insertion/deletion (I/D) (rs4646994) and Vegf polymorphism (+405G/C; rs2010963) in type II diabetic patients: Association with the risk of coronary artery disease. *J. Renin-Angiotensin-Aldosterone Syst.* **2015**, *16*, 672–680. [CrossRef]
10. Ismail, M.F.; Shaker, O.G.; Ashour, E.; Yousif, H.M.; Afify, M.; Gouda, W. The combined effect of ACE, TCF7L2, and PPARGC1A gene polymorphisms in diabetic nephropathy. *Egypt. J. Chem.* **2017**, *60*, 869–881.
11. Aggarwal, N.; Kare, P.K.; Varshney, P.; Kalra, O.P.; Madhu, S.V.; Banerjee, B.D.; Yadav, A.; Raizada, A.; Tripathi, A.K. Role of angiotensin converting enzyme and angiotensinogen gene polymorphisms in angiotensin converting enzyme inhibitor-mediated antiproteinuric action in type 2 diabetic nephropathy patients. *World J. Diabetes* **2017**, *8*, 112–119. [CrossRef] [PubMed]
12. Widodo, W.; Wisnasari, S.; Saifur Rohman, M.; Yunita, L.; Lukitasari, M.; Nuril, M.; Holil, K.; Laila Purwaningroom, D. Alu insertion/deletion of ACE gene polymorphism might not affect significantly the serum bradykinin level in hypertensive patients taking ACE inhibitors. *Egypt. J. Med. Hum. Genet.* **2017**, *18*, 187–191. [CrossRef]
13. Papadopoulou, A.; Fragkou, P.C.; Maratou, E.; Dimopoulou, D.; Kominakis, A.; Kokkinopoulou, I.; Kroupis, C.; Nikolaidou, A.; Antonakos, G.; Papaevangelou, V.; et al. Angiotensin-converting-enzyme insertion/deletion polymorphism, ACE activity, and COVID19: A rather controversial hypothesis. A case-control study. *J. Med. Virol.* **2022**, *94*, 1050–1059. [CrossRef] [PubMed]
14. Hye Khan, M.A.; Imig, J.D. Antihypertensive Drugs. In *Reference Module in Biomedical Sciences*; Elsevier: Amsterdam, The Netherlands, 2018.
15. Ceconi, C.; Francolini, G.; Olivares, A.; Comini, L.; Bachetti, T.; Ferrari, R. Angiotensin converting enzyme (ACE) inhibitors have different selectivity for bradykinin binding sites of human somatic ACE. *Eur. J. Pharmacol.* **2007**, *577*, 1–6. [CrossRef] [PubMed]
16. Fernandez, J.H.; Hayashi, M.A.; Camargo, A.C.; Neshich, G. Structural basis of the lisinopril-binding specificity in N- and C-domains of human somatic ACE. *Biochem. Biophys. Res. Commun.* **2003**, *308*, 219–226. [CrossRef] [PubMed]
17. Denti, P.; Sharp, S.K.; Kröger, W.L.; Schwager, S.L.; Mahajan, A.; Njoroge, M.; Gibhard, L.; Smit, I.; Chibale, K.; Wiesner, L.; et al. Pharmacokinetic evaluation of lisinopril-tryptophan, a novel C-domain ACE inhibitor. *Eur. J. Pharm. Sci.* **2014**, *56*, 113–119. [CrossRef] [PubMed]
18. Feng, J.; Wang, H.; Jing, Z.; Wang, Y.; Wang, W.; Jiang, Y.; Sun, W. Relationships of the Trace Elements Zinc and Magnesium with Diabetic Nephropathy-Associated Renal Functional Damage in Patients with Type 2 Diabetes Mellitus. *Front. Med.* **2021**, *8*, 626909. [CrossRef]
19. Ming, J.; Sana, S.R.G.L.; Deng, X. Identification of copper-related biomarkers and potential molecule mechanism in diabetic nephropathy. *Front. Endocrinol.* **2022**, *13*, 978601. [CrossRef]
20. Berman, H.M.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T.N.; Weissig, H.; Shindyalov, I.N.; Bourne, P.E. The Protein Data Bank. *Nucleic Acids Res.* **2000**, *28*, 235–242. [CrossRef]
21. Pettersen, E.F.; Goddard, T.D.; Huang, C.C.; Couch, G.S.; Greenblatt, D.M.; Meng, E.C.; Ferrin, T.E. UCSF Chimera—A visualization system for exploratory research and analysis. *J. Comput. Chem.* **2004**, *25*, 1605–1612. [CrossRef]
22. Eberhardt, J.; Santos-Martins, D.; Tillack, A.F.; Forli, S. AutoDock Vina 1.2.0: New Docking Methods, Expanded Force Field, and Python Bindings. *J. Chem. Inf. Model.* **2021**, *61*, 3891–3898. [CrossRef]
23. Trott, O.; Olson, A.J. AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* **2010**, *31*, 455–461. [CrossRef] [PubMed]
24. Biovia, D.S. *BIOVIA Discovery Studio Visualizer (2D Diagram and Scheme of the Interactions with Amino Acids)*; BIOVIA Workbook: San Diego, CA, USA, 2020.
25. Sagoo, M.K.; Gnudi, L. Diabetic Nephropathy: An Overview. *Methods Mol. Biol.* **2020**, *2067*, 3–7. [PubMed]

26. Kobori, H.; Kamiyama, M.; Harrison-Bernard, L.M.; Navar, L.G. Cardinal role of the intrarenal renin-angiotensin system in the pathogenesis of diabetic nephropathy. *J. Investig. Med.* **2013**, *61*, 256–264. [CrossRef] [PubMed]
27. Kim, S.S.; Kim, J.H.; Kim, I.J. Current Challenges in Diabetic Nephropathy: Early Diagnosis and Ways to Improve Outcomes. *Endocrinol. Metab.* **2016**, *31*, 245–253. [CrossRef]
28. ra Lee, S.; Moon, J.Y.; Lee, S.H.; Ihm, C.G.; Lee, T.W.; Kim, S.K.; Chung, J.H.; Kang, S.W.; Kim, T.H.; Park, S.J.; et al. Angiotensinogen polymorphisms and post-transplantation diabetes mellitus in Korean renal transplant subjects. *Kidney Blood Press. Res.* **2013**, *37*, 95–102. [CrossRef] [PubMed]
29. Azmandian, J.; Mohamadifar, M.; Rahmanian-Koshkaki, S.; Mehdipoor, M.; Nematollahi, M.H.; Saburi, A.; Mandegary, A. Study of the association between the donors and recipients angiotensin-converting enzyme insertion/deletion gene polymorphism and the acute renal allograft rejection. *J. Nephropathol.* **2015**, *4*, 62–68. [PubMed]
30. Bertoncello, N.; Moreira, R.P.; Arita, D.Y.; Aragão, D.S.; Watanabe, I.K.; Dantas, P.S.; Santos, R.; Mattar-Rosa, R.; Yokota, R.; Cunha, T.S.; et al. Diabetic Nephropathy Induced by Increased Ace Gene Dosage Is Associated with High Renal Levels of Angiotensin (1-7) and Bradykinin. *J. Diabetes Res.* **2015**, *2015*, 674047. [CrossRef]
31. de Alcantara Santos, R.; Guzzoni, V.; Silva, K.A.S.; Aragão, D.S.; de Paula Vieira, R.; Bertoncello, N.; Schor, N.; Aimbire, F.; Casarini, D.E.; Cunha, T.S. Resistance exercise shifts the balance of renin-angiotensin system toward ACE2/Ang 1-7 axis and reduces inflammation in the kidney of diabetic rats. *Life Sci.* **2021**, *287*, 120058. [CrossRef]
32. Al-Timimi, D.J.; Sulieman, D.M.; Hussen, K.R. Zinc status in type 2 diabetic patients: Relation to the progression of diabetic nephropathy. *J. Clin. Diagn. Res.* **2014**, *8*, CC04–CC08. [CrossRef]
33. Nie, P.; Lou, Y.; Bai, X.; Zhu, Y.; Guo, Q.; Luo, P.; Zhang, W.; Li, B. Influence of zinc levels and Nrf2 expression in the clinical and pathological changes in patients with diabetic nephropathy. *Nutr. Diabetes* **2022**, *12*, 37. [CrossRef]
34. Nikoobakht, M.R.; Pourmand, G.; Allameh, F.; Dialameh, H.; Sharifi, A.; Hashemiaghdam, A. Serum trace elements before and 3 months after renal transplantation in kidney recipients: An Iranian study. *Indian J. Transplant.* **2014**, *8*, 8–11. [CrossRef]
35. Muralidhara, K.; Kannan, S.; Ahamed, I.; Kishore, K.; Vincent, L.; Hegde, N. A prospective observational study on the traditional and novel risk factors associated with post-transplant diabetes mellitus in renal transplant recipients. *Biomedicine* **2021**, *41*, 482–488. [CrossRef]
36. Schüler, R.; Osterhoff, M.A.; Frahnow, T.; Möhlig, M.; Spranger, J.; Stefanovski, D.; Bergman, R.N.; Xu, L.; Seltmann, A.C.; Kabisch, S.; et al. Dietary Fat Intake Modulates Effects of a Frequent ACE Gene Variant on Glucose Tolerance with Association to Type 2 Diabetes. *Sci. Rep.* **2017**, *7*, 9234. [CrossRef]
37. ALkafaji, M.J.M.; Shehab, A.F.; Omair, H.A. ACE Polymorphisms Rs (4343) and Its Association with Ace Enzyme in Some of Patients with Diabetic Nephropathy in Some Population of Salah Al-Din Governorate in Iraq. *Pak. Heart J.* **2023**, *56*, 193–199.
38. Raza, S.T.; Abbas, S.; Siddiqi, Z.; Mahdi, F. Association between *ACE* (rs4646994), *FABP2* (rs1799883), *MTHFR* (rs1801133), *FTO* (rs9939609) Genes Polymorphism and Type 2 Diabetes with Dyslipidemia. *Int. J. Mol. Cell. Med.* **2017**, *6*, 121–130. [PubMed]
39. Zhao, Y.; Zhu, R.; Wang, D.; Liu, X. Genetics of diabetic neuropathy: Systematic review, meta-analysis and trial sequential analysis. *Ann. Clin. Transl. Neurol.* **2019**, *6*, 1996–2013. [CrossRef]
40. Bayoumy, N.; El-Shabrawi, M.M.; Leheta, O.F.; Omar, H.H. ACE (rs4646994) and MTHFR (rs1801133) single nucleotide polymorphisms in type 2 Diabetes Mellitus patients with dyslipidemia. *Rom. J. Diabetes Nutr. Metab. Dis.* **2022**, *29*, 401–406.
41. Sembach, F.E.; Fink, L.N.; Johansen, T.; Boland, B.B.; Secher, T.; Thrane, S.T.; Nielsen, J.C.; Fosgerau, K.; Vrang, N.; Jelsing, J.; et al. Impact of sex on diabetic nephropathy and the renal transcriptome in UNx db/db C57BLKS mice. *Physiol. Rep.* **2019**, *7*, e14333. [CrossRef]
42. Duan, J.; Wang, C.; Liu, D.; Qiao, Y.; Pan, S.; Jiang, D.; Zhao, Z.; Liang, L.; Tian, F.; Yu, P.; et al. Prevalence and risk factors of chronic kidney disease and diabetic kidney disease in Chinese rural residents: A cross-sectional survey. *Sci. Rep.* **2019**, *9*, 10408. [CrossRef]
43. Hussain, S.; Jamali, M.C.; Habib, A.; Hussain, M.S.; Akhtar, M.; Najmi, A.K. Diabetic kidney disease: An overview of prevalence, risk factors, and biomarkers. *Clin. Epidemiol. Glob. Health* **2021**, *9*, 2–6. [CrossRef]
44. Selmi, A.; Aydi, R.; Kammoun, O.; Bougatef, H.; Bougatef, A.; Miled, N.; Alghamdi, O.A.; Kammoun, M. Synthesis, crystal structure, molecular docking studies and biological evaluation of aryl substituted dihydroisoquinoline imines as a potent angiotensin converting enzyme inhibitor. *J. Mol. Struct.* **2021**, *1235*, 130230. [CrossRef]
45. Dey, T.K.; Chatterjee, R.; Mandal, R.S.; Roychoudhury, A.; Paul, D.; Roy, S.; Pateiro, M.; Das, A.K.; Lorenzo, J.M.; Dhar, P. ACE Inhibitory Peptides from *Bellamya bengalensis* Protein Hydrolysates: In Vitro and In Silico Molecular Assessment. *Processes* **2021**, *9*, 1316. [CrossRef]
46. Januszewicz, A.; Januszewicz, W.; Szczepańska-Sadowska, E.; Sznajderman, M. *Nadcis´nienie Te¸tnicze [Hypertension]*; Wydawnictwo Medycyna Praktyczna [Practical Medicine Publishing House]: Kraków, Poland, 2007; pp. 1079–1080.

Supplementary materials

Questionnaire S1: Sample of a questionnaire conducted among people suffering from diabetic nephropathy.

PART 1. PRELIMINARY INFORMATION

Sex [W/M]	City of residence	Age [years]	Height [cm]	Weight [kg]

QUESTION	ANSWER
Do you suffer from diabetes? If so, what type?	
Do you suffer from chronic diseases other than diabetes? If so, which ones?	
Do you suffer from diabetic nephropathy?	
Have you ever been or are you undergoing dialysis?	
Have you ever received a kidney transplant?	

PART 2. LIFESTYLE

Do you play sports? How many hours per week?	
Do you smoke cigarettes? How many years? How many packs a day?	
Do you consume alcohol? In what amount? How often?	
Do you take medications/dietary supplements? Which medications or dietary supplements?	

PART 3. DISEASES IN THE FAMILY

Do your parents suffer from diabetes?	
Do your siblings suffer from diabetes?	
Do your parents suffer from diabetic nephropathy?	
Do your siblings suffer from diabetic nephropathy?	

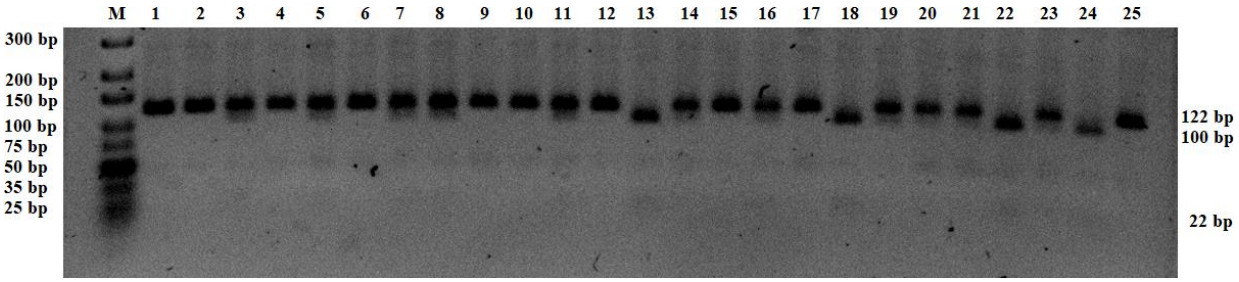


Figure S1. Example of electropherogram for rs4343 (*ACE*).

M – marker ladder; 1, 2, 4, 6, 9, 10, 12, 15, 17, 25 – G/G genotype; 3, 5, 7, 8, 11, 14, 16, 19, 20, 21, 23 – G/A genotype; 13, 18, 22, 24 – A/A genotype

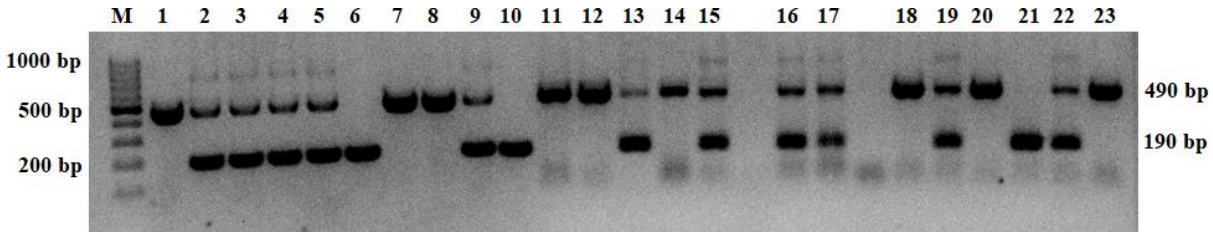
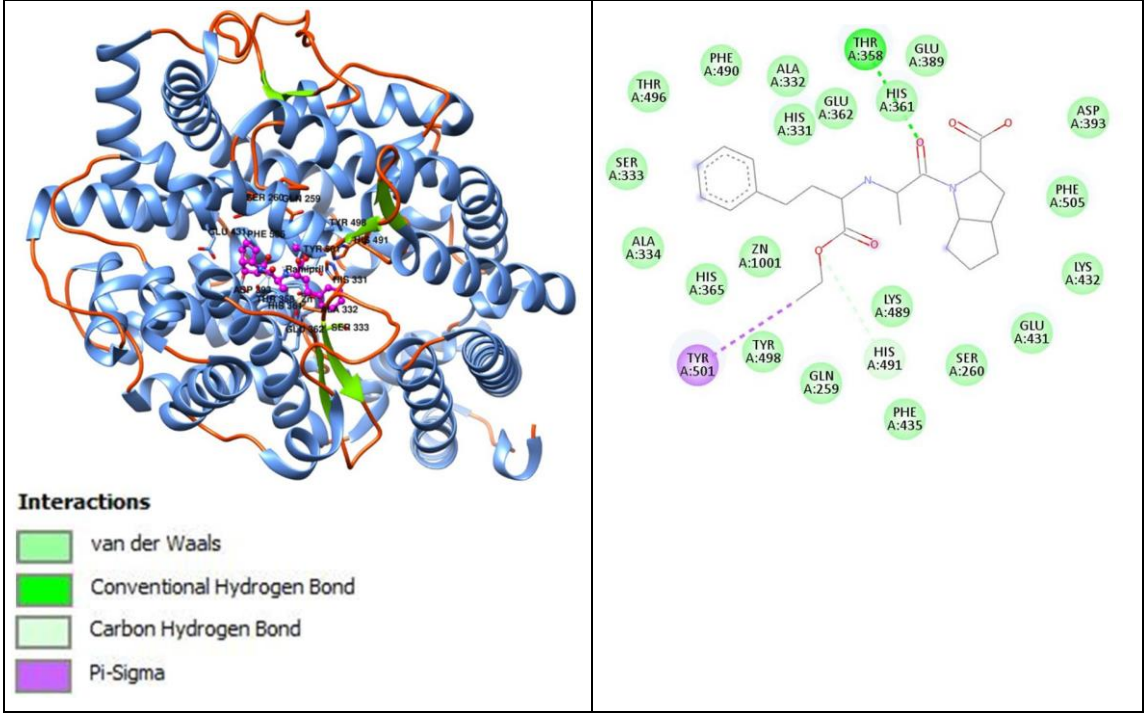


Figure S2. Example of electropherogram for rs4646994 (*ACE*).

M – marker ladder; 1, 7, 8, 11, 12, 14, 18, 20, 23 – I/I genotype; 2, 3, 4, 5, 9, 13, 15, 16, 17, 19, 22 – I/D genotype; 6, 10, 21 – D/D genotype

A.

N domain of ACE with ramipril



B.

