OSPEDALE Casa Sollievo della Sofferenza ISTITUTO DI RCOVERO E CURA A CARATTERE SCIENTIFICO	Data 16.9.2025

RICHIESTA ACQUISIZIONE MATERIALE DI CONSUMO
per acquisti da effettuarsi su fondi di ricerca scientifica

Dati Generali	
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Unità di riferimento	LRDE
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Numero identificativo RDA	41044
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OSPEDALE Casa Sollievo della Sofferenza ISTITUTO DI RCOVERO E CURA A CARATTERE SCIENTIFICO	Data 16.9.2025

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Indicare le alternative di fornitura secondo le seguenti indicazioni: Eventuale presenza di ditta produttrice esclusiva (compilare anche dichiarazione di unicità)

Quantità	Descrizione	Fornitore	Codice Prodotto Fornitore
Nr.1	CUSTOM KIT TRYPTOPHAN PERSONALIZE SIAL	SIAL	SIAL-CTP
Nr.10	100 X HUMAN Kynurenine CUSTOM KIT	SIAL	SIAL-EX1.2

Il Co-PI e RUP del Progetto
Dottoressa Claudia Menzaghi

Dottssa Claudia Menzaghi
CM 85561
Endocrinologia

OSPEDALE Casa Sollievo della Sofferenza
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OPERA DI SAN PIO DA PIETRELCINA
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DICHIARAZIONE DI UNICITA'

PER PROGETTI FINANZIATI DAI FONDI SIE-FSC-FESR E DA ENTI NAZIONALI ED INTERNAZIONALI

San Giovanni Rotondo, 16/09/2025

Al Responsabile Amministrativo
Dott. Michele Giuliani

DICHIARAZIONE DI UNICITA'
(resa ai sensi del D.P.R. n. 445/2000)

La sottoscritta Claudia Menzaghi, C.F. MNZCLD60S46A825N, in qualità di Co-PI e RUP del progetto:

- Titolo: "TRYPTOPHAN-RELATED METABOLIC PATHWAYS AND MORTALITY RATE IN PEOPLE WITH DIABETES. A COMPREHENSIVE APPROACH BASED ON OMICS AND CELL BIOLOGY STUDIES"
- Codice Identificativo Progetto: PNRR-MAD-2022-12375970
- Codice Commessa: RPNRR22DE01
- CUP: I23C22000720007

con riferimento alla richiesta di acquisto n. 41044 del 16.9.2025, relativa alla fornitura dei seguenti articoli:

- n. 1, Cod. SIAL-CTP-KIT-CUSTOM KIT TRYPTOPHAN PERSONALIZE SIAL

n. 10, Cod. SIAL-EX1.2-100X HUMAN Kynurenine CUSTOM KIT

- SIAL

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San Giovanni Rotondo, 16/09/2025

ipotesi di falsità in atti e dichiarazioni mendaci ivi indicate in merito alla presente dichiarazione.

Il Co-PI e RUP del Progetto
Timbro e Firma

Dott.ssa Claudia Menzaghi
CM 85561
Endocrinologia

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- Protocollo di ricerca – in stralcio - in cui viene citato il prodotto dichiarato infungibile;
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- Stralcio pubblicazioni scientifiche: J Clin Endocrinol Metab. 2025 Apr 22;110(5):e1323-e1333 e J Clin Endocrinol Metab. 2025 Apr 22;110(5):e1451-e1457

Protocollo di ricerca –	**CUSTOM KIT TRYPTOPHAN PERSONALIZE SIAL** **HUMAN Kynurenine CUSTOM KIT**
Description	Two enzyme immunoassays (ELISA) allowing the quantitative determination of L-Kynurenine to L-Tryptophan ratio in urine, plasma and serum samples
Format	2x 96-well plates
Samples	Plasma, serum, Cell culture supernatant, Urine
Minimal sample volume	20µL
Reactivity	Reacts with all species
Standard range	KYN: 0/100 – 10 000 ng/mL TRP: 2.5 – 250 µg/mL
Sensitivity	LoD KYN: < 47.5 ng/mL LoD TRP: < 1.2 µg/mL
Specificity	No significant cross-reactivity was observed with L-Tryptophan and L-Kynurenine analogs. See product pages for L-Kynurenine ELISA and L-Tryptophan ELISA
Assay time	Sample preparation (90min- 2h15min), Serum incubation overnight, revelation and read steps (1h)

A chi di interesse,

Roma, 6 settembre 2024

OGGETTO: Dichiarazione di distribuzione ESCLUSIVA – linea yourSIAL

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COMMENT

Genetic Variability in Insulin Action Inhibitor Ikkβ (*IKBKB*) Does Not Play a Major Role in the Development of Type 2 Diabetes

CLAUDIA MENZAGHI, NATTACHET PLENGVIDHYA, XIAOWEI MA, JAMES H. WARRAM, STEVEN E. SHOELSON, AND ALESSANDRO DORIA

Research Division, Joslin Diabetes Center, and Department of Medicine, Harvard Medical School, Boston, Massachusetts 02215

