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Lower frequency of the 5/5 homozygous *CNDP1* genotype in South Asian Surinamese

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Abstract

We investigated the frequency of the 5/5 homozygous *CNDP1* (carnosinase) genotype, which was found to be associated with a reduced risk of developing diabetic nephropathy, in three ethnic groups in the Netherlands. Particularly interesting were the South Asian Surinamese, who have a high prevalence of diabetic nephropathy. Furthermore, we investigated the association between this gene and carnosinase activity in South Asian Surinamese and whether carnosinase was expressed in the kidney.

We genotyped 290 South Asian Surinamese, 532 African Surinamese, and 472 white Dutch in a cross-sectional population study. Furthermore, an independent cohort of South Asian Surinamese was genotyped. In this population, carnosinase activity was measured in serum. Immunostaining and in-situ hybridization for *CNDP1* were performed on kidney tissue.

Both South Asian populations had lower frequencies of the 5/5 homozygous genotype than African Surinamese and white Dutch (23.0%, 27.2%, 38.2%, and 41.3%, respectively; chi-square, $p<0.001$). This genotype showed a lower carnosinase activity in South Asian Surinamese (Wilcoxon rank-sum, $p=0.03$). *CNDP1* was expressed in the kidney.

South Asian Surinamese have a lower frequency of the 5/5 homozygous genotype, which was associated with lower carnosinase activity. Our study provides an indication that South Asian Surinamese are genetically at risk for developing diabetic nephropathy.

Introduction

Diabetic nephropathy (DN) is the leading cause of end stage renal disease. Data from the UK prospective Diabetes Study group demonstrated that approximately 25% of patients with type 2 diabetes develop microalbuminuria or progressive DN within 10 years [1].

Several studies show a higher incidence of DN in certain families and races [2-4]. For example, the South Asian Surinamese, an ethnic minority who migrated from Suriname to the Netherlands but originally descended from India and Bangladesh, show an 8-times higher frequency of diabetes type 2 and a 3-fold higher risk of developing DN, a 40-fold higher risk of developing end-stage DN due to type 2 diabetes and a 1.5-fold faster disease progression than white Dutch [5,6]. The question arises whether these differences may in part be due to increased susceptibility because of a genetic predisposition.

As DN is a multi-genetic disease several susceptibility loci and candidate genes have been found (reviewed in [7]). One of the susceptibility loci for DN was identified by Vardarli *et al.* [4], who evaluated 18 Turkish families with type 2 diabetes and nephropathy in a family-based linkage study. A major susceptibility locus was found (LOD score 6.1) on chromosome 18q22.3-q23 for DN. This finding was confirmed by sib pair analysis on African Americans [8] and Pima Indians [4]. In a search for candidate genes within the quantitative trait locus (QTL) on chromosome 18q, Janssen *et al.* detected significant evidence for association between DN and *CNDP1*, which encodes for carnosine dipeptidase (also called serum carnosinase), in patients from Germany, the Netherlands, Qatar, and the Czech Republic [9]. Freedman *et al.* confirmed this finding in European Americans [10], but found a different polymorphism of the *CNDP1* gene in African Americans [11]. Interestingly, the association between DN and *CNDP1* could not be confirmed in patients with DN due to diabetes type 1 [12].Diabetes type 2 patients, who are homozygous for 5 copies of a trinucleotide repeat encoding for leucine in the leader peptide on exon 2, demonstrated a 2.56-fold reduced risk for DN compared to individuals with more leucine repeats (6-8 repeats) [9]. The presence of more than 5 leucine repeats has been shown to lead to higher serum carnosinase secretion [13] and higher carnosinase activity [9]. Serum carnosinase, a dipeptidase belonging to the M20-metalloprotease family, is the rate-limiting enzyme for hydrolysis of carnosine into beta-alanine and L-histidine. Carnosine is a reactive oxygen scavenger [14], exhibits

anti-AGE (advanced glycation end products) effects [15], a natural ACE (angiotensin converting enzyme) inhibitor [16] and reduces the synthesis of matrix components and TGF-β (transforming growth factor) in renal cell lines [9].