C domain of ACE with ramipril

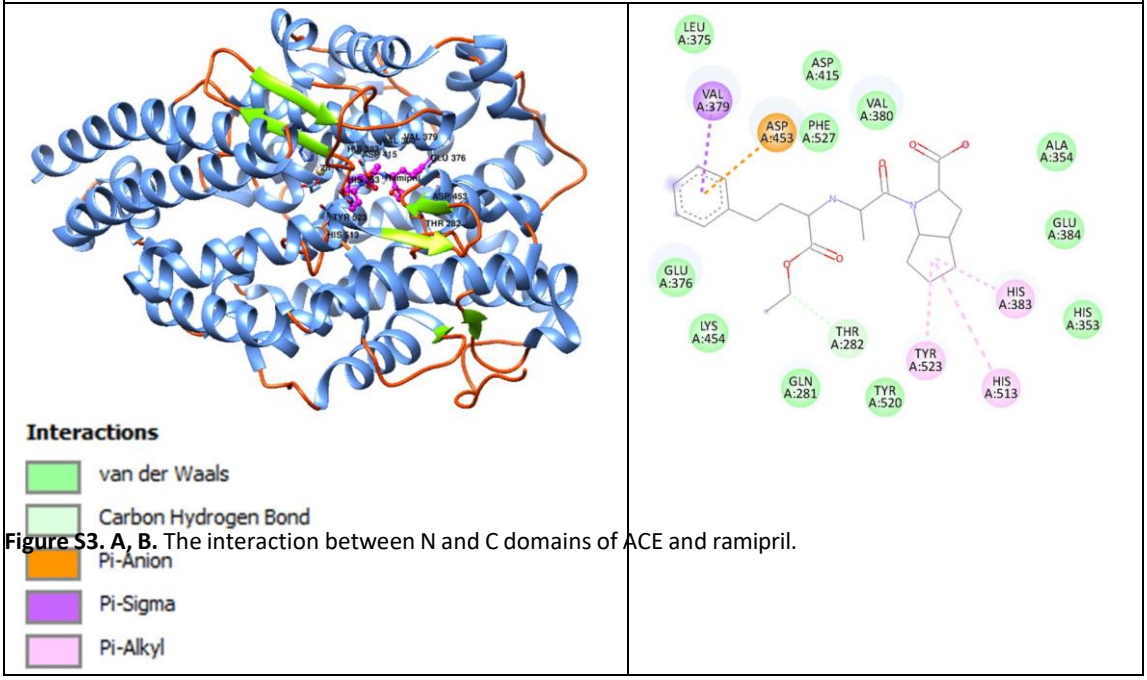
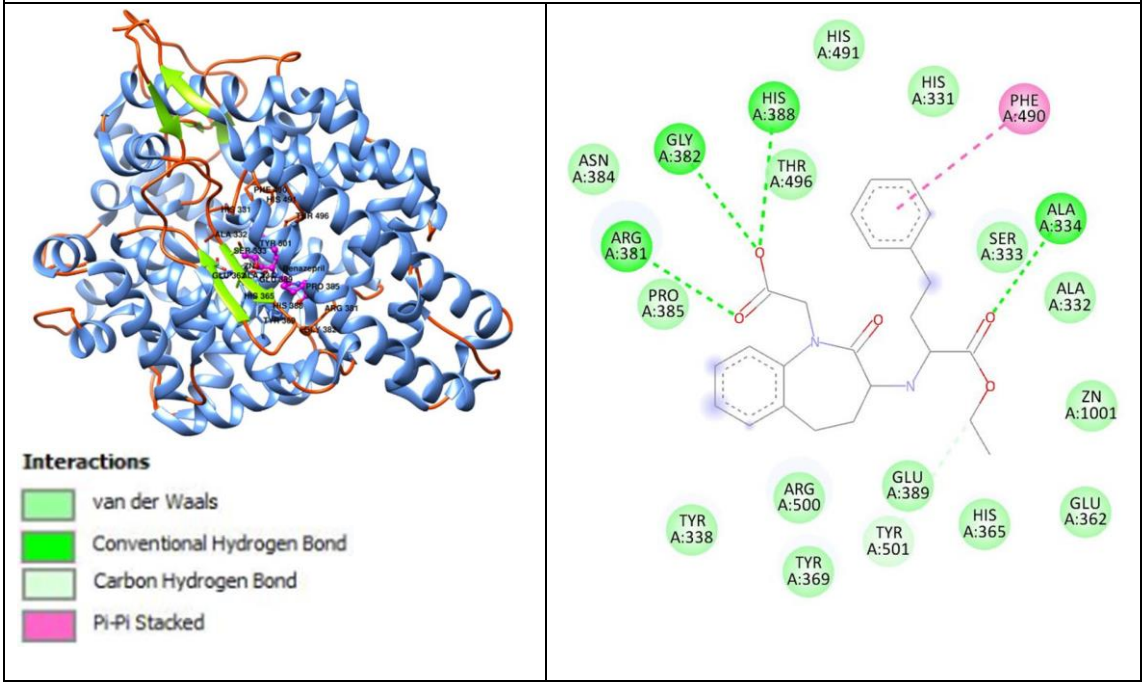


Figure S3. A, B. The interaction between N and C domains of ACE and ramipril.

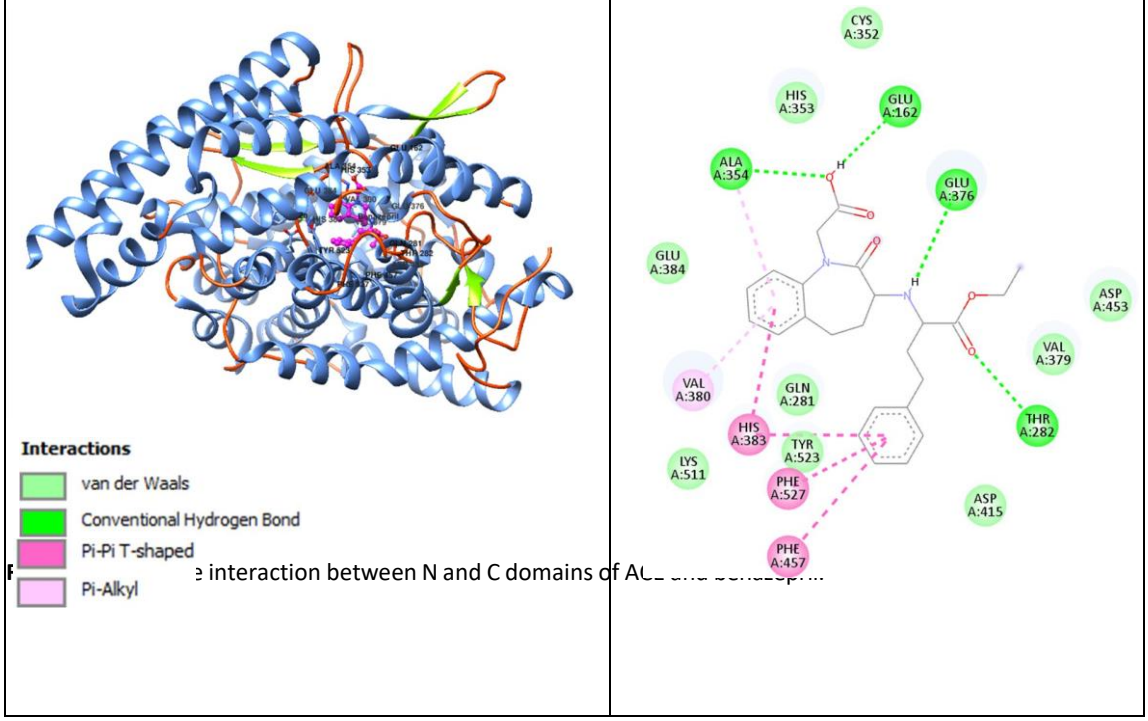
A.

N domain of ACE with benazepril



B.

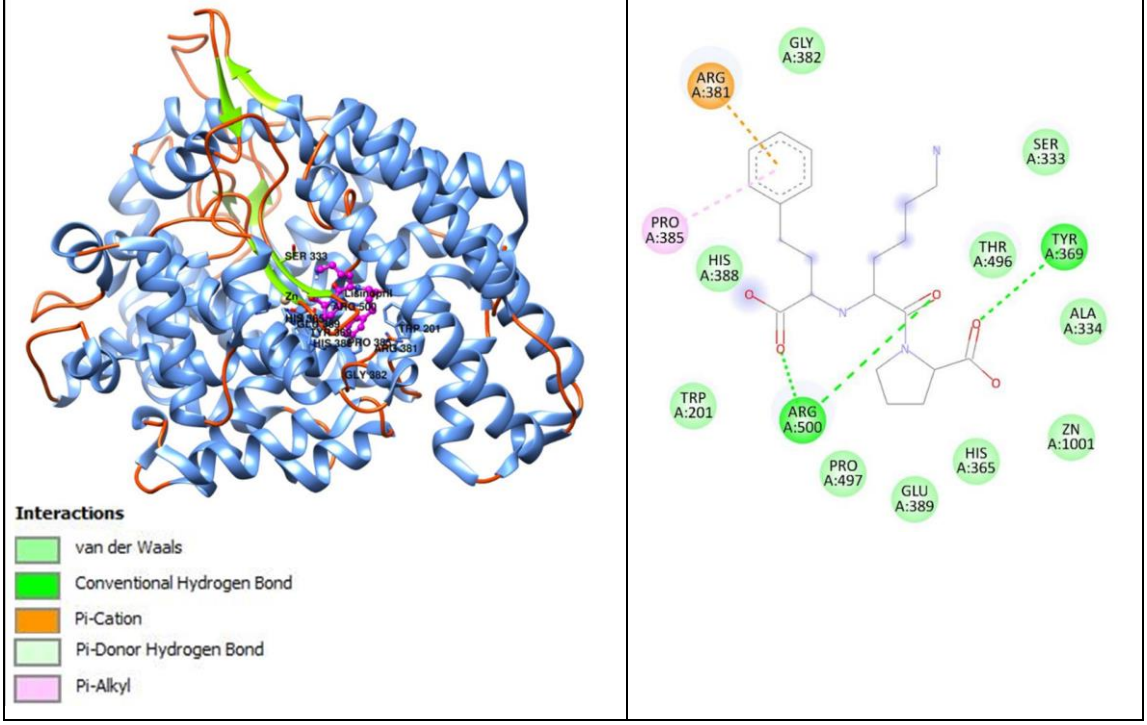
C domain of ACE with benazepril



F e interaction between N and C domains of ACE and benazepril

A.

N domain of ACE with lisinopril



B.

C domain of ACE with lisinopril

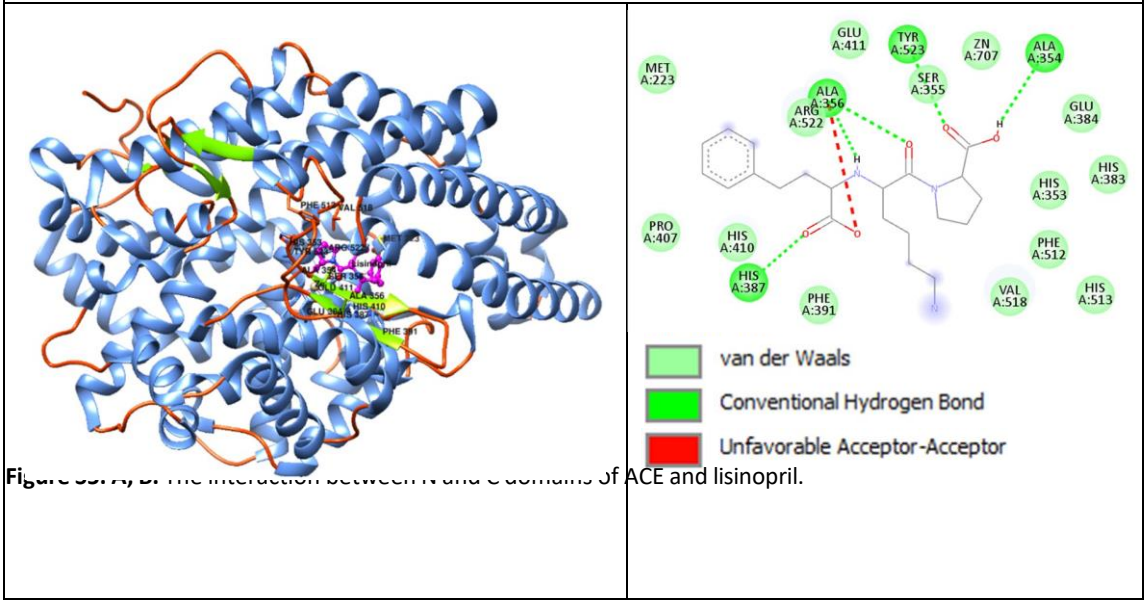


Figure 33. A, B. The interaction between N and C domains of ACE and lisinopril.

antioxidants

Article

The Effect of Selected Nitric Oxide Synthase Polymorphisms on the Risk of Developing Diabetic Nephropathy

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Abstract: Background: Nitric oxide synthase (NOS) is an enzyme that catalyzes the formation of nitric oxide (NO), the altered production of which is characteristic of diabetic nephropathy. NOS exists in three isoforms: NOS1, NOS2, and NOS3. Moreover, there are reports about the potential role of *NOS3* polymorphisms in the development of diabetes complications. The aim of this study was to assess the role of selected *NOS* polymorphisms—rs3782218 (*NOS1*), rs1137933 (*NOS2*), rs1799983, rs2070744, and rs61722009 (*NOS3*)—in the risk of developing diabetic nephropathy and in the likelihood of renal replacement therapy. Methods: The studied polymorphisms were analyzed in a group of 232 patients divided into three groups. Four polymorphisms (rs3782218, rs1137933, rs1799983, rs2070744) were genotyped using the PCR-RFLP, while the rs61722009 polymorphism was genotyped using the PCR. Results: The C/C genotype and the C allele of the rs3782218 polymorphism (*NOS1*) were associated with an increased risk of developing diabetic nephropathy and an increased likelihood of renal replacement therapy. In turn, the G allele of the rs1137933 polymorphism (*NOS2*) reduces the likelihood of renal replacement therapy. Conclusions: The specific genotypes or alleles of the rs3782218 (*NOS1*) and rs1137933 (*NOS2*) polymorphisms seem to be potential risk factors for diabetic nephropathy and renal replacement therapy.

Keywords: diabetes nephropathy; renal replacement therapy; single-nucleotide polymorphisms; NOS isoforms

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1. Introduction

In modern medicine, diabetes is classified as a lifestyle that cannot only significantly reduce the quality of life but also lead to serious complications [1–3]. One of the most common complications of type 1 and type 2 diabetes is diabetic nephropathy, which often leads to the need for renal replacement therapy [4–6]. To understand these somewhat complicated relationships, it is necessary to refer to the pro-/antioxidant balance disorders accompanying diabetes and its complications. Hyperglycemia increases the production of free radicals and reactive oxygen species (ROS), thus causing oxidative stress [7,8]. However, despite studies indicating that increased oxidative stress causes the progression of diabetic nephropathy, the accurate mechanisms are still not fully understood [9]. Analogous compounds to ROS are reactive nitrogen species (RNS)—a group of molecules derived from nitric oxide (NO). RNS have unpaired electrons; thus, they are characterized by high chemical reactivity [10]. Their excessive production causes the phenomenon of nitrosative stress, which also accompanies many diseases, including diabetes and its complications [11].

NO is produced in a reaction catalyzed by nitric oxide synthase (NOS). There are three known isoforms of this enzyme—NOS1, NOS2, and NOS3—and each of them is encoded by a different gene [12]. According to the literature, early nephropathy occurring in diabetes is associated with increased intrarenal NO production, mainly mediated by neuronal nitric oxide synthase (NOS1, nNOS) and endothelial nitric oxide synthase (NOS3, eNOS) [13].

Increased NO production may contribute to hyperfiltration as well as microalbuminuria, which are characteristic of the early stage of diabetic nephropathy [14]. On the other hand, most studies indicate that advanced nephropathy leads to severe proteinuria and that the deteriorating condition is associated with hypertension accompanied by progressive NO deficiency. Several factors, including hyperglycemia, increased formation of advanced glycation end products, increased oxidative stress, and the activation of certain proteins, contribute to reducing NO production or its availability [15,16].

The expression of all three NOS isoforms can be detected in pancreatic β cells. NOS1 can be found in insulin secretory granules [17]. In turn, in insulin resistance, NOS2 participates in the deregulation of metabolic processes in tissues by disturbing glucose and lipid homeostasis and causing endothelial dysfunction through the local and systemic formation of an inflammatory environment [18]. This is due to increased nitrosative stress, which affects the activity of proteins that are involved in maintaining glucose metabolism [19]. Moreover, polymorphisms of genes encoding NOS isoforms may also play a role in NO-related abnormalities that contribute to the development and progression of insulin resistance, type 2 diabetes (T2D), and diabetic nephropathy [20–23].

Garme et al. [24] showed that the C/T genotype of the rs1137933 polymorphism in *NOS2* was associated with an increased risk of developing T2D. In addition, the T allele of this polymorphism was also associated with an increased risk of progression of this disease. Other studies also demonstrated associations between the rs1137933 polymorphism and increased susceptibility to the development of T2D, but no similar relationship was observed in patients with diabetic nephropathy [25]. However, this study was only performed on the Chinese Han population, so it should be extended to include other populations to exclude or confirm their influence on the results.

The *NOS3* polymorphisms seem to be equally important in the development of T2D and its complications. The variable number of tandem repeat (VNTR) polymorphisms in the *NOS3* has been shown to be associated with the development of diabetic nephropathy. A study of 598 patients has shown that it may be associated with the progression of diabetes mellitus and, consequently, diabetic nephropathy [26]. Another study, which concerned the assessment of *NOS3* polymorphisms in the context of the risk of developing diabetic nephropathy, concerned the analysis of rs1799983, rs2070744, and 27-bp VNTR polymorphisms [27]. The T allele of the rs1799983 polymorphism and the C allele of the rs2070744 polymorphism were significantly more frequent in patients with diabetic nephropathy than in patients without nephropathy. However, in the case of 27-bp VNTR polymorphism, no significant change in NO concentrations was found. The obtained results suggest that *NOS* polymorphisms may indeed be genetic determinants of diabetic nephropathy in patients with type 2 diabetes.

The aim of this study was to assess the frequency of selected polymorphisms of genes encoding all three NOS isoforms—NOS1, NOS2, and NOS3—in patients with diabetic nephropathy, both after and without kidney transplantation. The following polymorphisms were analyzed: rs3782218 (*NOS1*), rs1137933 (*NOS2*), rs1799983, rs2070744, and rs61722009 (*NOS3*). The rs3782218 polymorphism is mainly associated with an increased risk of cardiovascular diseases, where the T allele shows an additive protective effect against coronary heart disease (CHD) and hypertension [28]. However, due to the fact that both diabetes and its complications are often accompanied by this type of disease, it was decided to include it in these studies. This polymorphism is located in a regulatory region of *NOS1*. It belongs to single-nucleotide polymorphisms (SNPs) and consists of replacing cytosine with thymine [29]. The rs1137933 polymorphism also belongs to the SNP type of polymorphism; it is located in exon 10 of *NOS2* and consists of replacing cytosine with thymine. This is a missense mutation [25]. The rs1799983 and rs2070744 polymorphisms also belong to the SNP-type polymorphisms. The first one is located in exon 7 of *NOS3* and consists of replacing guanine with thymine. This is a missense mutation [30,31]. The second polymorphism is located in the promoter region of *NOS3* and consists of replacing cytosine with thymine [31]. The last polymorphism—rs61722009—is a VNTR-type polymorphism

and is located in intron 4. It is often found under other names, such as 27-bp VNTR polymorphism or 4a/4b polymorphism [32].

Additionally, the relationship between specific genotypes of the above-mentioned polymorphisms and the concentration of individual NOS isoforms, creatinine, and C-reactive protein (CRP) in blood serum and glucose in blood plasma was also examined. The relationship between individual genotypes within the studied polymorphisms and the concentrations of zinc and copper was also taken into account, as these are elements that are of fundamental importance in the pathophysiology of diabetes and its complications [33,34].

2. Materials and Methods

2.1. Study Groups

The research material was blood samples obtained from 232 patients who participated in this study, divided into three groups: the control group ($N = 50$), the diabetic nephropathy group ($N = 85$), and the kidney transplant diabetic nephropathy group ($N = 97$). Consent of the Bioethics Committee at Wroclaw Medical University (no. KB 835/2021) was obtained for the use of the collected biological material for research purposes. All respondents were acquainted with research issues and gave their consent in writing to collect biological material. The sample size was determined by power analysis using preliminary data from previous studies, with assumptions of $\alpha = 0.05$ and a power of 80%. These data were obtained as part of unpublished research conducted on a group of patients with type 2 diabetes, the aim of which was to check the impact of the rs1799983 polymorphism in *NOS3* on the risk of developing this disease. In order to determine the sample size, differences in allele frequencies (in the context of the rs1799983 polymorphism) and biochemical parameters (concentrations of NOS3, glucose, CRP, zinc, and copper) were used. Based on the results obtained, it was estimated that the recruited sample size was adequate to observe the expected differences.

Biological samples were obtained from Łukasiewicz PORT—Polish Center for Technology Development (control group)—and from the Department and Clinic of Nephrology and Transplantation Medicine of the Wroclaw Medical University (diabetic nephropathy group and kidney transplant diabetic nephropathy group). Blood was collected into tubes with clotting activator (to obtain serum; cat. No.: BD 368815, Becton Dickinson, Franklin Lakes, NJ, USA) and tubes with EDTA (to obtain plasma and buffy coat; cat. No.: BD 367864, Becton Dickinson, USA). DNA was isolated from the buffy coat using a ready-made isolation kit (Syngen Blood/Cell DNA Mini Kit, cat. No.: SY221012, Syngen Biotech, Wrocław, Poland).

The control group consisted of respondents with excluded cardiovascular diseases, liver function disorders (measured by gamma-glutamyltransferase—GGT; alanine aminotransferase—ALT; and aspartate aminotransferase—AST activity), atherosclerosis, diabetes (based on insulin and fasting glucose measurements), hypertension (blood pressure measurements), inflammation (C-reactive protein concentration), and tumors. The use of drugs or dietary supplements in the last 6 months was also an exclusion criterion.