Our hypothesis is that the frequency of the leucine repeat in the *CNDP1* polymorphism differs with ethnic background and that South Asian Surinamese have an aberrant genotypic distribution of *CNDP1*, which could partly account for the reported higher risk for DN after the development of type 2 diabetes. Therefore, the primary objective was to investigate the frequency of the protective genotype, the 5/5 homozygous genotype, in South Asian Surinamese, African Surinamese (immigrants from Suriname who originally descended from Africa), and white Dutch in the Netherlands. The correlation between high carnosinase activity and more leucine repeats in the *CNDP1* gene has been found in whites, and the secondary objective of this study was to investigate whether the carnosinase activity was also correlated with the *CNDP1* genotype in South Asian Surinamese. Furthermore, to investigate the potential involvement in diabetic nephropathy we also determined whether carnosinase was expressed in the kidney.

Subjects, Materials and Methods

For this study, we analysed data from SUNSET (SUrinamese in the Netherlands: Study on Ethnicity and health) [17] and Hindinef (Hindustani Diabetic Nephropathy Study) [5]. Both studies have been approved by the institutional medical ethics committee in accordance with the Declaration of Helsinki.

SUNSET - SUNSET is a population-based, cross-sectional survey designed to obtain insight into the cardiovascular risk profile of three ethnic groups: South Asian Surinamese ('Hindustani', of South Asian origin), African Surinamese ('Creole' of African origin) and white Dutch [17,18]. Details of the recruitment, data collection and definitions of ethnicity are described elsewhere [19]. In brief, between 2001 and 2003 a random sample of non-institutionalised adults aged 35-60 years was selected from the Municipal Register of Amsterdam, the Netherlands. After informed consent, participants underwent an interview, followed by a physical examination, including donation of a fasting blood sample [19]. Ethnicity was subsequently determined based on self-identification.

Subjects who, did not participate in the interview and physical examination, who could not unequivocally be assigned to one of the three ethnic groups or who could not

be genotyped were excluded from the present study, leaving a total of 1294 persons for our present analysis. The 1294 that were genotyped consisted of 290 South Asian Surinamese, 532 African Surinamese and 472 white Dutch.

Hindinef - The Hindinef study is conducted in the Netherlands in the city of The Hague. The study was initially set up to investigate whether familial clustering of DN occurred in the South Asian Surinamese population. No evidence for familial aggregation of DN was found, and more South Asian Surinamese were collected but not yet described in this cohort.

The basic characteristics of the total cohort will be described in the results. The recruitment of the Hindinef study group were previously published [18].

Genotyping

DNA material from both SUNSET and Hindinef was genotyped as follows. A standard PCR protocol was used with primers 5-FAM-GCGGGGAGGGTGAGGAGAAC (forward) and GGTAACAGACCTTCTTGAGGAATTTGG (reverse). The denaturing, annealing and extension temperatures were 94°C, 60°C and 72°C, respectively. Fragment analysis was performed on the ABI3130 analyser (Perkin Elmer) to determine the fragment length corresponding with the different genotypes. Each peak corresponded with the number of leucine repeats on each allele. A 157 base pair product corresponded with 5 CTG codons encoding for 5 leucine repeats, and a product of 160 base pairs corresponds to 6 CTG codons encoding 6 leucine repeats. Homozygotes demonstrated a single peak, and homozygosity for the 5 leucine repeats in exon 2 is referred to as the protective genotype, 5/5 homozygous genotype. Samples with six or more leucine repeats were referred to as not having 5/5 homozygous genotype.