The selection of diabetic nephropathy patients was made on the basis of medical history, laboratory tests, and imaging tests (e.g., USG). The following parameters were measured in patients: creatinine, blood morphology, urine general examination (including the presence of protein), albuminuria, sodium/potassium, glucose, and eGFR (estimated glomerular filtration rate; calculated according to the abbreviated formula MDRD—Modification of Diet in Renal Disease). Qualification for this study required the presence of diabetes and one of the following: albuminuria, proteinuria, or increased creatinine levels. The exclusion criterion was the presence of other causes of kidney damage. In addition, patients received a questionnaire in which we collected information such as age, gender, anthropometric data (weight, height), other chronic diseases, the use of stimulants (smoking cigarettes, alcohol consumption), or medications (Questionnaire S1, Supplementary Materials). In order to characterize the groups, the following parameters were used: age, sex, BMI values, glucose and creatinine concentrations, eGFR values, and CRP concentrations. The

characteristics of the three studied groups have already been described in a previous study that focused on the role of *ACE* polymorphisms in assessing the risk of developing diabetic nephropathy [35].

2.2. Methods

2.2.1. Determination of NOS1, NOS2, NOS3, Glucose, Creatinine, eGFR, and CRP Concentrations

Serum NOS1 concentration was measured using the Human Nitric Oxide Synthase, Brain, NOS1 ELISA Kit (cat. No.: E0924Hu, Bioassay Technology Laboratory, Shanghai, China). Serum NOS2 concentration was measured using the Human Nitric Oxide Synthase, Inducible, INOS-NOS2 ELISA Kit (cat. No.: E4710Hu, Bioassay Technology Laboratory, Shanghai, China). Serum NOS3 concentration was measured using the Human Endothelial Nitric Oxide Synthase (eNOS) ELISA Kit (cat. No.: MBS265088, MyBioSource, Inc., San Diego, CA, USA). Glucose, creatinine, and CRP concentrations were measured in the hospital laboratory during routine patient visits. eGFR values were calculated according to the abbreviated MDRD formula.

2.2.2. Determination of Metal Concentrations

The metal concentrations (zinc—Zn; copper—Cu) in the blood serum were measured using the SOLAAR M6 atomic absorption spectrophotometer (Thermo Elemental Solaar House, Cambridge, UK) at the Department and Clinic of Internal and Occupational Diseases, Hypertension and Clinical Oncology, Wroclaw Medical University. To measure the concentrations of these metals, the flame atomic absorption spectrometry (FAAS) method was used in an air–acetylene flame.

2.2.3. Genotyping Analysis

The four polymorphisms (rs3782218, rs1137933, rs1799983, rs2070744) were determined using the polymerase chain reaction and restriction fragment length polymorphism analysis (PCR-RFLP). In turn, the rs61722009 polymorphism (*NOS3*), due to the fact that it is the VNTR polymorphism, was determined using the polymerase chain reaction (PCR). Primers were designed with the Primer-BLAST program based on gene sequences from the GenBank (National Center for Biotechnology Information). The sequences of the primers, reaction conditions, and the restriction enzyme used in this study are presented in Table 1.

Table 1. The conditions for PCR and restriction enzyme digestion.

SNP	Primers	PCR-RFLP Conditions
rs3782218 (*NOS1*)	Forward primer—5′ CTG AGA GCA GAA GGT GGG TG 3′ Reverse primer—5′ GTC CTG GAT GGG TTT CCC TG 3′	the initial denaturation—95 °C for 15 min denaturation—95 °C for 40 s annealing—62 °C for 35 s elongation—72 °C for 40 s the final elongation—72 °C for 10 min
	Restriction enzyme	**Restriction enzyme digestion conditions**
	Hpy99I	37 °C for 1 h
rs1137933 (*NOS2*)	**Primers**	**PCR-RFLP Conditions**
	Forward primer—5′ CTC ACC AAA AAG TCT TCA GAC TCA CA 3′ Reverse primer—5′ GGC CCC AGT TAA ATT GTG TCT ACC 3′	the initial denaturation—95 °C for 15 min denaturation—95 °C for 40 s annealing—59 °C for 35 s elongation—72 °C for 40 s the final elongation—72 °C for 10 min
	Restriction enzyme	**Restriction enzyme digestion conditions**
	Hin1I	37 °C for 16 h

Table 1. *Cont.*

rs1799983 (*NOS3*)	**Primers**	**PCR-RFLP Conditions**
	Forward primer—5′ GAC CCT GGA GAT GAA GGC AG 3′ Reverse primer—5′ CAT CCC ACC CAG TCA ATC CC 3′	the initial denaturation—95 °C for 5 min denaturation—95 °C for 40 s annealing—60.4 °C for 35 s elongation—72 °C for 40 s the final elongation—72 °C for 10 min
	Restriction enzyme	**Restriction enzyme digestion conditions**
	MboI	37 °C for 16 h
rs2070744 (*NOS3*)	**Primers**	**PCR-RFLP Conditions**
	Forward primer—5′ CTA GTG GCC TTT CTC CAG CC 3′ Reverse primer—5′ GCC CAG CAA GGA TGT AGT GA 3′	the initial denaturation—95 °C for 15 min denaturation—95 °C for 40 s annealing—62.0 °C for 35 s elongation—72 °C for 1 min the final elongation—72 °C for 10 min
	Restriction enzyme	**Restriction enzyme digestion conditions**
	MspI	37 °C for 16 h
rs61722009 (*NOS3*)	**Primers**	**PCR Conditions**
	Forward primer—5′ CTA TGG TAG TGC CTT GGC TGG AG 3′ Reverse primer—5′ GTC ACA GGC GTT CCA GTA ACT AAG 3′	the initial denaturation—95 °C for 15 min denaturation—95 °C for 20 s annealing—58.0 °C for 35 s elongation—72 °C for 40 s the final elongation—72 °C for 10 min

The digested DNA fragments were visualized using a 2% or 3% agarose gel with Green DNA Gel Stain (cat. No.: SY 521031, Syngen Biotech, Wrocław, Poland). Electropherograms showing restriction digest products are provided in Figures S1–S5 (Supplementary Materials).

2.2.4. Statistical Analysis

Statistical analyses were performed using the STATISTICA 13.3 package (Statsoft Polska, Sp. z o.o., Kraków, Poland) under the Wroclaw Medical University license.

The frequencies of genotypes were compared using the χ^2 test and Fisher's exact test. In order to check whether all populations are in Hardy–Weinberg equilibrium, the χ^2 test was performed.

Normality of distribution was checked using the Shapiro–Wilk test, and homogeneity of variances was checked using Levene's test. To test statistical significance between groups, the nonparametric Kruskal–Wallis test with Dunn's post hoc test was used.

Logistic regression analysis was performed to assess the significance of the effect of polymorphism genotypes on the risk of diabetic nephropathy and the likelihood of renal replacement therapy, expressed as odds ratios (OR) with a 95% confidence interval (CI). Statistical significance was considered for $p < 0.05$.

3. Results

3.1. Concentrations of the NOS Isoforms in the Studied Groups

Lower NOS1 and NOS2 concentrations were observed in the diabetic nephropathy group compared to the control group ($p = 0.002$, $p < 0.001$, respectively).

What is more, lower NOS3 concentrations were observed in patients with diabetic nephropathy ($p < 0.001$) and in patients with diabetic nephropathy after kidney transplantation ($p < 0.001$) compared to the control group. Similar relationships were not observed between the diabetic nephropathy group and the kidney transplant diabetic nephropathy

group (p = 1.000). The results are presented in Figures 1–3. Additionally, the results are shown in Table S1 (Supplementary Materials).

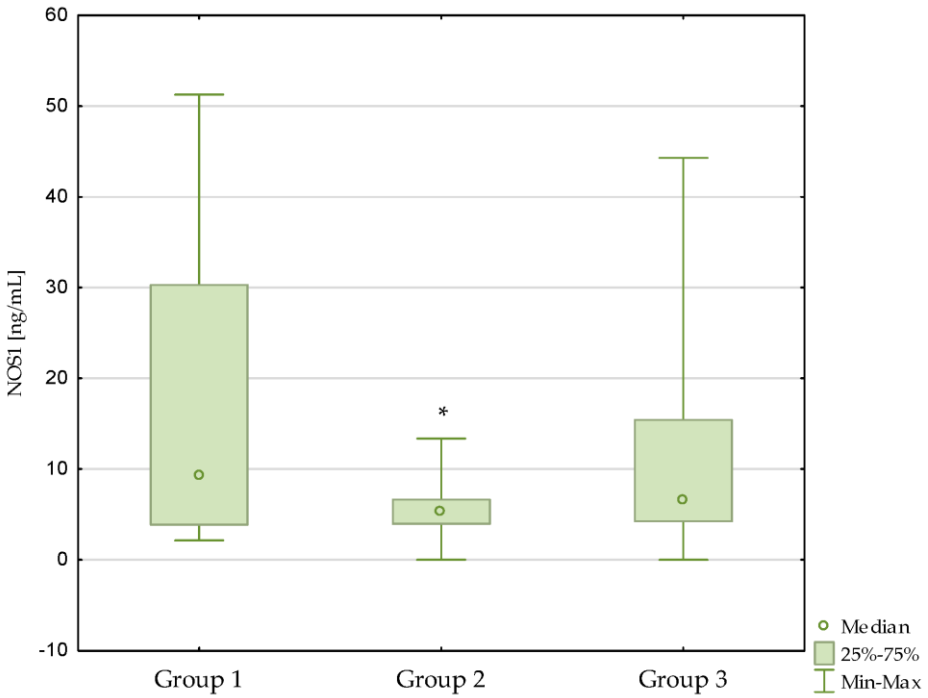

Figure 1. Concentrations of NOS1 in the studied groups. Group 1—control group; Group 2—diabetic nephropathy group; Group 3—kidney transplant diabetic nephropathy group. * $p < 0.05$—compared to the control group.

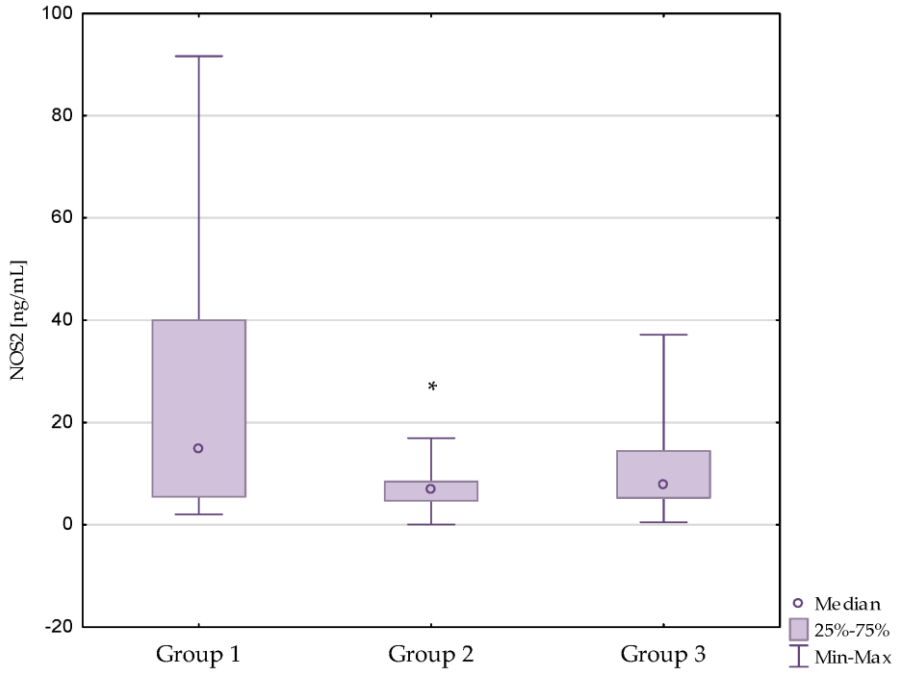

Figure 2. Concentrations of NOS2 in the studied groups. Group 1—control group; Group 2—diabetic nephropathy group; Group 3—kidney transplant diabetic nephropathy group. * $p < 0.05$—compared to the control group.

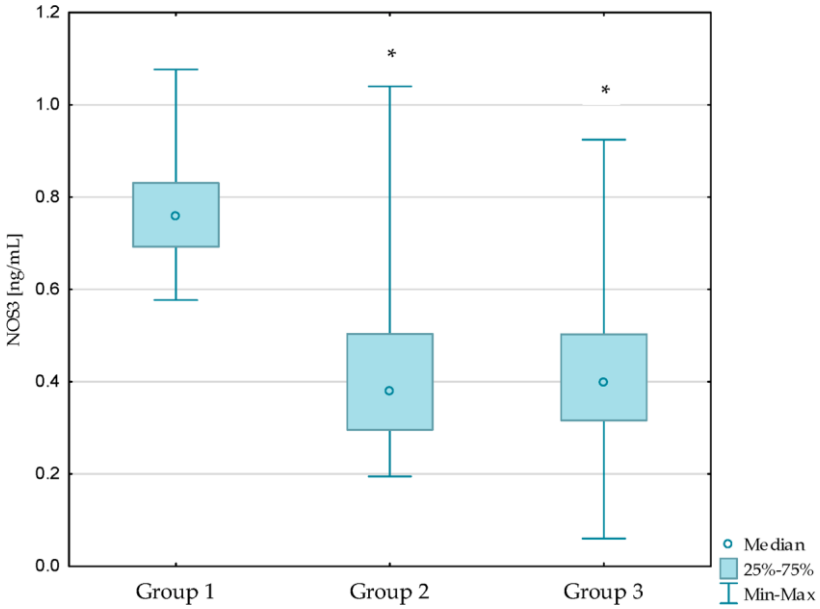

Figure 3. Concentrations of NOS3 in the studied groups. Group 1—control group; Group 2—diabetic nephropathy group; Group 3—kidney transplant diabetic nephropathy group. * $p < 0.05$—compared to the control group.

3.2. The Genotype Distribution of the NOS1, NOS2, and NOS3 Polymorphisms

No significant differences were observed in the genotype distribution in the studied groups for the rs1137933 polymorphism in *NOS2* ($p = 0.931$), the rs2070744 polymorphism in *NOS3* ($p = 0.495$), and the rs61722009 polymorphism in *NOS3* ($p = 0.839$). However, in the case of the rs3782218 polymorphism in *NOS1*, these differences were present ($p = 0.008$). The C/T genotype occurred much more often compared to other genotypes in the control group (68.09%), while in the remaining studied groups, it did not constitute such an advantage. Differences in the distribution of genotypes were also observed in the case of the rs1799983 polymorphism in *NOS3* ($p = 0.026$). The G/G genotype in the control group was the dominant genotype (52.00%), while in the other two groups, the G/T genotype was dominant (59.74% and 63.41%, respectively). The results are presented in Table 2 and Figures 4–8.

Table 2. The genotype distribution of the *NOS1*, *NOS2*, and *NOS3* polymorphisms in the studied groups.

Polymorphism (*Gene*)	Groups (N)	Genotype Frequencies (%)			*p*
		C/C	C/T	T/T	
rs3782218 (*NOS1*)	Control ($N = 47$)	$N = 4$ (8.51%)	$N = 32$ (68.09%)	$N = 11$ (23.40%)	0.008
	Diabetic Nephropathy ($N = 79$)	$N = 26$ (32.91%)	$N = 35$ (44.30%)	$N = 18$ (22.79%)	
	Kidney Transplant Diabetic Nephropathy ($N = 96$)	$N = 18$ (18.75%)	$N = 47$ (48.96%)	$N = 31$ (32.29%)	

Table 2. *Cont.*

Polymorphism (*Gene*)	Groups (N)	Genotype Frequencies (%)			*p*
		C/C	C/T	T/T	
rs1137933 (*NOS2*)	Control (*N* = 44)	*N* = 28 (63.64%)	*N* = 14 (31.82%)	*N* = 2 (4.54%)	0.931
	Diabetic Nephropathy (*N* = 83)	*N* = 55 (66.26%)	*N* = 24 (28.92%)	*N* = 4 (4.82%)	
	Kidney Transplant Diabetic Nephropathy (*N* = 97)	*N* = 58 (59.79%)	*N* = 34 (35.05%)	*N* = 5 (5.16%)	
Polymorphism (*Gene*)	**Groups (*N*)**	**Genotype Frequencies (%)**			***p***
		G/G	**G/T**	**T/T**	
rs1799983 (*NOS3*)	Control (*N* = 50)	*N* = 26 (52.00%)	*N* = 19 (38.00%)	*N* = 5 (10.00%)	0.026
	Diabetic Nephropathy (*N* = 77)	*N* = 29 (37.66%)	*N* = 46 (59.74%)	*N* = 2 (2.60%)	
	Kidney Transplant Diabetic Nephropathy (*N* = 82)	*N* = 24 (29.27%)	*N* = 52 (63.41%)	*N* = 6 (7.32%)	
Polymorphism (*Gene*)	**Groups (*N*)**	**Genotype Frequencies (%)**			***p***
		C/C	**C/T**	**T/T**	
rs2070744 (*NOS3*)	Control (*N* = 50)	*N* = 7 (14.00%)	*N* = 5 (10.00%)	*N* = 38 (76.00%)	0.495
	Diabetic Nephropathy (*N* = 84)	*N* = 6 (7.14%)	*N* = 13 (15.48%)	*N* = 65 (77.38%)	
	Kidney Transplant Diabetic Nephropathy (*N* = 97)	*N* = 12 (12.37%)	*N* = 9 (9.28%)	*N* = 76 (78.35%)	
Polymorphism (*Gene*)	**Groups (*N*)**	**Genotype Frequencies (%)**			***p***
		4a/4a	**4a/4b**	**4b/4b**	
rs61722009 (*NOS3*)	Control (*N* = 50)	*N* = 2 (4.00%)	*N* = 15 (30.00%)	*N* = 33 (66.00%)	0.839
	Diabetic Nephropathy (*N* = 85)	*N* = 2 (2.35%)	*N* = 31 (36.47%)	*N* = 52 (61.18%)	
	Kidney Transplant Diabetic Nephropathy (*N* = 97)	*N* = 3 (3.09%)	*N* = 38 (39.18%)	*N* = 56 (57.73%)	

It was also tested whether all populations, separated according to studied groups and polymorphisms, were in Hardy–Weinberg equilibrium. Several populations were found to have significant differences between observed and expected data, indicating that these populations are not in Hardy–Weinberg equilibrium. These were the following populations: the control group with the rs3782218 polymorphism; the diabetic nephropathy group and kidney transplant diabetic nephropathy group with the rs1799983 polymorphism; and the control group, diabetic nephropathy group, and kidney transplant diabetic nephropathy group with the rs2070744 polymorphism. Due to the fact that not all populations are in the Hardy–Weinberg equilibrium, the obtained results should be approached with caution. The results of the Hardy–Weinberg equilibrium χ^2 test are presented in Table S2 (Supplementary Materials).

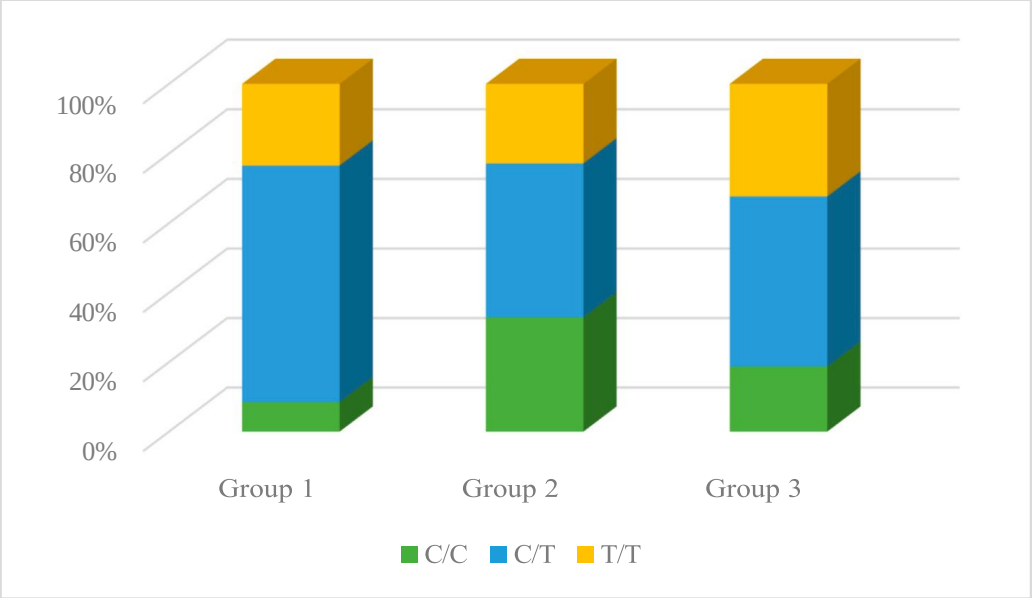

Figure 4. The genotype distribution of the rs3782218 polymorphism (*NOS1*) in the studied groups. Group 1—control group; Group 2—diabetic nephropathy group; Group 3—kidney transplant diabetic nephropathy group.