Determination of serum-carnosinase activity

Serum samples were obtained from a random group of 391 South Asian Surinamese of the Hindinef cohort. Serum-carnosinase activity was determined according to the method described by Teufel *et al.* [20]. Briefly, the reaction was initiated by addition of substrate (L-carnosine) to a serum sample and stopped after 10 minutes of incubation at 37°C by adding 1% sulphate salicylic acid. The maximum increase was used for determining the maximum activity. Liberated histidine was derivatized with *o*-phtaldialdehyde (OPA). Fluorescence was measured by excitation at 360 nm and emission at 460 nm.

The intra- and interassay variations were respectively 6% and 16%. The lowest carnosinase activity detectable was 0.117 µmol/ml/h.

CNDP1 expression in the kidney

Cadaveric donor kidneys were obtained from Eurotransplant. These kidneys were unsuitable for transplantation for technical or morphological reasons. For immunohistochemistry, paraffin slides were used. In brief, the slides were washed in PBS and incubated for 2 h at room temperature with the primary antibody (rabbit anti-*CNDP1*, generated as described by Teufel et al[20], diluted in 1% bovine serum albumin in PBS). After washing with PBS, the slides were incubated for 30 min with horseradish peroxidase–conjugated anti-rabbit Envision (DAKO, Glostrup, Denmark). The slides were again washed with PBS, and the staining was developed with diaminobenzidine. The RNA in situ hybridization was performed as previously described [21]. In brief, paraffin sections were pre-treated and hybridized with digoxigenin (DIG) labelled RNA probes for 16 hours. RNA hybrids were detected using mouse anti-DIG (Sigma-Aldrich Chemie, Steinham, Germany), rabbit anti-mouse Ig (1:50, Dako, Glostrup, Denmark), and mouse alkaline phosphatase anti-alkaline phosphatase (APAAP, Dako).

Glomeruli were isolated as described earlier [22]. Whole kidney and glomerular RNA was isolated using Trizol®, converted to cDNA using AMV reverse transcriptase (Roche Applied Science). Real-time polymerase chain reaction [23] was used in combination with the following *CNDP1* primerset: forward: TTCAATCCGTCTAGTCCCTCACATG, reverse: TGCAATCCACGGGTGTAGTCC. Melting curve and sequence analysis were performed to confirm that we amplified the appropriate product.

Statistical Analysis

First, the characteristics of the SUNSET population and the frequency of 5/5 homozygous genotype and not having 5/5 homozygous genotype in both SUNSET and Hindinef were described per ethnic group. The difference in frequency of the 5/5 homozygous genotype between ethnic groups were assessed by means of the chi-squared test.

When the genotypes were divided in three groups with increasing number of leucine repeats, the statistical significance between the groups in carnosinase activity was determined by the Kruskal-Wallis test. The Wilcoxon sum-rank test was used to compare the carnosinase activity between the lowest number of leucine repeat group and the highest number of leucine repeat group and to compare the 5/5 homozygous genotype with the other genotypes.

A linear regression model was used to adjust for age and sex. The statistical analyses were all performed using SPSS 16.0 and Graphpad prism. A p-value < 0.05 was considered to be statistically significant.

Results

Characteristics of the SUNSET population and Hindinef population

The characteristics of the study populations are presented in Table 1. The white Dutch were slightly older than the South Asian Surinamese and African Surinamese and had the highest proportion of smokers. The African Surinamese had the highest proportion of female participants, as well as the highest median BMI (27.7 IQR 24.5-31.5) and a high frequency of high blood pressure (28.3%) compared to the other ethnic groups.

South Asian Surinamese had the highest frequency of diabetes (26.2%) and highest waist-hip ratio (0.96 IQR 0.90-1.00) compared to African Surinamese and white Dutch.

South Asian Surinamese of the Sunset and Hindinef study have a similar age and percentage male, BMI and waist-hip ratio, prevalence of diabetes and high blood pressure. Only in the Hindinef study group there were slightly less smokers.

Table 1. Characteristics of South Asian Surinamese, African Surinamese and white Dutch participants in SUNSET

	South Asian Surinamese		**African Surinamese**	**White Dutch**
	SUNSET	Hindinef		
	N=290	N = 456	N= 532	N= 472
Age (years)	44 (39-50)	42 (35-50)	43 (39-48)	48 (42-54)
Sex (male)	130 (44.8%)	196 (42.8%)	170 (32.0%)	233 (49.4%)
Smoking (current)	103 (35.9%)	103 (27%)	214 (40.9%)	212 (45.0%)
BMI (kg/m2)	26.6 (23.9-29.3)	26.2 (23.5-29.4)	27.7 (24.5-31.5)	25.5 (23.0-28.4)
Waist-hip ratio	0.96 (0.90-1.00)	0.94 (0.88-1.0)	0.90 (0.84-0.95)	0.90 (0.83-0.96)
High blood pressure (yes)	91 (31.5%)	133 (29.0%)	150 (28.3%)	83 (17.6%)
Diabetes Mellitus (yes)	76 (26.2%)	118 (25.8%)	68 (12.8%)	33 (7.0%)

N (%) or median (IQR)

Analysis of CNDP1 genotype frequency in South Asian Surinamese, African Surinamese and white Dutch

The *CNDP1* genotype distribution is demonstrated for each ethnic group in Table 2. The various genotypes were ranked according to the corresponding enzyme activities (Figure 2). The different genotypes are determined by fragment analysis. An example of a 5/5 homozygous genotype (A), a homozygous 6/6 genotype (B), a 5/6 heterozygous (C) and a 6/7 heterozygous (D) are seen in Figure 1. The frequency of 5/5 homozygous genotype in South Asian Surinamese (27.2%) was significantly lower than the frequency among African Surinamese (38.2%) and white Dutch (41.3%) in the SUNSET population ($p < 0.001$, Table 1). No differences were observed in the frequency between men and women (data not shown). The frequency of 5/5 homozygous genotype among the South Asian Surinamese with familial susceptibility for diabetes, the Hindinef population, was 23.0 % (105/456), which was similar to the frequency among the South Asian Surinamese in SUNSET, and significantly lower than the frequency among the African Surinamese and White Dutch in SUNSET (Table 2).

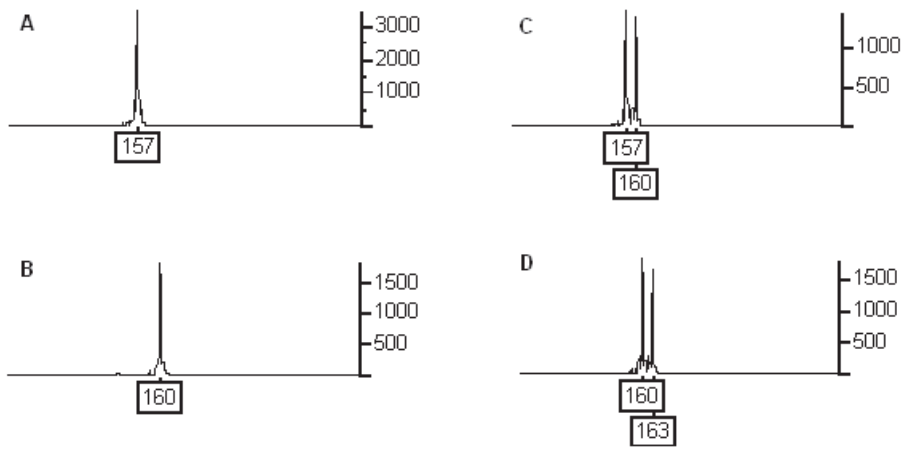

Figure 1. Measurement of the leucine repeat in exon 2 of the *CNDP1* gene

A = a fragment of 157 basepairs which corresponds to 5 leucine repeats in each allele (5/5 homozygous genotype), B = a fragment of 160 basepairs which corresponds to 6 leucine repeats in each allele, C = a fragment of 157 and 160 corresponding with 5 leucine repeats in one allele and 6 leucine repeats in the other, D = a fragment of 160 and 163 corresponding with 6 leucine repeats in one allele and 7 leucine repeats in the other.