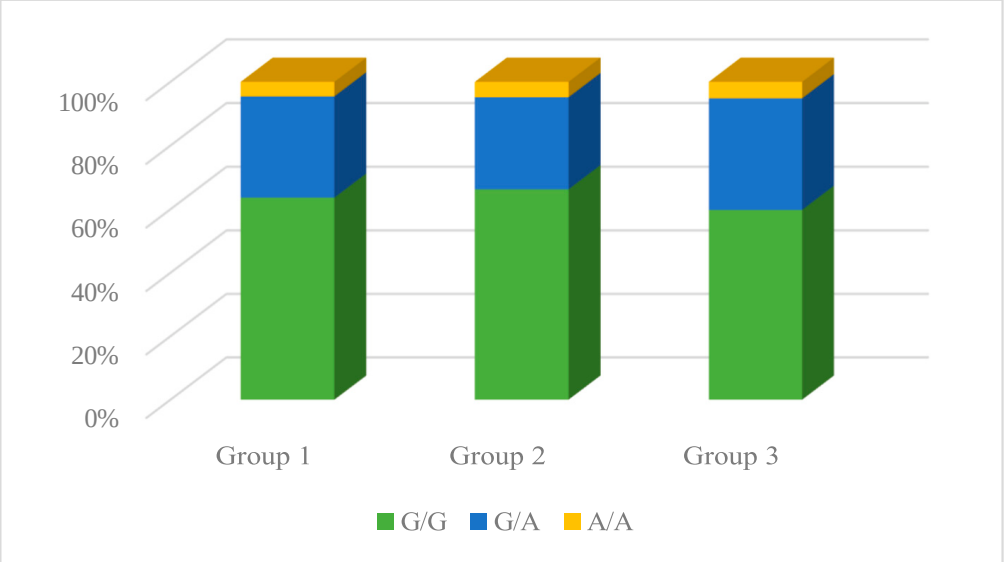

Figure 5. The genotype distribution of the rs1137933 polymorphism (*NOS2*) in the studied groups. Group 1—control group; Group 2—diabetic nephropathy group; Group 3—kidney transplant diabetic nephropathy group.

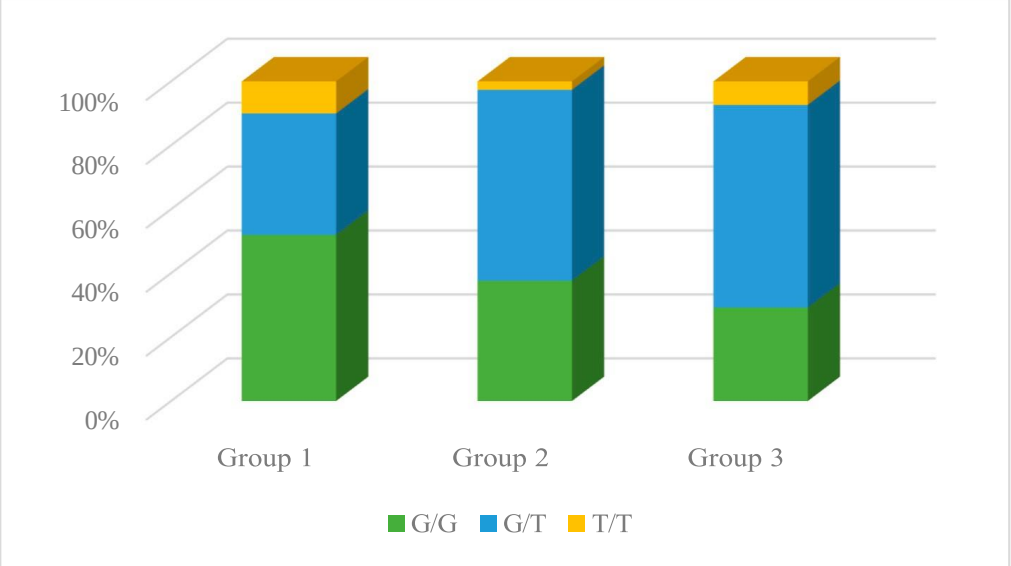

Figure 6. The genotype distribution of the rs1799983 polymorphism (*NOS3*) in the studied groups. Group 1—control group; Group 2—diabetic nephropathy group; Group 3—kidney transplant diabetic nephropathy group.

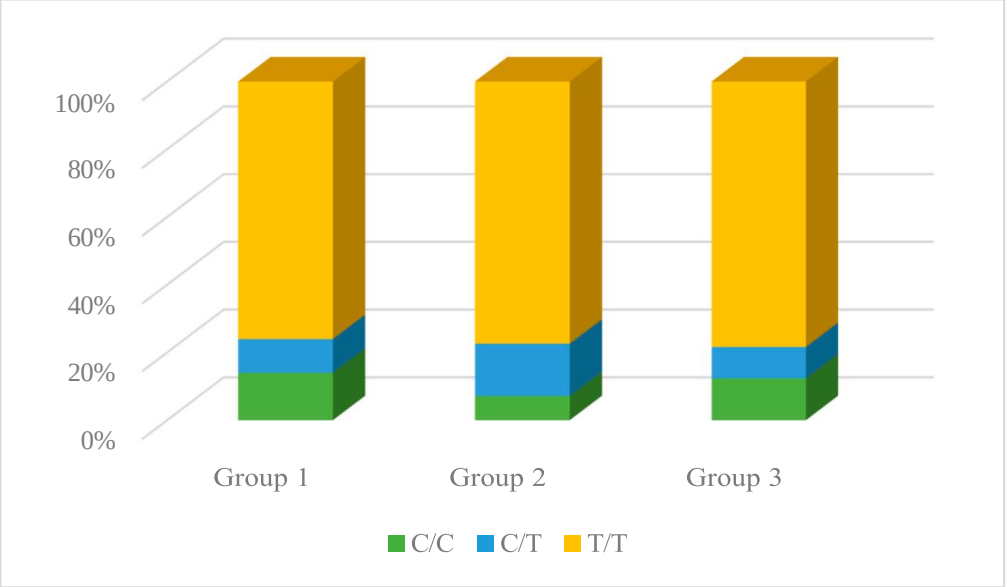

Figure 7. The genotype distribution of the rs2070744 polymorphism (*NOS3*) in the studied groups. Group 1—control group; Group 2—diabetic nephropathy group; Group 3—kidney transplant diabetic nephropathy group.

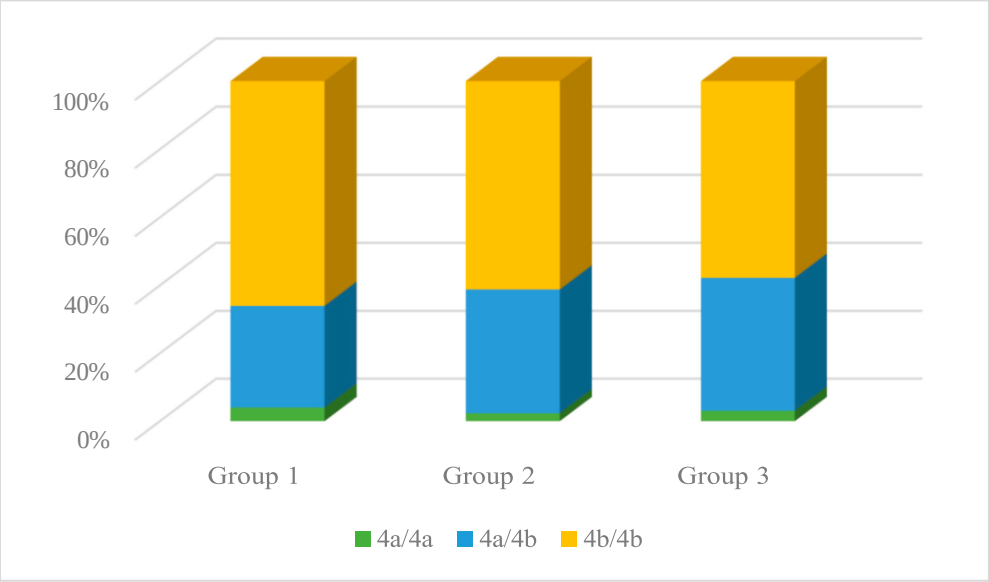

Figure 8. The genotype distribution of the rs61722009 polymorphism (*NOS3*) in the studied groups. Group 1—control group; Group 2—diabetic nephropathy group; Group 3—kidney transplant diabetic nephropathy group.

3.3. The Influence of NOS1, NOS2, and NOS3 Polymorphisms on the Risk of Occurrence of Diabetic Nephropathy or the Likelihood of Renal Replacement Therapy

The logistic regression was used to assess the risk of developing diabetic nephropathy or the likelihood of renal replacement therapy based on *NOS1*, *NOS2*, and *NOS3* polymorphisms. It was shown that the C/C genotype of the rs3782218 polymorphism in *NOS1* is associated with a more than 12-fold increase in the odds of developing diabetic nephropathy ($p = 0.035$). However, the wide confidence interval suggests low accuracy in estimating this parameter. Additionally, it has also been observed that age may be associated with the development of this complication of diabetes. The results suggest that each subsequent year increases the chance of developing this disease by 17.90% ($p < 0.001$).

In turn, the C allele of the rs3782218 polymorphism in *NOS1* seems to be associated with an increased likelihood of renal replacement therapy (approximately 2.06-fold, $p = 0.002$). In turn, the G allele of the rs1137933 polymorphism in *NOS2* is associated with a 2.28-fold lower likelihood of renal replacement therapy ($p = 0.007$). Moreover, age may also play an important role in this case because each subsequent year increases the likelihood of renal replacement therapy by 17.70% ($p < 0.001$).

The results are presented in Tables 3 and 4.

Table 3. The relationship between the selected parameters and the risk of developing diabetic nephropathy.

SNP (*Gene*)	Genotype	Diabetic Nephropathy Group	Control Group	*p*	OR	95% CI OR
rs3782218 (*NOS1*)	C/C	44	4	0.035	12.094	1.189–123.004
	C/T	82	32	0.740	0.813	0.239–2.768
	T/T	49	11	-	1.000	-
	C allele	170	40	0.300	1.275	0.850–2.019
	T allele	180	54	-	1.000	-

Table 3. *Cont.*

SNP (*Gene*)	Genotype	Diabetic Nephropathy Group	Control Group	*p*	OR	95% CI OR
rs1137933 (*NOS2*)	G/G	113	28	0.893	0.897	0.183–4.385
	G/A	58	14	0.921	0.921	0.179–4.744
	A/A	9	2	-	1.000	-
	G allele	284	70	0.892	0.961	0.540–1.710
	A allele	76	18	-	1.000	-
rs1799983 (*NOS3*)	G/G	53	26	0.412	2.219	0.331–14.895
	G/T	98	19	0.273	2.809	0.444–17.777
	T/T	8	5	-	1.000	-
	G allele	204	71	0.209	0.731	0.448–1.192
	T allele	114	29	-	1.000	-
rs2070744 (*NOS3*)	C/C	18	7	0.446	0.693	0.270–1.781
	C/T	22	5	0.747	1.186	0.421–3.338
	T/T	141	38	-	1.000	-
	C allele	58	19	0.480	0.813	0.459–1.443
	T allele	304	81	-	1.000	-
rs61722009 (*NOS3*)	4a/4a	5	2	0.754	0.764	0.142–4.122
	4a/4b	69	15	0.327	1.406	0.711–2.777
	4b/4b	108	33	-	1.000	-
	4a allele	79	19	0.558	1.182	0.676–2.065
	4b allele	285	81	-	1.000	-
Other Variables	**Category**	**Diabetic Nephropathy Group**	**Control Group**	***p***	**OR**	**95% CI OR**
Age	-	-	-	<0.001	1.179	1.113–1.249
BMI	-	-	-	0.774	1.019	0.894–1.162
Sex	Men	94	21	-	1.000	-
	Women	90	29	0.256	0.693	0.369–1.304

These results were obtained using logistic regression analysis.

Table 4. The relationship between the selected parameters and the likelihood of renal replacement therapy.

SNP (*Gene*)	Genotype	Kidney Transplant Diabetic Nephropathy Group	Control Group	*p*	OR	95% CI OR
rs3782218 (*NOS1*)	C/C	18	4	0.475	1.597	0.442–5.762
	C/T	47	32	0.120	0.521	0.229–1.185
	T/T	31	11	-	1.000	-
	C allele	83	40	0.002	2.056	1.296–3.262
	T allele	109	54	-	1.000	-

Table 4. *Cont.*

SNP (*Gene*)	Genotype	Kidney Transplant Diabetic Nephropathy Group	Control Group	*p*	OR	95% CI OR
rs1137933 (*NOS2*)	G/G	58	28	0.828	0.829	0.151–4.539
	G/A	34	14	0.974	0.971	0.168–5.613
	A/A	5	2	-	1.000	-
	G allele	150	70	0.007	0.438	0.242–0.795
	A allele	44	18	-	1.000	-
rs1799983 (*NOS3*)	G/G	24	26	0.531	0.767	0.334–1.761
	G/T	52	19	0.076	2.102	0.925–4.777
	T/T	6	5	-	1.000	-
	G allele	100	71	0.099	0.638	0.374–1.088
	T allele	64	29	-	1.000	-
rs2070744 (*NOS3*)	C/C	12	7	0.765	0.857	0.312–2.354
	C/T	9	5	0.856	0.900	0.282–2.872
	T/T	76	38	-	1.000	-
	C allele	33	19	0.672	0.874	0.468–1.632
	T allele	161	81	-	1.000	-
rs61722009 (*NOS3*)	4a/4a	3	2	0.896	0.884	0.140–5.567
	4a/4b	38	15	0.286	1.493	0.715–3.118
	4b/4b	56	33	-	1.000	-
	4a allele	44	19	0.467	1.251	0.685–2.283
	4b allele	150	81	-	1.000	-
Other Variables	**Category**	**Kidney Transplant Diabetic Nephropathy Group**	**Control Group**	***p***	**OR**	**95% CI OR**
Age	-	-	-	<0.001	1.177	1.111–1.246
BMI	-	-	-	0.785	0.982	0.864–1.117
Sex	Men	50	21	-	1.000	-
	Women	49	29	0.327	0.710	0.357–1.409

These results were obtained using logistic regression analysis.

3.4. The Influence of the Studied Polymorphisms in NOS1, NOS2, and NOS3 on the Concentrations of the NOS Isoforms and Some Selected Parameters

3.4.1. The Influence of the rs3782218 Polymorphism in *NOS1* on the Concentrations of the Selected Parameters

After subgrouping the population by genotype of the rs3782218 polymorphism in *NOS1*, no statistically significant differences were observed in the concentrations of NOS1 ($p = 0.477$), creatinine ($p = 1.000$), copper ($p = 0.089$), and eGFR values ($p = 1.000$).

However, higher glucose concentrations were found in the diabetic nephropathy group with the C/C genotype ($p = 0.045$), the C/T genotype ($p < 0.001$), and the T/T genotype ($p = 0.010$), as well as in the kidney transplant diabetic nephropathy group with the C/T genotype ($p < 0.001$) compared to control groups with the same genotypes.

Moreover, higher CRP concentrations were observed in the diabetic nephropathy group with the C/T genotype compared to the control group with the same genotype ($p = 0.009$), as well as in the kidney transplant diabetic nephropathy group with the C/C genotype compared to the control group with the same genotype ($p = 0.010$).

In the case of zinc concentration, lower concentrations of this metal were observed in the diabetic nephropathy group with the C/T genotype ($p = 0.007$) and in the kidney

transplant diabetic nephropathy group with the C/T genotype ($p < 0.001$) compared to the control group with the same genotype.

The results are presented in Table 5.

Table 5. Concentrations of the selected parameters in the studied groups in terms of the rs3782218 polymorphism in *NOS1*.

Parameter	Control Group (*N* = 47)			Diabetic Nephropathy Group (*N* = 79)			Kidney Transplant Diabetic Nephropathy Group (*N* = 96)		
	C/C (*N* = 4)	C/T (*N* = 32)	T/T (*N* = 11)	C/C (*N* = 26)	C/T (*N* = 35)	T/T (*N* = 18)	C/C (*N* = 18)	C/T (*N* = 47)	T/T (*N* = 31)
NOS1 (ng/mL)	{19.47; 40.24; 48.14}	{4.41; 8.15; 25.98}	{3.86; 11.38; 24.90}	{4.40; 5.88; 16.40}	{4.07; 5.69; 11.05}	{5.30; 7.02; 13.35}	{3.93; 9.34; 22.43}	{4.12; 6.32; 15.40}	{4.92; 7.76; 21.77}
Glucose (mg/dL)	{74.43; 81.99; 90.54}	{81.99; 86.04; 88.92}	{81.00; 86.49; 93.96}	{106.00; 141.00; 183.00} *	{103.00; 124.00; 154.00} **	{115.00; 145.00; 224.00} ***	{106.50; 121.50; 149.65}	{130.00; 153.00; 213.00} **	{104.00; 132.50; 153.00}
Creatinine (mg/dL)	-	-	-	{1.20; 1.34; 1.82}	{1.12; 1.30; 1.72}	{1.04; 1.35; 1.60}	{1.03; 1.54; 2.01}	{1.14; 1.32; 1.70}	{1.12; 1.22; 1.45}
eGFR (mL/min/1.73 m^2)	-	-	-	{30.00; 46.00; 55.00}	{36.00; 51.00; 61.00}	{39.00; 49.50; 61.00}	{35.00; 49.68; 60.50}	{40.00; 51.00; 62.00}	{51.00; 55.00; 65.50}
CRP (mg/L)	{015; 017; 0.31}	{0.34; 0.64; 0.97}	{0.50; 1.20; 1.40}	{0.24 1.54; 3.92}	{1.94; 2.38; 5.27} **	{1.03; 3.39; 4.49}	{1.49; 3.44; 8.12} *	{0.73; 1.63; 3.65}	{1.38; 2.69; 4.07}
Zn (µg/L)	{803.97; 856.71; 1101.53}	{853.50; 926.72; 1093.47}	{890.78; 964.51; 995.98}	{767.00; 835.00; 945.00}	{741.00; 826.00; 915.00} **	{739.00; 799.00; 840.00}	{632.00; 742.50; 887.00}	{733.00; 804.00; 854.00} **	{727.00; 815.00; 928.00}
Cu (µg/L)	{774.67; 827.77; 882.82}	{950.14; 995.71; 1081.33}	{1024.94; 1181.65; 1241.38}	{916.00; 1058.00; 1212.00}	{826.00; 1058.00; 1153.00}	{896.00; 1003.00; 1125.00}	{890.00; 1022.00; 1141.00}	{879.00; 1081.00; 1294.00}	{994.00; 1098.00; 1190.00}

Values are shown as {1st quartile; median; 3rd quartile}. * $p < 0.05$—compared to the control group with the C/C genotype; ** $p < 0.05$—compared to the control group with the C/T genotype; *** $p < 0.05$—compared to the control group with the T/T genotype.

3.4.2. The Influence of the rs1137933 Polymorphism in *NOS2* on the Concentrations of the Selected Parameters

After subgrouping the population by genotype of the rs1137933 polymorphism in *NOS2*, no statistically significant differences were observed in the concentrations of NOS2 ($p = 0.166$), creatinine ($p = 1.000$), copper ($p = 0.250$), and eGFR values ($p = 1.000$).

However, it was observed that patients with diabetic nephropathy and the G/G genotype had higher glucose concentrations compared to the control group with the same genotype ($p = 0.048$). It was similar in the case of the kidney transplant diabetic nephropathy group with the G/G genotype ($p = 0.017$). Higher glucose concentrations were also observed in patients with diabetic nephropathy and the G/A genotype ($p = 0.011$) and in patients after kidney transplantation with the G/A genotype ($p < 0.001$) compared to the control group with the same genotype.

Higher CRP concentrations were also found in the diabetic nephropathy group with the G/G genotype ($p < 0.001$) and in the kidney transplant diabetic nephropathy group with the G/G genotype ($p < 0.001$) compared to the control group with the same genotype. Higher concentrations of this protein were also observed in patients after kidney transplantation with the G/A genotype compared to the control group with the same genotype ($p = 0.003$).

Lower zinc concentrations were found in the diabetic nephropathy group with the G/G genotype compared to the control group with the same genotype ($p = 0.021$) and also in the kidney transplant diabetic nephropathy group with the G/A genotype compared to the control group with the same genotype ($p = 0.015$).

The results are presented in Table 6.

Table 6. Concentrations of the selected parameters in the studied groups in terms of the rs1137933 polymorphism in *NOS2*.

Parameter	Control Group (*N* = 44)			Diabetic Nephropathy Group (*N* = 83)			Kidney Transplant Diabetic Nephropathy Group (*N* = 97)		
	G/G (*N* = 28)	G/A (*N* = 14)	A/A (*N* = 2)	G/G (*N* = 55)	G/A (*N* = 24)	A/A (*N* = 4)	G/G (*N* = 58)	G/A (*N* = 34)	A/A (*N* = 5)
NOS2 (ng/mL)	{6.47; 13.86; 30.92}	{4.32; 7.74; 33.37}	{40.93; 66.29; 91.66}	{5.46; 7.51; 12.11}	{4.72; 8.36; 21.64}	{1.57; 7.80; 16.41}	{5.69; 8.08; 16.42}	{4.88; 8.55; 21.79}	{9.13; 31.68; 54.22}
Glucose (mg/dL)	{80.46; 83.46; 87.48}	{84.96; 88.92; 91.08}	{75.96; 76.95; 77.94}	{101.00; 112.50; 171.50} *	{101.00; 111.50; 150.00} **	{102.00; 142.00; 228.00}	{121.00; 143.00; 173.00} *	{103.00; 139.50; 176.00} **	{110.00; 140.00; 147.00}
Creatinine (mg/dL)	-	-	-	{1.13; 1.29; 1.82}	{1.21; 1.38; 1.55}	{1.30; 1.52; 1.72}	{1.14; 1.30; 1.61}	{1.07; 1.28; 1.80}	{1.46; 1.58; 2.07}
eGFR (mL/min/1.73 m²)	-	-	-	{34.00; 48.00; 58.00}	{37.50; 49.50; 59.50}	{35.00; 42.00; 53.00}	{46.00; 53.00; 62.00}	{36.00; 55.50; 63.00}	{26.00; 47.00; 52.00}
CRP (mg/L)	{0.38; 0.65; 1.13}	{0.20; 0.64; 0.93}	{0.15; 0.60; 1.05}	{0.67; 3.07; 5.35} *	{0.71; 1.14; 2.38}	{1.76; 2.79; 3.14}	{0.66; 2.22; 4.70} *	{1.49; 2.87; 3.71} **	{2.66; 3.36; 3.82}
Zn (µg/L)	{843.22; 913.53; 1018.25}	{924.03; 981.37; 1031.87}	{852.79; 1077.01; 1301.23}	{752.00; 817.00; 927.00} *	{739.00; 796.00; 876.50}	{830.50; 929.50; 960.50}	{718.00; 813.50; 910.00}	{720.00; 751.00; 829.00} **	{733.00; 791.00; 902.00}
Cu (µg/L)	{866.96; 979.66; 1128.35}	{996.68; 1076.99; 1231.94}	{747.10; 1423.73; 2100.35}	{896.00; 1058.00; 1205.00}	{867.50; 1011.50; 1123.50}	{817.00; 919.00; 1009.00}	{899.00; 1080.50; 1200.00}	{935.00; 1097.50; 1254.00}	{993.00; 1025.00; 1079.00}

Values are shown as {1st quartile; median; 3rd quartile}. * $p < 0.05$—compared to the control group with the G/G genotype; ** $p < 0.05$—compared to the control group with the G/A genotype.

3.4.3. The Influence of the rs1799983 Polymorphism in *NOS3* on the Concentrations of the Selected Parameters

After subgrouping the population by genotype of the rs1799983 polymorphism in *NOS3*, no statistically significant differences were observed in the concentrations of creatinine ($p = 1.000$) and eGFR values ($p = 1.000$).

However, lower NOS3 concentrations were observed in the diabetic nephropathy group with the G/G genotype ($p < 0.001$) and in the kidney transplant diabetic nephropathy group with the G/G genotype ($p = 0.001$) compared to the control group with the same genotype. It was similar in the case of patients with the G/T genotype, where lower NOS3 concentrations were also observed in the diabetic nephropathy group ($p < 0.001$) and in the kidney transplant diabetic nephropathy group ($p < 0.001$) compared to the control group.

Higher glucose concentrations were found in the diabetic nephropathy group with the G/G genotype ($p < 0.001$) and in the kidney transplant diabetic nephropathy group with the G/G genotype ($p < 0.001$) compared to the control group with the same genotype. Higher glucose concentrations were also observed in patients with diabetic nephropathy with the G/T genotype ($p < 0.001$) and in patients after kidney transplantation with the G/T genotype ($p < 0.001$) compared to the control group with the same genotype.

In the case of CRP concentration, higher concentrations of this protein were found in patients with diabetic nephropathy and the G/G genotype compared to the control group with the same genotype ($p = 0.011$), and its higher concentrations were also observed in patients after kidney transplantation with the G/T genotype compared to the control group with the same genotype ($p = 0.008$).

Significant dependencies were also noticed in the case of the concentrations of the studied metals. Patients with diabetic nephropathy and the G/G genotype ($p = 0.002$) and patients after kidney transplantation with the G/G genotype ($p = 0.001$) had lower zinc concentrations compared to the control group with the same genotype. Also, patients

after kidney transplantation with the G/T genotype had lower concentrations of this element compared to the control group with the same genotype ($p = 0.008$). Lower copper concentrations were also observed in the control group with the G/T genotype compared to the same group but with the G/G genotype ($p = 0.027$).

The results are presented in Table 7.

Table 7. Concentrations of the selected parameters in the studied groups in terms of the rs1799983 polymorphism in *NOS3*.

Parameter	Control Group (*N* = 50)			Diabetic Nephropathy Group (*N* = 77)			Kidney Transplant Diabetic Nephropathy Group (*N* = 82)		
	G/G (*N* = 26)	G/T (*N* = 19)	T/T (*N* = 5)	G/G (*N* = 29)	G/T (*N* = 46)	T/T (*N* = 2)	G/G (*N* = 24)	G/T (*N* = 52)	T/T (*N* = 6)
NOS3 (ng/mL)	{0.69; 0.77; 0.87}	{0.69; 0.75; 0.82}	{0.76; 0.79; 0.83}	{0.30; 0.38; 0.51} *	{0.30; 0.37; 0.49} **	{0.30; 0.67; 1.04}	{0.35; 0.43; 0.47} *	{0.30; 0.37; 0.53} **	{0.41; 0.46; 0.54}
Glucose (mg/dL)	{81.90; 85.50; 90.00}	{79.92; 84.51; 88.92}	{81.00; 86.94; 88.92}	{101.00; 135.00; 187.00} *	{101.00; 108.50; 146.00} **	{110.00; 127.50; 145.00}	{115.00; 129.00; 153.00} *	{111.50; 150.00; 173.00} **	{89.00; 134.00; 179.00}
Creatinine (mg/dL)	-	-	-	{1.07; 1.44; 1.80}	{1.16; 1.29; 1.59}	{1.60; 2.15; 2.70}	{1.14; 1.35; 2.00}	{1.07; 1.28; 1.59}	{1.44; 1.84; 2.23}
eGFR (mL/min/1.73 m^2)	-	-	-	{34.50; 43.50; 54.50}	{36.00; 49.00; 58.00}	{24.00; 37.00; 50.00}	{36.00; 44.50; 54.00}	{45.50; 58.50; 64.00}	{25.00; 40.00; 55.00}
CRP (mg/L)	{0.34; 0.61; 1.10}	{0.15; 0.80; 1.01}	{0.53; 0.94; 1.21}	{1.94; 5.42; 7.15} *	{0.71; 1.24; 2.38}	{2.38; 2.91; 3.43}	{0.63; 2.43; 5.28}	{1.06; 2.66; 4.11} **	{0.99; 2.54; 4.09}
Zn (µg/L)	{895.37; 987.03; 1081.96}	{854.21; 964.51; 1020.85}	{803.53; 825.70; 890.78}	{744.00; 789.00; 898.00} *	{777.00; 839.00; 927.00}	{762.00; 799.00; 836.00}	{711.50; 811.00; 891.00} *	{737.50; 808.00; 918.50} **	{672.00; 730.00; 749.00}
Cu (µg/L)	{978.17; 1049.14; 1282.70}	{802.23; 912.33; 1024.94} *	{1046.20; 1100.73; 1184.39}	{930.00; 1070.00; 1205.00}	{851.00; 970.50; 1133.00}	{876.00; 1073.50; 1271.00}	{905.50; 1087.50; 1162.50}	{915.00; 1066.00; 1195.00}	{993.00; 1144.00; 1200.00}

Values are shown as {1st quartile; median; 3rd quartile}. * $p < 0.05$—compared to the control group with the G/G genotype; ** $p < 0.05$—compared to the control group with the G/T genotype.

3.4.4. The Influence of the rs2070744 Polymorphism in *NOS3* on the Concentrations of the Selected Parameters

After subgrouping the population by genotype of the rs2070744 polymorphism in *NOS3*, no statistically significant differences were observed in the concentrations of creatinine ($p = 1.000$), copper ($p = 0.619$), and eGFR values ($p = 1.000$).

Higher values of NOS3 concentration were observed in the group of patients with diabetic nephropathy and the C/C genotype ($p = 0.049$ and the T/T genotype ($p < 0.001$) and in the group of patients after kidney transplantation with the genotype T/T ($p < 0.001$) compared to control groups with the same genotypes.

It was very similar in the case of glucose, where significantly higher concentrations of this parameter were observed in the kidney transplant diabetic nephropathy group with the C/C genotype ($p = 0.022$), in the diabetic nephropathy group with the T/T genotype ($p < 0.001$) and in the kidney transplant diabetic nephropathy group with the T/T genotype ($p < 0.001$) compared to control groups with the same genotypes.

Also, higher CRP concentrations were found in the kidney transplant diabetic nephropathy group with the T/T genotype compared to the control group with the same genotype ($p = 0.001$).

Lower zinc concentrations were also observed in the diabetic nephropathy group with the T/T genotype ($p < 0.001$) and in the kidney transplant diabetic nephropathy group with the T/T genotype ($p < 0.001$) compared to the control group with the same genotype.

The results are presented in Table 8.

Table 8. Concentrations of the selected parameters in the studied groups in terms of the rs2070744 polymorphism in *NOS3*.

Parameter	Control Group (*N* = 50)			Diabetic Nephropathy Group (*N* = 84)			Kidney Transplant Diabetic Nephropathy Group (*N* = 97)		
	C/C (*N* = 7)	C/T (*N* = 5)	T/T (*N* = 38)	C/C (*N* = 6)	C/T (*N* = 13)	T/T (*N* = 65)	C/C (*N* = 12)	C/T (*N* = 9)	T/T (*N* = 76)
NOS3 (ng/mL)	{0.76; 0.79; 0.84}	{0.69; 0.72; 0.75}	{0.69; 0.78; 0.83}	{0.30; 0.39; 0.41} *	{0.31; 0.33; 0.44}	{0.30; 0.39; 0.52} **	{0.36; 0.42; 0.75}	{0.31; 0.41; 0.50}	{0.32; 0.41; 0.54} **
Glucose (mg/dL)	{81.00; 83.52; 86.94}	{77.04; 91.98; 93.06}	{81.90; 85.50; 88.92}	{110.50; 127.50; 142.50}	{110.00; 126.00; 154.00}	{105.00; 145.00; 180.00} **	{115.00; 152.00; 183.00} *	{120.00; 146.00: 177.00}	{112.00; 136.00; 173.00} **
Creatinine (mg/dL)	-	-	-	{0.97; 1.20; 1.63}	{1.17; 1.55; 2.22}	{1.14; 1.33; 1.59}	{1.24; 1.32; 1.95}	{1.22; 1.30; 1.75}	{1.08; 1.28; 1.67}
eGFR (mL/min/1.73 m²)	-	-	-	{39.50; 57.00; 72.00}	{26.50; 45.50; 63.00}	{36.00; 49.00; 57.00}	{36.00; 46.00; 62.00}	{41.50; 61.00; 69.00}	{42.00; 53.50; 63.00}
CRP (mg/L)	{0.15; 0.40; 1.21}	{0.37; 0.78; 1.01}	{0.34; 0.72; 1.15}	{2.16; 2.80; 3.44}	{3.60; 3.84; 5.70}	{0.71; 1.71; 4.49}	{0.77; 3.59; 8.12}	{1.67; 2.20; 4.07}	{0.97; 2.46; 4.09} **
Zn (µg/L)	{803.53; 927.92; 995.98}	{907.67; 924.03; 1020.85}	{856.07; 972.73; 1043.34}	{788.00; 906.50; 945.00}	{759.00; 838.00; 912.00}	{744.00; 812.00; 887.00} **	{677.00; 751.00; 811.50}	{854.00; 902.00: 967.00}	{718.00; 804.50; 874.50} **
Cu (µg/L)	{868.53; 1046.20; 1100.73}	{994.74; 996.68; 1024.94}	{912.33; 998.18; 1184.39}	{961.00; 1120.50; 1205.00}	{973.00; 1145.00; 1226.00}	{851.00; 1009.00; 1129.00}	{862.50; 1053.50; 1239.50}	{1014.00; 1165.00; 1215.00}	{910.00; 1080.50; 1199.00}

Values are shown as {1st quartile; median; 3rd quartile}. * $p < 0.05$—compared to the control group with the C/C genotype; ** $p < 0.05$—compared to the control group with the T/T genotype.

3.4.5. The Influence of the rs61722009 Polymorphism in *NOS3* on the Concentrations of the Selected Parameters

After subgrouping the population by genotype of the rs61722009 polymorphism in *NOS3*, no statistically significant differences were observed in the concentrations of creatinine ($p = 1.000$), copper ($p = 0.777$), and eGFR values ($p = 1.000$).

Significantly lower NOS3 concentrations were observed in patients with diabetic nephropathy and the 4a/4b genotype ($p < 0.001$) and in patients after kidney transplantation and with the 4a/4b genotype ($p < 0.001$) compared to the control group with the same genotype. It was similar in the case of genotype 4b/4b, where lower NOS3 concentrations in patients with diabetic nephropathy ($p < 0.001$) were also observed and in patients after kidney transplantation ($p < 0.001$) compared to the control group with the same genotype.

Moreover, higher glucose concentrations were found in the diabetic nephropathy group with the 4a/4b genotype ($p < 0.001$) and the 4b/4b genotype ($p < 0.001$), as well as in the kidney transplant diabetic nephropathy group with the 4a/4b genotype ($p < 0.001$) and the 4b/4b genotype ($p < 0.001$) compared to the control groups with the same genotypes.

Higher CRP concentrations were also observed in the diabetic nephropathy group with the 4b/4b genotype (p = 0.028) and in the kidney transplant diabetic nephropathy group with the 4a/4b genotype (p = 0.002) and the 4b/4b genotype (p = 0.014) compared to the control groups with the same genotypes.

Significant differences were also noticed in the case of zinc concentration. Lower concentrations of this metal were found in the diabetic nephropathy group with the 4b/4b genotype (p = 0.003) and in the kidney transplant diabetic nephropathy group with the 4a/4b genotype (p = 0.049) and the 4b/4b genotype (p < 0.001) compared to the control groups with the same genotypes.

The results are presented in Table 9.

Table 9. Concentrations of the selected parameters in the studied groups in terms of the rs61722009 polymorphism in *NOS3*.

Parameter	Control Group (*N* = 50)			Diabetic Nephropathy Group (*N* = 85)			Kidney Transplant Diabetic Nephropathy Group (*N* = 97)		
	4a/4a (*N* = 2)	4a/4b (*N* = 15)	4b/4b (*N* = 33)	4a/4a (*N* = 2)	4a/4b (*N* = 31)	4b/4b (*N* = 52)	4a/4a (*N* = 3)	4a/4b (*N* = 38)	4b/4b (*N* = 56)
NOS3 (ng/mL)	{0.61; 0.73; 0.84}	{0.69; 0.75; 0.87}	{0.70; 0.77; 0.82}	{0.27; 0.30; 0.32}	{0.26; 0.33; 0.46} *	{0.32; 0.40; 0.52} **	{0.35; 0.41; 0.47}	{0.32; 0.39; 0.45} *	{0.32; 0.44; 0.58} **
Glucose (mg/dL)	{86.04; 88.02; 90.00}	{79.92; 86.04; 93.06}	{81.00; 83.97; 88.92}	{110.00; 125.00; 140.00}	{101.00; 145.00; 191.00} *	{101.00; 107.00; 142.00} **	{90.00; 93.50; 97.00}	{115.00; 143.00; 173.00} *	{116.00; 139.00; 183.00} **
Creatinine (mg/dL)	-	-	-	{1.08; 1.17; 1.25}	{1.14; 1.45; 1.83}	{1.15; 1.32; 1.58}	{1.14; 1.19; 1.24}	{1.02; 1.16; 1.32}	{1.18; 1.41; 1.80}
eGFR (mL/min/1.73 m^2)	-	-	-	{45.00; 48.50; 52.00}	{30.00; 45.00; 61.00}	{39.00; 50.00; 58.00}	{46.00; 60.50; 75.00}	{43.00; 59.00; 66.00}	{36.00; 51.00; 58.00}
CRP (mg/L)	{0.33; 1.16; 1.99}	{0.20; 0.64; 0.93}	{0.37; 0.78; 1.20}	{2.38; 2.91; 3.43}	{0.57; 1.03; 5.42}	{0.74; 1.91; 4.49} **	{0.77; 1.22; 1.67}	{1.49; 3.06; 5.28} *	{0.97; 2.20; 4.11} **
Zn (µg/L)	{927.92; 954.86; 981.79}	{856.07; 918.00; 1115.95}	{846.35; 964.51; 1020.85}	{788.00; 797.50; 807.00}	{737.00; 847.00; 937.00}	{755.50; 821.50; 886.50} **	{739.00; 980.00; 1015.00}	{742.00; 808.00; 921.00} *	{714.00; 787.50; 846.50} **
Cu (µg/L)	{1067.96; 1124.81; 1181.65}	{853.30; 996.68; 1232.50}	{920.91; 1018.42; 1155.12}	{1058.00; 1139.00; 1220.00}	{840.00; 1070.00; 1205.00}	{880.00; 1009.00; 1146.00}	{794.00; 1349.00; 1547.00}	{904.00; 1084.50; 1187.00}	{910.00; 1080.50; 1195.00}

Values are shown as {1st quartile; median; 3rd quartile}. * p < 0.05—compared to the control group with the 4a/4b genotype; ** p < 0.05—compared to the control group with the 4b/4b genotype.

4. Discussion

Due to its prevalence among diabetic patients, diabetic nephropathy constitutes a significant challenge for modern medicine. It can develop for years, and despite the treatment, the patient may still need a kidney transplant in the future [36,37]. The literature has discussed the role of the *NOS* polymorphisms in the development of this disease, but the influence of the *NOS3* polymorphisms is mainly emphasized [38,39]. Therefore, the aim of this study was to investigate the impact of selected polymorphisms of all NOS isoforms on the risk of developing diabetic nephropathy or on the likelihood of renal replacement therapy. Such a comprehensive summary of the role of the *NOS* polymorphisms in the pathophysiology of diabetic complications could contribute to their better understanding and, thus, possibly the development of a faster method of diagnosing them.

The studied polymorphisms of the *NOS1* and *NOS2* (rs3782218 and rs1137933, respectively) showed no effect on the concentrations of NOS1 and NOS2. However, it was noted that individual genotypes within the *NOS3* polymorphisms (rs1799983, rs2070744, and rs61722009) were associated with differences in NOS3 concentrations between the diabetic nephropathy group and the kidney transplant diabetic nephropathy group and the control group. However, this was most likely due to the fact that respondents from the control group showed higher concentrations of this parameter compared to the other two groups (even without genotype division). Altered NO levels are often observed in patients with diabetic nephropathy [39]. There is evidence that the *NOS3* polymorphisms can influence the NO concentration, consequently leading to increased or decreased production of this compound, thereby promoting various pathological progressions [40,41]. For example, the rs1799983 polymorphism is associated with reduced NOS3 expression or activity, which corresponds well with this research. These relationships may result from the role calmodulin plays in the pathogenesis of diabetic nephropathy. Calmodulin forms the Ca^{2+}/calmodulin complex, on which the activity of two NOS isoforms (NOS1, NOS3) depends [12]. Yuzawa et al. [42] proved that pancreatic β cells calmodulin-overexpressing transgenic (CaMTg) mice develop most of the changes characteristic of human diabetic nephropathy. In turn, other studies emphasized that in diabetic nephropathy there is an increase in the level of calcium–calmodulin-dependent protein kinase II (CaMKII) [43,44]. Moreover, the rs1799983 polymorphism in *NOS3* is characterized by a change of guanine (G) to thymine (T), which results in a change of glutamine (Glu) to aspartate (Asp) in the NOS3 protein. This change reduces the binding of NOS3 to caveolin-1, thereby reducing the availability of the protein for calcium-activated calmodulin activation [45,46]. This phenomenon explains the reduced activity of NOS3.

Significantly higher glucose concentrations were observed in the diabetic nephropathy group and the kidney transplant diabetic nephropathy group compared to the control group, which was a phenomenon independent of the occurring genotypes. This fact should not be surprising because these are diabetic patients with complications, and in most of them, the complications last for years [47].

This study also showed a relationship between zinc concentration and the studied *NOS* polymorphisms. However, as with NOS3 concentrations, this is most likely the result of ongoing disease and not genetic factors. Each time, these differences were concerned with lower zinc concentrations in the groups of patients with diabetic nephropathy or patients after kidney transplantation compared to the control group. Indeed, there are many examples in the literature confirming lower concentrations of this metal in patients with diabetic complications [48–51]. Zinc deficiency may be associated with inflammation or oxidative stress that accompanies many chronic diseases, including diabetes and diabetic nephropathy [48]. Al-Timimi et al. [48] indicated the role of zinc deficiency in kidney damage associated with diabetic nephropathy resulting from type 2 diabetes. The cause of this phenomenon was progressive inflammation, which was closely correlated with reduced zinc concentrations. Therefore, in patients with diabetes and diabetic nephropathy, special attention should be paid to zinc supplementation and the measurement of this parameter as part of diagnostics because long-term deficiency of this element may contribute to kidney damage [51].