Table 2. Distribution of *CNDP1* leucine repeats of South Asian Surinamese, African Surinamese and white Dutch participants

	South Asian Surinamese				**Creole Surinamese**		**White Dutch**	
	(SUNSET)		(Hindinef)					
	n=290	%	n = 456	%	n= 532	%	n= 472	%
4/4, 4/5, 4/6	3	1	0	0	18	3.4	2	0.4
5/5	79	27.2*	105	23.0*	203	38.2	195	41.3
5/6, 5/7	117	40.3	204	44.7	239	44.9	222	47
6/6, 6/7	91	31.4	147	32.2	71	13.3	51	10.8
7/7	0	0	0	0	1	0.2	2	0.4

* $p < 0.001$ chi-squared, comparing South Asian Surinamese with white Dutch and African Surinamese

Determination of serum carnosinase activity

The carnosinase activity was measured in 391 South Asian Surinamese of the Hindinef study. The genotypes were divided into three groups, the 5/5 homozygous genotype, the 5/6 and 5/7 heterozygous genotype, the 6/6 and 6/7 genotype. A clear correlation was found between carnosinase enzyme levels and the number of leucine repeats in the *CNDP1* gene (Figure 2). The carnosinase activity was significantly different between the three leucine repeat groups ($p = 0.03$). The 5/5 genotype differed significantly from the other genotypes ($p = 0.03$). The highest difference was found between the 6/6, 6/7 leucine repeat group and the 5/5 homozygous genotype ($p = 0.009$). The carnosinase activity did not seem to significantly differ with age and sex. When adjusted for age and sex in a linear regression model the association remained significant ($p = 0.04$). The apparent difference of significances is not due to age and sex variation but is a result of the different statistical tests.

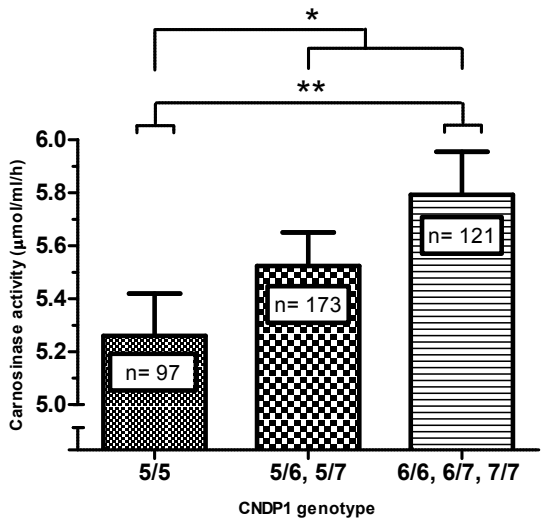

Figure 2. Mean serum-carnosinase activity in South Asian Surinamese categorised by amount of leucine repeats in both alleles.

Mean serum carnosinase activity with standard error of the mean divided in 3 genotype groups. '5-5' consists of 5-5 homozygous genotype, '5-6/5-7' consists of the genotype with 5 leucine repeats in one allele and 6 or 7 in the other allele, '6-6/6/7' consists of 6 leucine repeats in one allele and 6 or 7 in the other allele.

* < 0.05, ** < 0.01

CNDP1 expression in human kidney

Immunohistochemical staining and mRNA expression by in situ hybridization were performed on kidney tissue (Figure 3). Both protein and mRNA for *CNDP1* is expressed in glomerular cells and tubular epithelial cells. Real time PCR showed a specific expression of *CNDP1* in whole kidney, isolated glomeruli and mRNA.