However, the main aim of this study was to evaluate the selected *NOS* polymorphisms—rs3782218 (*NOS1*), rs1137933 (*NOS2*), rs1799983, rs2070744, and rs61722009 (*NOS3*)—as factors that may increase the risk of developing diabetic nephropathy or the increased likelihood of renal replacement therapy in patients with already developed diabetic nephropathy. Based on the logistic regression results, it was noticed that the rs3782218 polymorphism (*NOS1*) could potentially be used to predict the risk of developing diabetic nephropathy. The presence of the C/C genotype seems to be associated with a more than ten times greater risk of developing this complication of diabetes. This is quite interesting because most of the research related to these topics focuses on the role of the *NOS3* polymorphisms, while the rs3782218 polymorphism has not been considered an effective tool in assessing the de-

velopment of this risk [52,53]. However, due to the wide confidence interval, the obtained results should be treated with caution. Moreover, it was observed that the C genotype (rs3782218, *NOS1*) is associated with an increased likelihood of renal replacement therapy, which could confirm the previously obtained result related to the C/C genotype and the development of diabetic nephropathy. In turn, the G allele (rs1137933, *NOS2*) would be associated with a reduced likelihood of this type of therapy. Unfortunately, similar information cannot be found in other studies. Perhaps this polymorphism is still understudied in the context of diabetes complications. Age also seems to be an important factor that may influence the increased risk of developing diabetic nephropathy or the increased likelihood of renal replacement therapy, which is confirmed in other studies [54,55].

5. Conclusions

In summary, this study indicates the potential use of the rs3782218 polymorphism (*NOS1*) in assessing the risk of diabetic nephropathy or the likelihood of renal replacement therapy. The C/C genotype and the C allele appear to be associated with an increased likelihood of diabetic nephropathy or the need for renal replacement therapy. In addition, the G allele (rs1137933, *NOS2*) appears to be associated with a reduced likelihood of renal replacement therapy. It is also confirmed that age is a factor that increases the risk of these complications. Although the role of the *NOS3* polymorphisms in the risk of developing diabetes complications has not been demonstrated, lower NOS3 concentrations were observed in patients with diabetic nephropathy or after kidney transplantation.

However, the above results should be treated as preliminary research because in order to confirm these relationships, cohort studies should be carried out, taking into account the interdependencies between these and additional factors. Nevertheless, research related to the influence of *NOS* polymorphisms on the development of diabetes complications seems to be interesting and worth continuing, as a better understanding of the pathomechanism of diabetic nephropathy may allow for its earlier diagnosis or more effective treatment.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/antiox13070838/s1. Questionnaire S1: Sample of a questionnaire conducted among people suffering from diabetic nephropathy; Table S1: Concentrations of NOS1, NOS2, and NOS3 in the studied groups; Table S2: The Hardy–Weinberg equilibrium for studied groups; Figure S1: Example of electropherogram of the rs3782218 polymorphism in *NOS1*; Figure S2: Example of electropherogram of the rs1137933 polymorphism in *NOS2*; Figure S3: Example of electropherogram of the rs1799983 polymorphism in *NOS3*; Figure S4: Example of electropherogram of the rs2070744 polymorphism in *NOS3*; Figure S5: Example of electropherogram of the rs61722009 polymorphism in *NOS3*.

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References

1. Golicki, D.; Dudzińska, M.; Zwolak, A.; Tarach, J.S. Quality of life in patients with type 2 diabetes in Poland—Comparison with the general population using the EQ-5D questionnaire. *Adv. Clin. Exp. Med.* **2015**, *24*, 139–146. [CrossRef] [PubMed]
2. Goyal, Y.; Verma, A.K.; Bhatt, D.; Rahmani, A.H.; Yasheshwar; Dev, K. Diabetes: Perspective and challenges in modern era. *Gene Rep.* **2020**, *20*, 100759. [CrossRef]
3. Mukhtar, Y.; Galalain, A.M.; Yunusa, U.M. A modern overview on diabetes mellitus: A chronic endocrine disorder. *Eur. J. Biol.* **2020**, *5*, 1–14. [CrossRef]
4. Madziarska, K.; Banasik, M. Chorzy na cukrzyce˛ w programach hemodializy i dializy otrzewnowej—Zagroz˙enia, których moz˙na unikna˛c´ [Diabetes patients in hemodialysis and peritoneal dialysis programs—Risks that can be avoided]. *Probl. Lek.* **2006**, *45*, 240–241.
5. Sagoo, M.K.; Gnudi, L. Diabetic Nephropathy: An Overview. *Methods Mol. Biol.* **2020**, *2067*, 3–7. [PubMed]
6. Samsu, N. Diabetic Nephropathy: Challenges in Pathogenesis, Diagnosis, and Treatment. *Biomed. Res. Int.* **2021**, *2021*, 1497449. [CrossRef] [PubMed]
7. Miranda-Díaz, A.G.; Pazarín-Villaseñor, L.; Yanowsky-Escatell, F.G.; Andrade-Sierra, J. Oxidative Stress in Diabetic Nephropathy with Early Chronic Kidney Disease. *J. Diabetes Res.* **2016**, *2016*, 7047238. [CrossRef] [PubMed]
8. Hojs, N.V.; Bevc, S.; Ekart, R.; Hojs, R. Oxidative Stress Markers in Chronic Kidney Disease with Emphasis on Diabetic Nephropathy. *Antioxidants* **2020**, *9*, 925. [CrossRef] [PubMed]
9. Sagoo, M.K.; Gnudi, L. Diabetic nephropathy: Is there a role for oxidative stress? *Free. Radic. Biol. Med.* **2018**, *116*, 50–63. [CrossRef]
10. Ferreira, C.A.; Ni, D.; Rosenkrans, Z.T.; Cai, W. Scavenging of reactive oxygen and nitrogen species with nanomaterials. *Nano Res.* **2018**, *11*, 4955. [CrossRef]
11. Pérez-Torres, I.; Manzano-Pech, L.; Rubio-Ruíz, M.E.; Soto, M.E.; Guarner-Lans, V. Nitrosative Stress and Its Association with Cardiometabolic Disorders. *Molecules* **2020**, *25*, 2555. [CrossRef] [PubMed]
12. Król, M.; Kepinska, M. Human Nitric Oxide Synthase—Its Functions, Polymorphisms, and Inhibitors in the Context of Inflammation, Diabetes and Cardiovascular Diseases. *Int. J. Mol. Sci.* **2020**, *22*, 56. [CrossRef] [PubMed]
13. Dellamea, B.S.; Leitão, C.B.; Friedman, R.; Canani, L.H. Nitric oxide system and diabetic nephropathy. *Diabetol. Metab. Syndrome* **2014**, *6*, 17. [CrossRef] [PubMed]
14. Evans, T.C.; Capell, P. Diabetic nephropathy. *Clin. Diabetol.* **2021**, *2*, 15–23.
15. Adela, R.; Nethi, S.K.; Bagul, P.K.; Barui, A.K.; Mattapally, S.; Kuncha, M.; Patra, C.R.; Reddy, P.N.; Banerjee, S.K. Hyperglycaemia Enhances Nitric Oxide Production in Diabetes: A Study from South Indian Patients. *PLoS ONE* **2015**, *10*, e0125270. [CrossRef] [PubMed]
16. Ren, X.; Ren, L.; Wei, Q.; Shao, H.; Chen, L.; Liu, N. Advanced glycation end-products decreases expression of endothelial nitric oxide synthase through oxidative stress in human coronary artery endothelial cells. *Cardiovasc. Diabetol.* **2017**, *16*, 52. [CrossRef] [PubMed]
17. Mezghenna, K.; Pomiès, P.; Chalançon, A.; Castex, F.; Leroy, J.; Niclauss, N.; Nadal, B.; Cambier, L.; Cazevieille, C.; Petit, P.; et al. Increased neuronal nitric oxide synthase dimerisation is involved in rat and human pancreatic beta cell hyperactivity in obesity. *Diabetologia* **2011**, *54*, 2856–2866. [CrossRef] [PubMed]
18. Muniyappa, R.; Sowers, J.R. Role of Insulin Resistance in Endothelial Dysfunction. *Rev. Endocr. Metab. Disord.* **2013**, *14*, 5. [CrossRef] [PubMed]
19. Kaneko, Y.K.; Ishikawa, T. Dual role of nitric oxide in pancreatic β-cells. *J. Pharmacol. Sci.* **2013**, *123*, 295–300. [CrossRef]
20. He, Y.; Fan, Z.; Zhang, J.; Zhang, Q.; Zheng, M.; Li, Y.; Zhang, D.; Gu, S.; Yang, H. Polymorphisms of eNOS gene are associated with diabetic nephropathy: A meta-analysis. *Mutagenesis* **2011**, *26*, 339–349. [CrossRef]
21. Zeng, R.; Duan, L.; Sun, L.; Kong, Y.; Wu, X.; Wang, Y.; Xin, G.; Yang, K. A meta-analysis on the relationship of eNOS 4b/a polymorphism and diabetic nephropathy susceptibility. *Ren. Fail.* **2014**, *36*, 1520–1535. [CrossRef] [PubMed]
22. Raina, P.; Sikka, R.; Gupta, H.; Matharoo, K.; Bali, S.K.; Singh, V.; Bhanwer, A.J.S. Association of eNOS and MCP-1 Genetic Variants with Type 2 Diabetes and Diabetic Nephropathy Susceptibility: A Case–Control and Meta-Analysis Study. *Biochem. Genet.* **2021**, *59*, 966–996. [CrossRef]
23. Padhi, U.N.; Mulkalwar, M.; Saikrishna, L.; Verma, H.K.; Bhaskar, L.V.K.S. NOS_3 gene intron 4 a/b polymorphism is associated with ESRD in autosomal dominant polycystic kidney disease patients. *J. Bras. Nefrol.* **2022**, *44*, 224–231. [CrossRef] [PubMed]
24. Garme, Y.; Moudi, M.; Saravani, R.; Galavi, H. Nitric Oxide Synthase 2 Polymorphisms (rs2779248T/C and rs1137933C/T) and the Risk of Type 2 Diabetes in Zahedan, Southeastern Iran. *Iran. J. Public Health* **2018**, *47*, 1734. [PubMed]
25. Chen, F.; Li, Y.M.; Yang, L.Q.; Zhong, C.G.; Zhuang, Z.X. Association of NOS_2 and NOS_3 gene polymorphisms with susceptibility to type 2 diabetes mellitus and diabetic nephropathy in the Chinese Han population. *IUBMB Life* **2016**, *68*, 516–525. [CrossRef]
26. Yigit, S.; Nursal, A.F.; Uzun, S.; Rustemoglu, H.; Dashatan, P.A.; Soylu, H.; Atmaca, A.; Rustemoglu, A.; Kuruca, N.; Karakus, N. Impact of Endothelial NOS VNTR Variant on Susceptibility to Diabetic Neuropathy and Type 2 Diabetes Mellitus. *Curr. Neurovasc. Res.* **2020**, *17*, 700–705. [CrossRef] [PubMed]
27. Shoukry, A.; Shalaby, S.M.; Abdelazim, S.; Abdelazim, M.; Ramadan, A.; Ismail, M.I.; Fouad, M. Endothelial nitric oxide synthase gene polymorphisms and the risk of diabetic nephropathy in type 2 diabetes mellitus. *Genet. Test. Mol. Biomark.* **2012**, *16*, 574–579. [CrossRef] [PubMed]

28. Levinsson, A.; Olin, A.C.; Björck, L.; Rosengren, A.; Nyberg, F. Nitric oxide synthase (NOS) single nucleotide polymorphisms are associated with coronary heart disease and hypertension in the INTERGENE study. *Nitric Oxide* **2014**, *39*, 1–7. [CrossRef] [PubMed]
29. Sarginson, J.E.; Deakin, J.F.; Anderson, I.M.; Downey, D.; Thomas, E.; Elliott, R.; Juhasz, G. Neuronal Nitric Oxide Synthase (NOS1) Polymorphisms Interact with Financial Hardship to Affect Depression Risk. *Neuropsychopharmacology* **2014**, *39*, 2857–2866. [CrossRef]
30. Adibmanesh, A.; Bijanzadeh, M.; Mohammadzadeh, G.; Alidadi, R.; Rashidi, M.; Talaiezadeh, A. The Correlation of Endothelial Nitric Oxide Synthase Gene rs1799983 Polymorphisms with Colorectal Cancer. *Int. J. Cancer Manag.* **2020**, *13*, 97220. [CrossRef]
31. Heidari, M.M.; Khatami, M.; Tahamtan, Y. Molecular Analysis of rs2070744 and rs1799983 Polymorphisms of NOS_3 Gene in Iranian Patients with Multiple Sclerosis. *Basic Clin. Neurosci.* **2017**, *8*, 279. [CrossRef] [PubMed]
32. Fishchuk, L.; Rossokha, Z.; Pokhylko, V.; Cherniavska, Y.; Dubitska, O.; Vershyhora, V.; Tsvirenko, S.; Kovtun, S.; Gorovenko, N. NOS3 (rs61722009) gene variants testing in prediction of COVID-19 pneumonia severity. *Nitric Oxide* **2023**, *134–135*, 44–48. [CrossRef]
33. Kubota, M.; Matsuda, S.; Matsuda, M.; Yamamoto, K.; Yoshii, Y. Association of Serum Zinc Level with severity of chronic kidney disease in diabetic patients: A cross-sectional study. *BMC Nephrol.* **2022**, *23*, 1–8. [CrossRef]
34. Ming, J.; Sana, S.R.G.L.; Deng, X. Identification of copper-related biomarkers and potential molecule mechanism in diabetic nephropathy. *Front. Endocrinol.* **2022**, *13*, 978601. [CrossRef] [PubMed]
35. Król-Kulikowska, M.; Abramenko, N.; Jakubek, M.; Banasik, M.; Kepinska, M. The Role of Angiotensin-Converting Enzyme (ACE) Polymorphisms in the Risk of Development and Treatment of Diabetic Nephropathy. *J. Clin. Med.* **2024**, *13*, 995. [CrossRef]
36. Selby, N.M.; Bmbs, B.; Dm, M.; Taal, M.W.; Mmed, B. An updated overview of diabetic nephropathy: Diagnosis, prognosis, treatment goals and latest guidelines. *Diabetes Obes. Metab.* **2020**, *22*, 3–15. [CrossRef] [PubMed]
37. Pelle, M.C.; Provenzano, M.; Busutti, M.; Porcu, C.V.; Zaffina, I.; Stanga, L.; Arturi, F. Up-Date on Diabetic Nephropathy. *Life* **2022**, *12*, 1202. [CrossRef] [PubMed]
38. Varghese, S.; Kumar, S.G. Role of eNOS and TGFβ1 gene polymorphisms in the development of diabetic nephropathy in type 2 diabetic patients in South Indian population. *Egypt. J. Med. Hum. Genet.* **2022**, *23*, 1–10. [CrossRef]
39. Dobrijevic´, Z.; Stevanovic´, J.; Robajac, D.; Penezic´, A.; Cˇ etic´, D.; Baralic´, M.; Nedic´, O. Association between nitric oxide synthase (NOS3) gene polymorphisms and diabetic nephropathy: An updated meta-analysis. *Mol. Cell. Endocrinol.* **2024**, *586*, 112197. [CrossRef]
40. Varghese, S.; Kumar, S.G. Association between genetic variants of NOS3, TGF-β and susceptibility of diabetic nephropathy: A meta-analysis. *Meta Gene* **2019**, *21*, 100573. [CrossRef]
41. Oliveira-Paula, G.H.; Lacchini, R.; Tanus-Santos, J.E. Endothelial nitric oxide synthase: From biochemistry and gene structure to clinical implications of NOS_3 polymorphisms. *Gene* **2016**, *575*, 584–599. [CrossRef] [PubMed]
42. Yuzawa, Y.; Niki, I.; Kosugi, T.; Maruyama, S.; Yoshida, F.; Takeda, M.; Tagawa, Y.; Kaneko, Y.; Kimura, T.; Kato, N.; et al. Overexpression of Calmodulin in Pancreatic β Cells Induces Diabetic Nephropathy. *J. Am. Soc. Nephrol.* **2008**, *19*, 1701–1711. [CrossRef] [PubMed]
43. Mikhailov, A.V.; Liu, Y.; Cheng, H.J.; Lin, J.J.; Cheng, C.P. Calmodulin-dependent protein kinase II activation promotes kidney mesangial expansion in streptozotocin-induced diabetic mice. *Heliyon* **2022**, *8*, e11653. [CrossRef] [PubMed]
44. Benchoula, K.; Mediani, A.; Hwa, W.E. The functions of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) in diabetes progression. *J. Cell Commun. Signal.* **2023**, *17*, 25–34. [CrossRef] [PubMed]
45. Cotta Filho, C.K.; Oliveira-Paula, G.H.; Rondon Pereira, V.C.; Lacchini, R. Clinically relevant endothelial nitric oxide synthase polymorphisms and their impact on drug response. *Expert Opin. Drug Metab. Toxicol.* **2020**, *16*, 927–951. [CrossRef] [PubMed]
46. Saito, Y.; Otaki, Y.; Watanabe, T.; Wanezaki, M.; Kutsuzawa, D.; Kato, S.; Tamura, H.; Nishiyama, S.; Arimoto, T.; Takahashi, H.; et al. Effects of Nitric Oxide Synthase 3 Gene Polymorphisms on Cardiovascular Events in a General Japanese Population—The Yamagata (Takahata) Study. *Circ. Rep.* **2022**, *4*, 222–229. [CrossRef] [PubMed]
47. Shahid, S.M.; Nawab, S.N.; Shaikh, R.; Mahboob, T. Glycemic control, dyslipidemia and endothelial dysfunction in coexisted diabetes, hypertension and nephropathy. *Pak. J. Pharm. Sci.* **2012**, *25*, 123–129. [PubMed]
48. Al-Timimi, D.J.; Sulieman, D.M.; Hussen, K.R. Zinc Status in Type 2 Diabetic Patients: Relation to the Progression of Diabetic Nephropathy. *J. Clin. Diagn. Res.* **2014**, *8*, CC04. [CrossRef]
49. Barman, S.; Pradeep, S.R.; Srinivasan, K. Zinc supplementation alleviates the progression of diabetic nephropathy by inhibiting the overexpression of oxidative-stress-mediated molecular markers in streptozotocin-induced experimental rats. *J. Nutr. Biochem.* **2018**, *54*, 113–129. [CrossRef]
50. Feng, J.; Wang, H.; Jing, Z.; Wang, Y.; Wang, W.; Jiang, Y.; Sun, W. Relationships of the Trace Elements Zinc and Magnesium with Diabetic Nephropathy-Associated Renal Functional Damage in Patients with Type 2 Diabetes Mellitus. *Front. Med.* **2021**, *8*, 626909. [CrossRef]
51. Ume, A.C.; Wenegieme, T.Y.; Adams, D.N.; Adesina, S.E.; Williams, C.R. Zinc Deficiency: A Potential Hidden Driver of the Detrimental Cycle of Chronic Kidney Disease and Hypertension. *Kidney360* **2023**, *4*, 398–404. [CrossRef] [PubMed]
52. Mohammadi, M.; Yaghooti, H.; Adibmanesh, A.; Mohammadtaghvaie, N.; Akhormeh, A.K.; Eslami, M. Endothelial Nitric Oxide Synthase 4a/B Polymorphism and Its Interaction with Enos G894T Variants in Type 2 Diabetic Patients: Modifying the Risk of Diabetic Nephropathy. *Iran. J. Public Health* **2022**, *51*, 219. [PubMed]

53. Li, H.; Shu, G.; Gao, H. Epithelial nitric oxide synthases (eNOS) 894 G < T polymorphism and diabetic nephropathy susceptibility: A meta-Analysis. *Pteridines* **2022**, *33*, 49–57.
54. Duan, J.-Y.; Duan, G.-C.; Wang, C.-J.; Liu, D.-W.; Qiao, Y.-J.; Pan, S.-K.; Jiang, D.-K.; Liu, Y.; Zhao, Z.-H.; Liang, L.-L.; et al. Prevalence and risk factors of chronic kidney disease and diabetic kidney disease in a central Chinese urban population: A cross-sectional survey. *BMC Nephrol.* **2020**, *21*, 1–13. [CrossRef]
55. Natesan, V.; Kim, S.J. Diabetic Nephropathy—A Review of Risk Factors, Progression, Mechanism, and Dietary Management. *Biomol. Ther.* **2021**, *29*, 365. [CrossRef]

Supplementary materials

Questionnaire S1: Sample of a questionnaire conducted among people suffering from diabetic nephropathy.

PART 1. PRELIMINARY INFORMATION

Sex [W/M]	City of residence	Age [years]	Height [cm]	Weight [kg]

QUESTION	ANSWER
Do you suffer from diabetes? If so, what type?	
Do you suffer from chronic diseases other than diabetes? If so, which ones?	
Do you suffer from diabetic nephropathy?	
Have you ever been or are you undergoing dialysis?	
Have you ever received a kidney transplant?	

PART 2. LIFESTYLE

Do you play sports? How many hours per week?	
Do you smoke cigarettes? How many years? How many packs a day?	
Do you consume alcohol? In what amount? How often?	