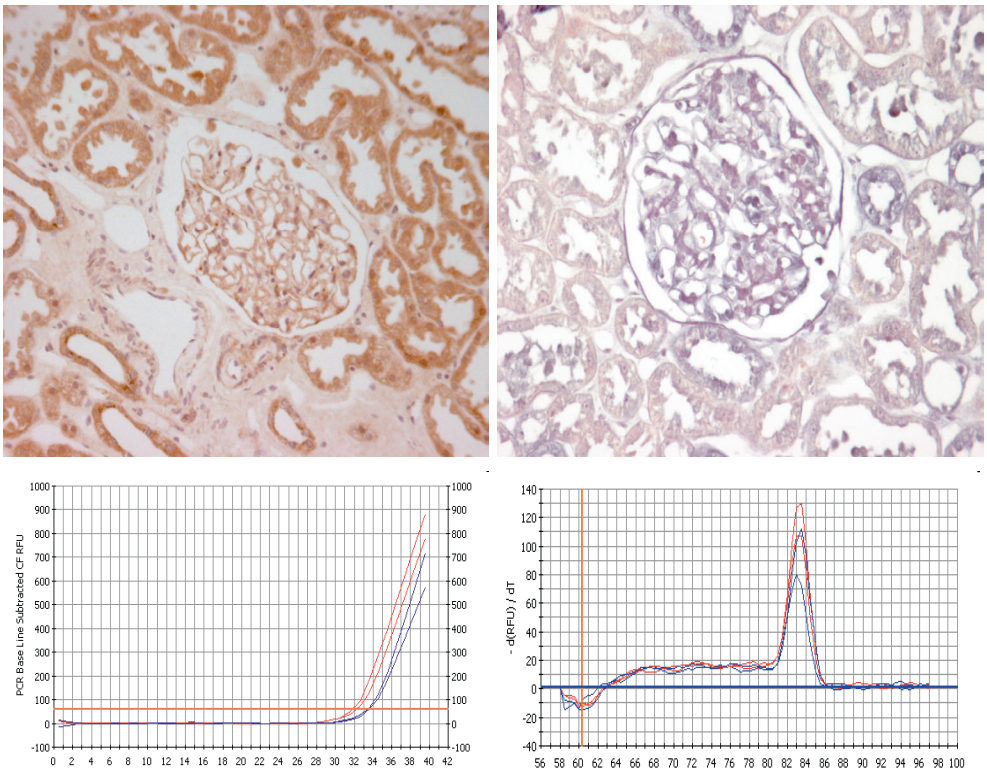

Figure 3. *CNDP1* expression in human kidney

Immunohistochemical staining (A) and mRNA expression by in situ hybridization (B) of *CNDP1* in normal kidney. Both protein and mRNA for *CNDP1* is expressed in glomerular cells and tubular epithelial cells. Real time PCR showed expression of *CNDP1* in both whole kidney (red lines) and isolated glomerular (blue lines) mRNA (C). Melting curve analysis showed specific amplification of *CNDP1* (D).

Discussion

This study reports the *CNDP1* polymorphism distribution in three ethnic groups in the Netherlands. Our study provides the first data on the frequency of 5/5 homozygous genotype among a population of South Asian origin. We found that the frequency of the protective genotype, the 5/5 homozygous genotype, was significantly lower in South Asian Surinamese compared to white Dutch and African Surinamese. The low frequency among South Asian Surinamese was confirmed in an independent South

Asian Surinamese cohort. Furthermore, we showed that, similar to Europeans, the carnosinase activity increases with the amount of leucine repeats among South Asian Surinamese and carnosinase was expressed in the kidney. This suggests a possible role in the development of DN in this population.

The white Dutch in our study show a frequency of the 5/5 homozygous genotype similar to that of healthy European control groups in previously published studies (41.3% in this study versus 36.8% in the study by Janssen *et al.* [9] and 38.6% in Freedman *et al.* [10].)