Do you take medications/dietary supplements? Which medications or dietary supplements?

PART 3. DISEASES IN THE FAMILY

Do your parents suffer from diabetes?	
Do your siblings suffer from diabetes?	
Do your parents suffer from diabetic nephropathy?	
Do your siblings suffer from diabetic nephropathy?	

Table S1: Concentrations of NOS1, NOS2 and NOS3 in the studied groups.

Parameter	Control Group (*N* = 50)	Diabetic Nephropathy Group (*N* = 85)	Kidney Transplant Diabetic Nephropathy Group (*N* = 97)	*p*
NOS1 [ng/mL]	{3.85; 9.35; 30.28}	{3.95; 5.30; 6.62}*	{4.22; 6.64; 15.39}	**0.002**
NOS2 [ng/mL]	{5.51; 14.82; 39.99}	{4.68; 6.87; 8.46}*	{5.28; 7.86; 14.46}	**<0.001**
NOS3 [ng/mL]	{0.69; 0.76; 0.83}	{0.30; 0.38; 0.50}*	{0.32; 0.40; 0.50}*	**<0.001**

Values are shown as {1st quartile; median; 3rd quartile}. * $p < 0.05$ – compared to the control group.

Table S2: The Hardy-Weinberg equilibrium for studied groups.

Polymorphism (*Gene*)	**Groups (N)**	**The χ2 test values**
rs3782218 (*NOS1*)	Control (*N* = 47)	**7.24**
	Diabetic Nephropathy (*N* = 79)	0.87
	Kidney Transplant Diabetic Nephropathy (*N* = 96)	<0.01
Polymorphism (*Gene*)	**Groups (*N*)**	**The χ2 test values**
rs1137933 (*NOS2*)	Control (*N* = 44)	0.02
	Diabetic Nephropathy (*N* = 83)	0.42
	Kidney Transplant Diabetic Nephropathy (*N* = 97)	<0.01
Polymorphism (*Gene*)	**Groups (*N*)**	**The χ2 test values**
rs1799983 (*NOS3*)	Control (*N* = 50)	0.30
	Diabetic Nephropathy (*N* = 77)	**10.11**
	Kidney Transplant Diabetic Nephropathy (*N* = 82)	**9.07**
Polymorphism (*Gene*)	**Groups (*N*)**	**The χ2 test values**
rs2070744 (*NOS3*)	Control (*N* = 50)	**22.79**
	Diabetic Nephropathy (*N* = 84)	**12.72**
	Kidney Transplant Diabetic Nephropathy (*N* = 97)	**43.72**
Polymorphism (*Gene*)	**Groups (*N*)**	**The χ2 test values**
rs61722009 (*NOS3*)	Control (*N* = 50)	0.03

	Diabetic Nephropathy (N = 85)	1.13
	Kidney Transplant Diabetic Nephropathy (N = 97)	1.33

In order to calculate the Hardy-Weinberg equilibrium, the following values were adopted: df = 1, p = 0.05. The critical value was 3.84.

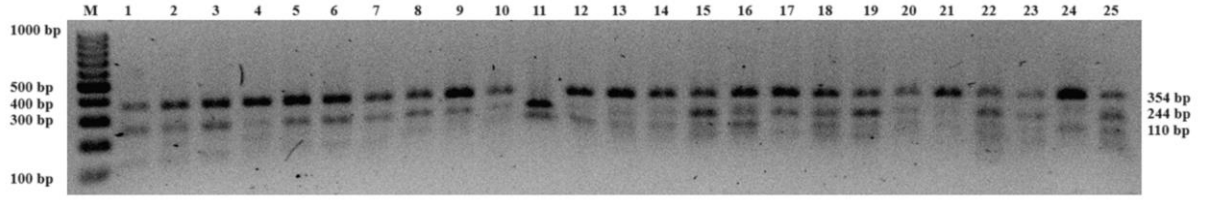

Figure S1: Example of electropherogram of the rs3782218 polymorphism in *NOS1*.
M – marker ladder; 11 – C/C genotype; 1, 2, 3, 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22, 23, 24, 25 – C/T genotype; 4, 21 – T/T genotype

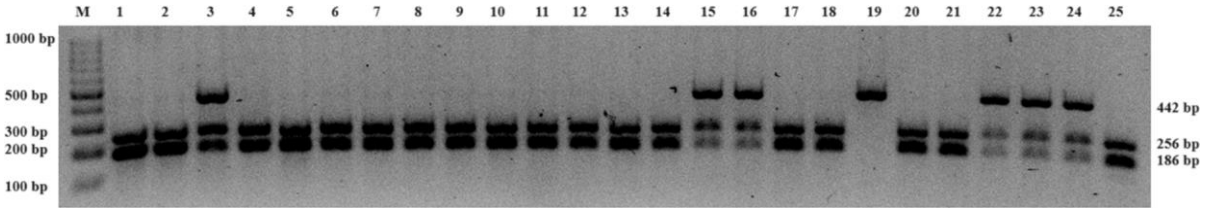

Figure S2: Example of electropherogram of the rs1137933 polymorphism in *NOS2*.
M – marker ladder; 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 17, 18, 20, 21, 25 – G/G genotype; 3, 15, 16, 22, 23, 24 – G/A genotype; 19 – A/A genotype

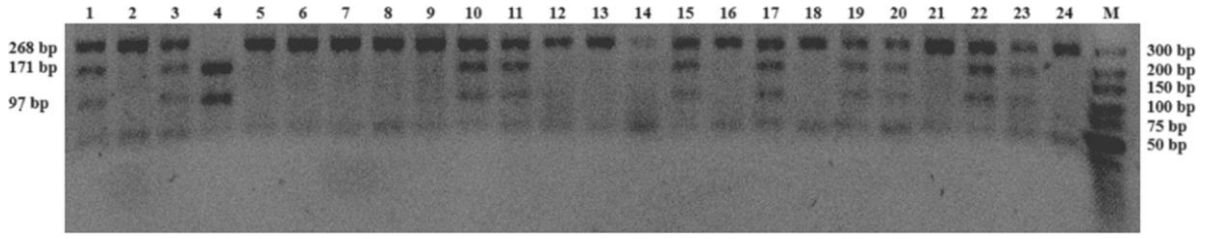

Figure S3: Example of electropherogram of the rs1799983 polymorphism in *NOS3*.
M – marker ladder; 2, 5, 6, 7, 8, 9, 12, 13, 16, 18, 21, 24 – G/G genotype; 1, 3, 10, 11, 14, 15, 17, 19, 20, 22, 23 – G/T genotype; 4 – T/T genotype

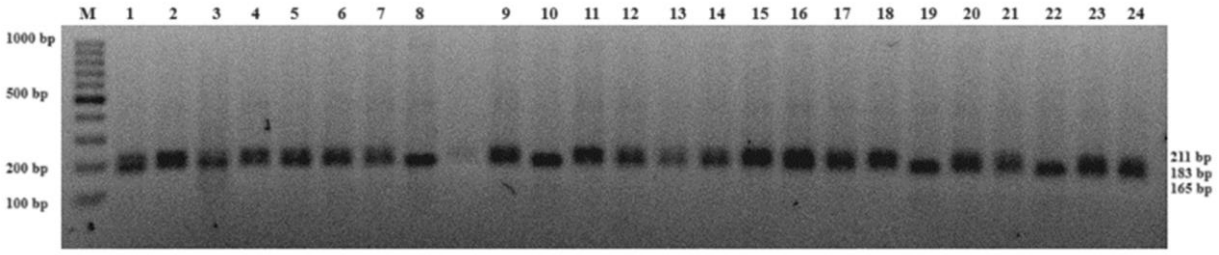

Figure S4: Example of electropherogram of the rs2070744 polymorphism in *NOS3*.
M – marker ladder; 8, 10, 19, 22 – C/C genotype; 3 – C/T genotype; 1, 2, 4, 5, 6, 7, 9, 11, 12, 13, 14, 15, 16, 17, 18, 20, 21, 23, 24 – T/T genotype

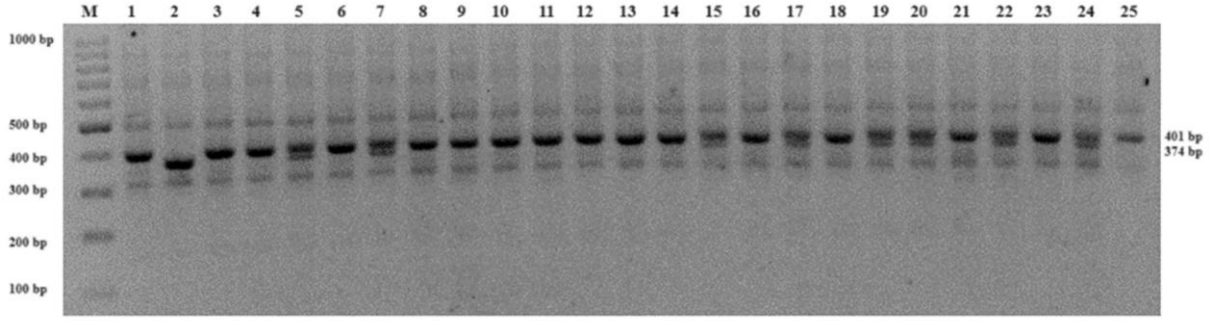

Figure S5: Example of electropherogram of the rs61722009 polymorphism in *NOS3*.
M – marker ladder; 2 – 4a/4a genotype; 5, 7, 15, 17, 19, 20, 22, 24 – 4a/4b genotype; 1, 3, 4, 6, 8, 9, 10, 11, 12, 13, 14, 16, 18, 21, 23, 25 – 4b/4b genotype

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2. Król-Kulikowska Magdalena, Abramenko Nikita, Jakubek Milan, Banasik Mirosław, Kepinska Marta: The role of angiotensin-converting enzyme (ACE) polymorphisms in the risk of development and treatment of diabetic nephropathy. Journal of Clinical Medicine, 2024, vol. 13, nr 4, art.995 [17 s.], DOI: 10.3390/jcm13040995

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3. **Król-Kulikowska** Magdalena, Banasik Mirosław, Kepinska Marta: The effect of selected nitric oxide synthase polymorphism on the risk of developing diabetic nephropathy. Antioxidants, 2024, vol. 13, nr 7, art.838 [23 s.], DOI:10.3390/antiox13070838

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mój udział polegał na wyselekcjonowaniu pacjentów z cukrzycową chorobą nerek i pacjentów po przeszczepie nerki, korekcie ostatecznej wersji manuskryptu, a także pozyskaniu źródeł finansowania;

2. Król-Kulikowska Magdalena, Banasik Mirosław, Kepinska Marta: The effect of selected nitric oxide synthase polymorphism on the risk of developing diabetic nephropathy. Antioxidants, 2024, vol. 13, nr 7, art.838 [23 s.], DOI:10.3390/antiox13070838

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mój udział polegał na częściowym opracowaniu metodologii oraz korekcie i edycji gotowego manuskryptu.

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OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, że w pracy:

1. **Król-Kulikowska Magdalena,** Abramenko Nikita, Jakubek Milan, Banasik Mirosław, Kepinska Marta: The role of angiotensin-converting enzyme (ACE) polymorphisms in the risk of development and treatment of diabetic nephropathy. Journal of Clinical Medicine, 2024, vol. 13, nr 4, art.995 (17 s.], DOI: 10.3390/jcm13040995

mój udział polegał na c2tściowym opracowaniu metodologii, zarządzaniu danymi badawczymi, wykonaniu analizy in *silico* oraz opìsaniu części metod i wyników.

podpis współautora

podpis promotora

4. Załącznik 4. Życiorys naukowy autorki rozprawy doktorskiej.

ŻYCIORY NAUKOWY

Imię i nazwisko: Magdalena Król-Kulikowska
Data urodzenia: 27 lipca 1996
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Wykształcenie:

- studia magisterskie na kierunku Analityka medyczna; Wydział Farmaceutyczny, Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu; 10.2015-07.2020
  Temat pracy magisterskiej: "Wpływ wybranych polimorfizmów genu *INS* na stężenie insuliny w surowicy w kontekście występowania cukrzycy typu drugiego i/lub otyłości"
- uczestnik Szkoły Doktorskiej; Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu; 10.2020-09.2024
  Temat rozprawy doktorskiej: "Znaczenie wybranych polimorfizmów genów syntaz tlenku azotu (*NOS*) oraz genu kodującego enzym konwertujący angiotensynę (*ACE*) w ocenie ryzyka wystąpienia cukrzycowej choroby nerek"
- studia podyplomowe na kierunku Biologia molekularna; Wydział Biochemii, Biofizyki i Biotechnologii, Uniwersytet Jagielloński, Kraków; 10.2023-06.2024

Doświadczenie zawodowe:

- Pomoc laboratoryjna; Diagnostyka S.A., Wrocław; 07.2019-12.2019
  Zakres obowiązków: pobieranie materiału biologicznego, wykonywanie podstawowych badań laboratoryjnych
- Asystent w grupie badawczo-dydaktycznej; Katedra i Zakład Biochemii Farmaceutycznej, Wydział Farmaceutyczny, Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu; 03.2023-obecnie
  Zakres obowiązków: prowadzenie zajęć dydaktycznych dla studentów kierunków analityka medyczna, farmacja oraz dietetyka; prowadzenie badań naukowych; pisanie publikacji

- Diagnosta laboratoryjny; Klinika Nefrologii, Medycyny Transplantacyjnej i Chorób Wewnętrznych, Uniwersytecki Szpital Kliniczny im. Jana Mikulicza-Radeckiego we Wrocławiu; 08.2024-obecnie
  Zakres obowiązków: wykonywanie badań laboratoryjnych, autoryzacja wyników

WYKAZ PULIKACJI I DONIESIEŃ NAUKOWYCH

1. Publikacje w czasopismach z IF

Lp.	Opis bibliograficzny	IF	Punkty MNiSW/MEiN
1.	Czyż Anna, **Król Magdalena**, Przybyszewski Oskar, Radajewska Anna, Moreira Helena, Barg Ewa: Transformacje nowotworowe komórek hematopoetycznych u osób z trisomią 21 chromosomu, Postępy Biologii Komórki, 2019, vol. 46, nr 2, s. 111-127	0,163	20
2.	**Król Magdalena**, Kepinska Marta: Human nitric oxide synthase – its functions, polymorphisms, and inhibitors in the context of inflammation, diabetes and cardiovascular diseases, International Journal of Molecular Sciences, 2021, vol. 22, nr 1, art.56 [18 s.], DOI:10.3390/ijms22010056	6,208	140
3.	Martuszewski Adrian, Paluszkiewicz Patrycja, **Król Magdalena**, Banasik Mirosław, Kepinska Marta: Donor-derived cell-free DNA in kidney transplantation as a potential rejection biomarker: a systematic literaturę review, Journal of Clinical Medicine, 2021, vol. 10, nr 2, art. 193 [19 .], DOI:10.3390/jcm10020193	4,964	140
4.	Kosendiak Aureliusz, **Król Magdalena**, Ligocka Marta, Kepinska Marta: Eating habits and nutritional knowledge among amateur ultrarunners, Frontiers in Nutrition, 2023, vol. 10, art.1137412 [11 s.], DOI:10.3389/fnut.2023.1137412	4,000	70
5.	Grussy Katarzyna, Łaska Magdalena, Moczurad Wiktoria, **Król-Kulikowska Magdalena**, Ściskalska Milena: The importance of polymorphism in the genes encoding glutathione S-transferase isoenzymes in development of selected cancers and cardiovascular diseases, Molecular Biology Reports, 2023, vol. 50, s. 9649-9661, DOI:10.1007/s11033-023-08894-4	2,600	70
6.	**Król-Kulikowska Magdalena**, Banasik Mirosław, Kepinska Marta: The effect of selected nitric oxide synthase polymorphism on the risk of developing diabetic nephropathy, Antioxidants, 2024, vol. 13, nr 7, art.838 [23 s.], DOI:10.3390/antiox13070838	6,000	100
7.	Kunachowicz Dominika, **Król-Kulikowska Magdalena**, Raczycka Wiktoria, Sleziak Jakub, Błażejewska Marta, Kulbacka Julita, Heat shock proteins, a double-edged sword: significance in cancer progression, chemotherapy resistance and novel therapeutic perspectives, Cancers, 2024, vol. 16, nr 8, art.1500 [38 s.], DOI:10.3390/cacers16081500	4,500	140

8.	**Król-Kulikowska Magdalena**, Abramenko Nikita, Jakubek Milan, Banasik Mirosław, Kepinska Marta: The role of angiotensin-converting enzyme (ACE) polymorphisms in the risk of development and treatment of diabetic nephropathy, Journal of Clinical Medicine, 2024, vol. 13, nr 4, art.995 [17 s.], DOI: 10.3390/jcm13040995	3,000	140
9.	**Król-Kulikowska Magdalena**, Urbanowicz Iwona, Kepinska Marta: The concentrations of interleukin-6, insulin, and glucagon in the context of obesity and type 2 diabetes and single nucleotide polymorphisms in *IL6* and *INS* genes, Journal of Obesity, 2024, vol. 2024, art.7529779 [13 s.], DOI:10.1155/2024/7529779	3,800	100
	Podsumowanie	**35,235**	**920**

2. Publikacje w czasopismach bez IF

Lp.	Opis bibliograficzny	Punkty MNiSW/MEiN
1.	Kosendiak Aureliusz, **Król Magdalena**, Ściskalska Milena, Kepinska Marta: The changes in stress coping, alcohol use, cigarette smoking and physical activity during COVID-19 related lockdown in medical students in Poland, International Journal of Environmental Research and Public Health, 2022, vol. 9, nr 1, art.302 [15 s.], DOI:10.3390/ijerph19010302	140
	Podsumowanie	**140**

3. Doniesienia naukowe

Lp.	Opis bibliograficzny
1.	**Król Magdalena**, Przybyszewski Oskar: Zbrodnia zapisana w genach, czyli o wpływie ekspresji genu MAO na skłonność do agresji, W: IV Ogólnopolska Studencka Konferencja Kryminalistyczna „50 Twarzy Zbrodni", Wrocław, 23-25 marca 2018. – praca prezentowana w czasie realizacji studiów magisterskich
2.	**Król Magdalena**, Lewoń Dominika: Przykłady mutacji i innych markerów molekularnych jako czynniki predykcyjne w chorobach nowotworowych, W: IX Wykłady Otwarte z cyklu Spotkania Młodych z Nauką w Poznaniu, Poznań, 7-8 kwietnia 2018. – praca prezentowana w czasie realizacji studiów magisterskich
3.	**Król Magdalena**, Lewandowski Łukasz, Milnerowicz Halina: Co w… herbacie piszczy. Kombucha – płynne zdrowie czy potencjalna trucizna?, W: Ogólnopolska Studencko-Doktorancka Konferencja Naukowa „Epidemie Wielkie i Małe", Wrocław, 10 maja 2018. – praca prezentowana w czasie realizacji studiów magisterskich
4.	**Król Magdalena**, Przybyszewski Oskar: Badania genetyczne w służbie sprawiedliwości, czyli jak odnaleźć przestępcę dzięki konsumenckim bazom danych DNA, W: V Ogólnopolska Konferencja Kryminalistyczna „50 Twarzy Zbrodni",

	Poznań, 7-9 grudnia 2018. – praca prezentowana w czasie realizacji studiów magisterskich
5.	**Król Magdalena**, Przybyszewski Oskar: W jaki sposób odnaleźć przestępcę dzięki bazom danych DNA?, W: III Ogólnopolska Konferencja Naukowa „24h kryminalistyki", Wrocław, 14-15 grudnia 2018. – praca prezentowana w czasie realizacji studiów magisterskich
6.	**Król Magdalena**, Blachel Milena: Cheiloskopia – ślady kryminalnego pocałunku, W: III Ogólnopolska Konferencja Naukowa „24h kryminalistyki", Wrocław, 14-15 grudnia 2018. – praca prezentowana w czasie realizacji studiów magisterskich
7.	**Król Magdalena**, Kepinska Marta, Lewandowski Łukasz, Milnerowicz Halina: The influence of insulin gene polymorphisms on its concentration in a selected group of people, W: *3rd Wroclaw Scientific Meetings*. Wrocław, 1st-2nd March 2019, (red.) Julita Kulbacka, Nina Rembiałkowska, Joanna Weżgowiec, Wrocław 2019, Wydawnictwo Naukowe TYGIEL sp. z o.o., 110 poz.P56, ISBN 978-83-65932-64-8 – praca prezentowana w czasie realizacji studiów magisterskich
8.	Czyż Anna, **Król Magdalena**, Przybyszewski Oskar, Radajewska Anna: Acute leukemia in people with trisomy 21 chromosome, W: *3rd Wroclaw Scientific Meetings*. Wrocław, 1st-2nd March 2019, (red.) Julita Kulbacka, Nina Rembiałkowska, Joanna Weżgowiec, Wrocław 2019, Wydawnictwo Naukowe TYGIEL sp. z o.o., 147 poz.P93, ISBN 978-83-65932-64-8 – praca prezentowana w czasie realizacji studiów magisterskich
9.	**Król Magdalena**, Pluta Dominika: Odkrywamy tajemnice starożytnych władców – współczesne metody badań egipskich mumii, W: II Ogólnopolska Naukowa Konferencja Antropologiczno-Archeologiczna „Możliwości badawcze w antropologii i archeologii", Wrocław, 9-10 marca 2019. – praca prezentowana w czasie realizacji studiów magisterskich
10.	**Król Magdalena**, Janiszewska Ewa: Wspomnienia o poległych wiecznie żywe, czyli o profilowaniu genetycznym ofiar zbrodni systemów totalitarnych i wojen, W: IV Międzynarodowa Studencka Konferencja Nauk o Człowieku „W pierścieniu wspomnień", Wrocław, 15-17 marca 2019. – praca prezentowana w czasie realizacji studiów magisterskich
11.	**Król Magdalena**: Profilowanie genetyczne ofiar zbrodni systemów totalitarnych i wojen, W: Sympozjum „Genetyka w muzealnictwie" oraz Warsztaty „Niedestrukcyjne techniki izolacji DNA z eksponatów muzealnych", Wrocław, 1-2 czerwca 2019. – praca prezentowana w czasie realizacji studiów magisterskich
12.	**Król Magdalena**, Urbanowicz Iwona, Lewandowski Łukasz, Kepinska Marta, Milnerowicz Halina: Relationship between the concentration of IL-6, insulin activity, glycated haemoglobin in human blood and the development of type 2 diabetes and/or obesity, W: *4th International Wroclaw Scientific Meetings*. Wrocław, 09-10 October 2020, (red.) Julita Kulbacka, Nina Rembiałkowska, Joanna Weżgowiec, Wrocław 2020, Wydawnictwo Naukowe TYGIEL sp. z o.o., 33-34 poz.O1, ISBN 978-83-66489-37-0
13.	**Król Magdalena**, Kepinska Marta: Porównanie metod oznaczania aktywności syntazy tlenku azotu w próbkach biologicznych, W: V Ogólnopolska Konferencja Naukowa "Współczesne zastosowanie metod analitycznych w farmacji i medycynie". [Online], 27 listopada 2020 r. Książka abstraktów 2020, s. 35-36

14.	**Król Magdalena**, Urbanowicz Iwona, Kepinska Marta: Ocena genotypów w obrębie wybranych polimorfizmów genów *INS* i *NOS3* u pacjentów z cukrzycą typu 2 z wykorzystaniem metody PCR-RFLP, W: VI Ogólnopolska Konferencja Naukowa "Współczesne zastosowanie metod analitycznych w farmacji i medycynie". Wrocław, 03 grudnia 2021 r. Książka abstraktów, Wrocław 2021, s. 18
15.	**Król Magdalena**, Urbanowicz Iwona, Kepinska Marta: Wpływ polimorfizmu rs1800795 w genie interleukiny-6 (*IL6*) na ryzyko rozwoju otyłości i/lub cukrzycy typu 2, W: V Śląskie Farmaceutyczne Spotkanie Naukowe "Od nauki do pacjenta" – konferencja. Sosnowiec, 9 grudnia 2022, (red.) Grażyna Janikowska, Kazimiera Klementys 2022, Polskie Towarzystwo Farmaceutyczne, 103 poz.S.V.P_2, ISBN 978-83-64968-29-7
16.	Kunachowicz Dominika, **Król Magdalena**, Ściskalska Milena, Kepinska Marta: Egzosomy – w poszukiwaniu odpowiedzi na kluczowe pytania biomedycyny, W: VII Ogólnopolska Konferencja Naukowa "Współczesne zastosowanie metod analitycznych w farmacji i medycynie". Wrocław, 15 grudnia 2022 roku. Książka abstraktów, Wrocław 2022, s. 10-11
17.	**Król Magdalena**, Kunachowicz Dominika, Kepinska Marta: Analiza *in silico* wiązań enzymu konwertującego angiotensynę (ACE) z jego inhibitorami, W: VII Ogólnopolska Konferencja Naukowa "Współczesne zastosowanie metod analitycznych w farmacji i medycynie". Wrocław, 15 grudnia 2022 roku. Książka abstraktów, Wrocław 2022, s. 31
18.	Klęczar Michalina, Klońska Aleksandra, **Król-Kulikowska Magdalena**, Kunachowicz Dominika, Kepinska Marta: The effect of the Mediterranean diet on insulin concentration, Advances in Clinical and Experimental Medicine, 2023, vol. 32, nr 3 spec., s. 76, [*5th International Wroclaw Scientific Meetings*. Wrocław, Poland, October 19-21, 2023. Abstract book]
19.	Klońska Aleksandra, Klęczar Michalina, **Król-Kulikowska Magdalena**, Kepinska Marta: Choosing the right diet in the prevention and treatment of cancer, Advances in Clinical and Experimental Medicine, 2023, vol. 32, nr 3 spec., s. 77, [*5th International Wroclaw Scientific Meetings*. Wrocław, Poland, October 19-21, 2023. Abstract book]
20.	**Król-Kulikowska Magdalena**, Banasik Mirosław, Kepinska Marta: The role of angiotensin converting enzyme polymorphisms in the development of diabetic nephropathy, Advances in Clinical and Experimental Medicine, 2023, vol. 32, nr 3 spec., s. 84, [*5th International Wroclaw Scientific Meetings*. Wrocław, Poland, October 19-21, 2023. Abstract book]
21.	Klęczar Michalina, Klońska Aleksandra, **Król-Kulikowska Magdalena**, Kunachowicz Dominika, Kepinska Marta: Wpływ diety na stężenie kortyzolu we krwi, W: VIII Ogólnopolska Konferencja Naukowa "Współczesne zastosowanie metod analitycznych w farmacji i medycynie". Wrocław, 06 grudnia 2023 roku. Książka abstraktów, Wrocław 2023, s. 17

POZOSTAŁE OSIĄGNIĘCIA

1. Projekty naukowe

Lp.	Nazwa projektu	Rola
1.	Cukrzyca jako choroba cywilizacyjna – znaczenie polimorfizmu syntazy tlenku azotu 3 – rs799983 w ocenie ryzyka wystąpienia cukrzycy typu 2. Fundusz Aktywności Studenckiej (FAST), Wrocławskie Centrum Akademickie, 2020 r.	**Kierownik projektu**
2.	Analiza wybranych parametrów przydatnych w ocenie wielkości zaburzeń w funkcjonowaniu organizmu w stanie zapalnym trzustki i cukrzycowej chorobie nerek. Zadanie badawcze w ramach subwencji, SUBZ.D022.22.033, kierownik projektu: dr Milena Ściskalska, 2022 r.	**Wykonawca projektu**
3.	Wpływ ksenobiotyków na zaburzenia równowagi pro/antyoksydacyjnej – badanie *ex vivo* i *in vitro*. Zadanie badawcze w ramach subwencji, SUBZ.D020.23.008, kierownik projektu: dr hab. inż. Marta Kepinska, prof. UMW, 2023 r.	**Wykonawca projektu**
4.	Parametry oksydacyjne w ocenie regulacji chorób o podłożu zapalnym. Zadanie badawcze w ramach subwencji, kierownik projektu: dr hab. inż. Marta Kepinska, prof. UMW, SUBZ.D020.24.071, 2024 r.	**Wykonawca projektu**
5.	Nowe markery w diagnostyce cukrzycowej choroby nerek – opracowanie modelu predykcyjnego w oparciu o polimorfizmy genów kodujących fetuinę-A i hemooksygenazę 1 oraz stężenia tych parametrów. „Studenckie koła naukowe tworzą innowacje", Ministerstwo Nauki i Szkolnictwa Wyższego, SKN.D020.24.003, 2024-2025 r.	**Opiekun projektu**
6.	Genetyczne warianty dysmutazy ponadtlenkowej SOD2 a przeżywalność pacjentów z rakiem niedrobnokomórkowym płuca. Subwencja UMW, SUBK.D040.24.018, kierownik projektu: mgr Mateusz Witkowski, 2024 r.	**Wykonawca projektu**

2. Nagrody i wyróżnienia

Lp.	Nazwa osiągnięcia	Rok
1.	Nagroda Komitetu Naukowego przyznana na konferencji V Ogólnopolska Konferencja Kryminalistyczna „50 Twarzy Zbrodni" za wystąpienie pt. „Badania genetyczne w służbie sprawiedliwości, czyli jak odnaleźć przestępcę dzięki konsumenckim bazom danych DNA" (Poznań).	**2018**
2.	Trzecie miejsce na konferencji *4th International Wroclaw Scientific Meetings* za wystąpienie pt. „Relationship between the	**2020**

	concentration of IL-6, insulin activity, glycated haemoglobin in human blood and the development of type 2 diabetes and/or obesity" (Wrocław).	
3.	Drugie miejsce na konferencji VII Ogólnopolska Konferencja Naukowa „Współczesne zastosowanie metod analitycznych w farmacji i medycynie" za poster pt. „Analiza *in silico* wiązań enzymu konwertującego angiotensynę (ACE) z jego inhibitorami" (Wrocław).	**2022**

3. Staże naukowe

Lp.	Rodzaj stażu	Rok
1.	Staż we Wrocławskim Parku Technologicznym w ramach programu „Dolnośląscy Liderzy Medycyny – realizacja zintegrowanego programu podnoszenia kompetencji studentów, doktorantów oraz kadry dydaktycznej i administracyjnej"	**2019 (3 tygodnie)**
2.	Staż w ramach programu Erasmus+: „Traineeship on the topic of drug protein interactions in the BIOCEV"; Charles University (1st Faculty of Medicine – BIOCEV), Praga, Czechy	**2022 (2 miesiące)**
3.	Staż w ramach programu Erasmus+; International Hellenic University, Saloniki, Grecja	**2024 (tydzień)**

4. Inne aktywności

Lp.	Rodzaj aktywności	Rok
1.	Członek Studenckiego Towarzystwa Diagnostów Laboratoryjnych	**2015-2024**
2.	Współorganizacja konferencji *VIII International Student's Conference of Young Medical Researchers*, Wrocław	**2018**
3.	Współorganizacja zajęć w ramach XXI, XXII, XXIV, XXV, XXVI i XXVII Dolnośląskiego Festiwalu Nauki	**2018-2024**
4.	Członek Rady Dyscypliny Nauki Farmaceutyczne	**2020-2024**
5.	Członek Polskiego Towarzystwa Biochemicznego	**od 2020**
6.	Współorganizacja Dnia Otwartego na Uniwersytecie Medycznym im. Piastów Śląskich we Wrocławiu	**2021 i 2024**
7.	Ukończenie szkolenia dla osób uczestniczących w wykonywaniu procedur na zwierzętach doświadczalnych	**2021**
8.	Opiekun SKN przy Zakładzie Biochemii Farmaceutycznej (K 214)	**od 2023**
9.	Współorganizacja konferencji *5th International Wroclaw Scientific Meetings*, Wrocław	**2023**

10.	Realizacja studiów podyplomowych na kierunku Biologia molekularna, Uniwersytet Jagielloński	**2023 i 2024**
11.	Wolontariat w ramach Wielkiej Orkiestry Świątecznej Pomocy	**2024**
12.	Wygłoszenie wystąpienia w ramach Targów pracy dla studentów analityki medycznej UMW	**2024**
13.	Wolontariat w Stowarzyszeniu TRATWA – odbieranie i segregacja darów dla powodzian	**2024**

5. Załącznik 5. Wykaz publikacji i abstraktów potwierdzony przez bibliotekę.

Wrocław, 23.09.2024 r.

Król-Kulikowska Magdalena

Wykaz publikacji

1. Publikacje w czasopismach naukowych

1.1 Publikacje w **czasopiśmie** z IF

Lp.	Opis bibliograficzny	IF	Punkty
1	Czyż Anna, Król Magdalena, Przybyszewski Oskar, Radajewska Anna, Moreira Helena, Barg Ewa: Transformacje nowotworowe komórek hematopoetycznych u osób z trisomią 21 chromosomu, Postępy Biologii Komórki, 2019, vol. 46, nr 2, s. 111-127	0,163	20
2	Król Magdalena, Kepinska Marta: Human nitric oxide synthase - its functions, polymorphisms, and inhibitors in the context of inflammation, diabetes and cardiovascular diseases, International Journal of Molecular Sciences, 2021, vol. 22, nr 1, art.56 [18 s.], DOI:10.3390/ijms22010056	6,208	140
3	Martuszewski Adrian, Paluszkiewicz Patrycja, Król Magdalena, Banasik Mirosław, Kepinska Marta: Donor-derived cell-free DNA in kidney transplantation as a potential rejection biomarker: a systematic literature review, Journal of Clinical Medicine, 2021, vol. 10, nr 2, art.193 [19 s.], DOI:10.3390/jcm10020193	4,964	140
4	Kosendiak Aureliusz, Król Magdalena, Ligocka Marta, Kepinska Marta: Eatìng habits and nutritional knowledge among amateur ultrarunners, Frontiers in Nutrition, 2023, vol. 10, art.1137412 [11 s.], DOI:10.3389/fnut.2023.1137412	4	70
5	Grussy Katarzyna, Łaska Magdalena, Moczurad Wiktoria, **Król-Kulikowska** Magdalena, Ściskalska Milena: The importance of polymorphisms in the genes encoding glutathione S-transferase isoenzymes in development of selected cancers and cardiovascular diseases, Molecular Biology Reports, 2023, vol. 50, s. 9649-9661, DOI:10.1007/s11033-023-08894-4	2,6	70
6	**Król-Kulikowska Magdalena,** Banasik Mirosław, Kepinska Marta: The effect of selected nitric oxide synthase polymorphisms on the risk of developing diabetic nephropathy, Antioxidants, 2024, vol. 13, nr 7, art.838 [23 s.], DOI:10.3390/antiox13070838	6*	100
7	Kunachowicz Dominika, **Król-Kulikowska Magdalena,** Raczycka Wiktoria, Sleziak Jakub, Błażejewska Marta, Kulbacka Julita: Heat shock proteins, a double-edged sword: significance in cancer progression, chemotherapy resistance and novel therapeutic perspectives, Cancers, 2024, vol. 16, nr 8, art.1500 [38 s.], DOI:10.3390/cancers16081500	4,5*	140

Lp.	Opis bibliograficzny	IF	Punkty
8	**Król-Kulikowska Magdalena,** Abramenko Nikita, Jakubek Milan, Banasik Mirosław, Kepinska Marta: The role of angiotensin-converting enzyme (ACE) polymorphisms in the risk of development and treatment of diabetic nephropathy, Journal of Clinical Medicine, 2024, vol. 13, nr 4, art.995 [17 s.], DOI:10.3390/jcm13040995	3*	140
9	**Król-Kulikowska Magdalena,** Urbanowicz Iwona, Kepinska Marta: The concentrations of interleukin-6, insulin, and glucagon in the context of obesity and type 2 diabetes and single nucleotide polymorphisms in IL6 and INS genes, Journal of Obesity, 2024, vol. 2024, art.7529779 [13 s.], DOI:10.1155/2024/7529779	3,8*	100

' IF 2023

1.2 Publikacje w czasopiśmie bez IF

Lp.	Opis bibliograficzny	Punkty
1	Kosendiak Aureliusz, Król Magdalena, Ściskalska Milena, Kepinska Marta: The changes in stress coping, alcohol use, cigarette smoking and physical activity during COVID-19 related lockdown in medical students in Poland, International Journal of Environmental Research and Public Health, 2022, vol. 9, nr 1, art.302 [15 s.], DOI:10.3390/ijerph19010302	140

1.3 Publikacje w czasopiśmie - prace kontrybutorskie -

2. Abstrakty

Lp.	Opis bibliograficzny
1	Król Magdalena, Kepinska Marta, Lewandowski Łukasz, Milnerowicz Halina: The influence of insulin gene polymorphisms on its concentration in a selected group of people, W: 3rd Wroclaw Scientific Meetings. Wrocław, 1st-2nd March 2019, (red.) Julita Kulbacka, Nina Rembiałkowska, Joanna Weżgowiec, Wrocław 2019, Wydawnictwo Naukowe TYGIEL sp. z o.o., 110 poz.P56, ISBN 978-83-65932-64-8
2	Czyż A., Król Magdalena, Przybyszewski 0., Radajewska A.: Acute leukemia in people with trisomy 21 chromosome, W: 3rd Wrocław Scientific Meetings. Wrocław, 1st-2nd March 2019, (red.) Julita Kulbacka, Nina Rembiałkowska, Joanna Weżgowiec, Wrocław 2019, Wydawnictwo Naukowe TYGIEL sp. z o.o., 147 poz.P93, ISBN 978-83-65932-64-8
3	Król Magdalena, Urbanowicz Iwona, Lewandowski Łukasz, Kepinska Marta, Milnerowicz Halina: Relationship between the concentration of IL-6, insulin activity, glycated haemoglobin in human blood and the development of type 2 diabetes and/or obesity, W: 4th International Wroclaw Scientific Meetings. Wrocław, 09-10 October 2020, (red.) Julita Kulbacka, Nina Rembiałkowska, Joanna Weżgowiec, Wrocław 2020, Wydawnictwo Naukowe TYGIEL sp. z o.o., 33-34 poz.01, ISBN 978-83-66489-37-0

Lp.	Opis bibliograficzny
4	Król Magdalena, Kepinska Marta: Porównanie metod oznaczania aktywności syntazy tlenku azotu w próbkach biologicznych, W: V Ogólnopolska Konferencja Naukowa "Współczesne zastosowanie metod analitycznych w farmacji i medycynie". [Online], 27 listopada 2020 r. Książka abstraktów 2020, s. 35-36
5	Król Magdalena, Urbanowicz Iwona, Kepinska Marta: Ocena genotypów w obrębie wybranych polimorfizmów genów INS i NOS3 u pacjentów z cukrzycą typu 2 z wykorzystaniem metody PCR-RFLP, W: VI Ogólnopolska Konferencja Naukowa "Współczesne zastosowanie metod analitycznych w farmacji i medycynie". Wrocław, 03 grudnia 2021 r. Książka abstraktów, Wrocław 2021, s. 18
6	Król Magdalena, Urbanowicz Iwona, Kepinska Marta: Wpływ polimorfizmu rs1800795 w genie interleukiny-6 (IL6) na ryzyko rozwoju otyłości i/lub cukrzycy typu 2, W: V Śląskie Farmaceutyczne Spotkanie Naukowe "Od nauki do pacjenta" - konferencja. Sosnowiec, 9 grudnia 2022, (red.) Grażyna Janikowska, Kazimiera Klementys 2022, Polskie Towarzystwo Farmaceutyczne, 103 poz.S.V.P_2, ISBN 978-83-64968-29-7
7	Kunachowicz Dominika, Król Magdalena, Ściskalska Milena, Kepinska Marta: Egzosomy - w poszukiwaniu odpowiedzi na kluczowe pytania biomedycyny, W: VII Ogólnopolska Konferencja Naukowa "Współczesne zastosowanie metod analitycznych w farmacji i medycynie". Wrocław, 15 grudnia 2022 roku. Książka abstraktów, Wrocław 2022, s. 10-11
8	Król Magdalena, Kunachowicz Dominika, Kepinska Marta: Analiza in silico wiązań enzymu konwertującego angiotensynę (ACE) z jego inhibitorami, W: VII Ogólnopolska Konferencja Naukowa "Współczesne zastosowanie metod analitycznych w farmacji i medycynie". Wrocław, 15 grudnia 2022 roku. Książka abstraktów, Wrocław 2022, s. 31
9	Klęczar Michalina, Klońska Aleksandra, **Król-Kulikowska Magdalena,** Kunachowicz Dominika, Kepinska Marta: The effect of the Mediterranean diet on insulin concentration, Advances in Clinical and Experimental Medicine, 2023, vol. 32, nr 3 spec., s. 76, [Sth International Wroclaw Scientific Meetings. Wrocław, Poland, October 19-21, 2023. Abstract book]
10	Klońska Aleksandra, Klęczar Michalina, **Król-Kulikowska Magdalena,** Kepinska Marta: Choosing the right diet in the prevention and treatment of cancer, Advances in Clinical and Experimental Medicine, 2023, vol. 32, nr 3 spec., s. 77, [Sth International Wroclaw Scientific Meetings. Wrocław, Poland, October 19-21, 2023. Abstract book]
11	**Król-Kulikowska Magdalena,** Banasik Miroslaw, Kepinska Marta: The role of angiotensin converting enzyme polymorphisms in the development of diabetic nephropathy, Advances in Clinical and Experimental Medicine, 2023, vol. 32, nr 3 spec., s. 84, [Sth International Wroclaw Scientific Meetings. Wroctaw, Poland, October 19-21, 2023. Abstract book]
12	Klęczar Michalina, Klońska Aleksandra, **Król-Kulikowska Magdalena,** Kunachowicz Dominika, Kepinska Marta: Wpływ diety na stężenie kortyzolu we krwi, W: VIII Ogólnopolska Konferencja Naukowa "Współczesne zastosowanie metod analitycznych w farmacji i medycynie". Wrocław, 06 grudnia 2023 roku. Książka abstraktów, Wrocław 2023, s. 17

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