In African Americans, no association between reduced risk of DN and 5/5 homozygous genotype was found [10]. The frequency of 5/5 homozygous genotype among African Surinamese is similar to that of African Americans (38.2% and 35.9%, respectively). Other polymorphisms in the *CNDP1* gene have been found to play a role in the development of DN in African Americans [11]. These other polymorphisms in the *CNDP1* might play a role in African Surinamese as well, but were not included in this study.

The low frequency of the 5/5 homozygous genotype found in South Asian Surinamese observed in our study may be associated with the higher occurrence of DN in South Asian populations.

Janssen *et al.* found an association between the leucine repeat in the *CNDP1* gene and DN, and this finding was confirmed in a large study conducted by Freedman *et al.* in European American patients with DN and end-stage DN [9,10].

Functional studies provide evidence for the mechanism behind the association between 5/5 homozygous genotype and DN. The absence of 5/5 homozygous genotype has shown to lead to higher carnosinase levels and activity, thereby decreasing levels of carnosine [13]. Carnosine levels in blood are very low and vary during the day and therefore cannot be reliably ascertained (unpublished results). In our study we confirmed this increase in serum carnosinase activity with increasing number of leucine repeats in South Asian Surinamese. Carnosine has been shown to delay senescence in cultured human fibroblasts and temporarily reverse the senescence phenotype [24,25]. Moreover, several studies have demonstrated a likely anti-aging effect in vivo [26-28], which may also be related to the mechanism underlying vascular damage observed in DN. In that respect, in vitro supplementation of blood and plasma with carnosine has been shown to have an anti-oxidant effect is a promising finding [14]. If further developed, the implications of this discovery are potentially large, suggesting that

carnosine supplementation might be a possible therapeutic modality in the future for South Asian Surinamese subjects.

Before reaching a final conclusion, limitations deserve to be discussed. Selective recruitment or participation could have affected the reported frequency. The Hindinef population was originally selected for detecting familial clustering of DN. However, the frequency of 5/5 homozygous genotype was similar to the frequency in the South Asian population in SUNSET. We are therefore confident that our conclusion that the frequency of the 5/5 homozygous genotype is lower in South Asian Surinamese than in other ethnic groups living in the Netherlands is justified.

This is a population-based study and therefore provides no direct relation between diabetic nephropathy and the *CNDP1* gene in South Asian Surinamese but an indirect relation. Still, other investigators have found a clear relation between the *CNDP1* gene and diabetic nephropathy in Europeans [9] and European Americans [10]. South Asian Surinamese have found to have a significantly higher risk of diabetes type II and diabetic nephropathy [5,18]. The results of this study strongly suggest that the *CNDP1* polymorphism, given its distribution across ethnic groups, contribute to the higher risk of nephropathy in South Asian Surinamese.

Furthermore, DN is a multi-genetic disease and therefore it does seem unlikely that this gene is the only reason for the higher risk of developing DN of South Asian Surinamese. However, it might be one of the genes involved in the pathogenesis of DN in this ethnic minority in the Netherlands.

On the other hand, a powerful feature of this paper is that it presents data from a population-based survey among three ethnic groups in the same city. Selection due to clinic location and setting therefore did not occur. Another strong feature is that the findings from the SUNSET study could be confirmed in a second, independent study that was carried out in a different location (Hindinef). The similarity of the findings in both studies increase the power of the conclusions drawn concerning the frequency of the 5/5 homozygous genotype among the South Asian Surinamese.

In conclusion, South Asian Surinamese living in the Netherlands have a lower frequency of the protective genotype, 5/5 homozygous genotype, associated with a lower carnosinase activity. This polymorphism has proven to be protective for DN in Europeans and European-Americans and we showed that carnosinase was expressed in the kidney.

The specific role of 5/5 homozygous genotype in the occurrence of DN among South Asian origin populations remains to be further investigated, as carnosines or carnosine enhancers have the potential to become a therapeutic modality with great clinical relevance.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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