Recent evidence indicates that IκB kinase β (Ikkβ) may be a mediator of acquired forms of insulin-resistance. In this study, we examined whether genetic variability at the Ikkβ locus (*IKBKB*) contributes to the development of genetic forms of early-onset type 2 diabetes transmitted with an autosomal dominant mode of inheritance. Linkage with four markers flanking the *IKBKB* gene was evaluated in 32 multigenerational families. Included in the study were 233 diabetic (mean age at Dx = 37 ± 18) and 152 nondiabetic subjects. The overall LOD scores were negative (−54.9 and −46.2 on the centromeric and telomeric sides, respectively) indicating that variability in *IKBKB* was not a major determinant of diabetes in these families. Positive values, however, were observed for selected pedigrees. All 17 families for which linkage with the *IKBKB* locus could not be excluded were screened for sequence differences in the 22 exons and 1.6 kb of the 5′ flanking region by dideoxyfingerprinting or direct sequencing. Polymorphisms were identified in the 5′ flanking region (−1775del/insC and −1547T > A), exon 11 (c.1083A > G, L361L) and in intron 12 (IVS12+14t > a). However, no mutations segregating with diabetes could be found in these families. Furthermore, all four polymorphisms had similar allele frequencies in the 32 family probands, 171 individuals with common, later-onset type 2 diabetes, and 182 nondiabetic controls. We conclude that sequence differences in the *IKBKB* gene do not play a major role in either early-onset, autosomal dominant type 2 diabetes, or common forms with a later-onset. (*J Clin Endocrinol Metab* 87: 1894–1897, 2002)

GENETIC FACTORS ARE important determinants of insulin resistance and type 2 diabetes, but the genes that are involved remain mostly unknown (1). Until recently, IκB kinase β (Ikkβ) was known primarily for its role in phosphorylating IκB and activating NF-κB in response to stimuli related to inflammation and cell survival (2). New evidence, however, points to Ikkβ as an important mediator of insulin resistance. Inhibition of Ikkβ with salicylates or through targeted gene disruption causes a dramatic improvement of insulin sensitivity in animal models of insulin resistance such as ob/ob mice and obese Zucker fatty rats (3, 4). Reports in the literature of blood glucose lowering after high dose aspirin in type 2 diabetic subjects strongly suggest that Ikkβ acts as a crucial regulator of insulin-sensitivity also in humans (5). The specific mechanisms underlying this Ikkβ action are unclear at this time, but an increased serine/threonine phosphorylation of IRS1 appears to be involved. Because of this inhibitory effect on insulin action, Ikkβ mutations/polymorphisms determining gain of function may contribute to genetic forms of insulin-resistance and type 2 diabetes. In the present study, we examined whether genetic variability at the Ikkβ locus (*IKBKB*) is involved in the etiology of early-onset, autosomal dominant type 2 diabetes—a subtype of diabetes that is more strongly determined by genetic factors than common type 2 diabetes but is also characterized by the presence of insulin resistance (6).

Abbreviations: ddF, Dideoxyfingerprinting; Ikkβ, IκB kinase β; SNP, single nucleotide polymorphism.

Subjects and Methods

Families

Thirty-two families with early-onset, autosomal dominant type 2 diabetes were included in this study. The ascertainment of these families has been previously described (6). Diabetes was diagnosed: 1) if an individual was treated with insulin or oral agents, 2) if results of an oral glucose tolerance test met World Health Organization criteria, or 3) if the level of HbA1c was >7.0% in individuals who declined an oral glucose tolerance test or were not fasting when examined. Families were of Caucasian origin with the exception of four Hispanic and two African-American pedigrees. Included were 233 family members with diabetes, impaired glucose tolerance or previous gestational diabetes mellitus and 152 nonaffected members. All families were negative for mutations in the *HNF-4α*, *HNF-1α*, *IPF1*, and *NEUROD*1 genes. Analysis with markers flanking the glucokinase locus also excluded linkage with diabetes (Doria, A., unpublished results). The clinical characteristics of diabetes in 29 of these families have been previously reported in detail (6). This form of diabetes is on average diagnosed at an older age than maturity-onset of diabetes of the young, and is frequently associated with obesity and insulin resistance (6). The study protocol and informed consent procedures were approved by the Human Subjects Committee of the Joslin Diabetes Center.

Radiation hybrid mapping

The *IKBKB* gene was mapped by screening the Stanford G3 Radiation Hybrid panel (Research Genetics, Inc., Huntsville, AL) with an STS derived from BAC RPCI-11–384-C-8 in the GenBank GSS division (entry

no. AQ533730), one end of which was found to contain bp 1522–1722 of the *IKBKB* cDNA. Screening was performed by standard PCR using primers 5′-TTTGGGATCAGTG AGTGTGC-3′ and 5′-CACCCCCTTG-GTAAGTTCC3-3′. Scores were submitted to the Stanford on line RH Server for a two-point statistical analysis with markers in the version 2.0 SHGC G3 map.

Linkage studies

Marker genotypes were determined by ^{32}P-labeled PCR followed by denaturing PAGE and autoradiography. Multipoint parametric and nonparametric analyses were performed using GENEHUNTER (version 1.2.1) (7). The parametric analysis assumed an autosomal dominant mode of inheritance with a disease allele frequency of 0.001 and four age-related liability classes, as previously described (8).

Exon-intron boundaries

A BAC clone (RPCI-11-384-C-8) containing the portion of the *IKBKB* gene 3′ to bp 1522 was identified through a BLAST search of the GSS division of GenBank. Another BAC clone overlapping with RPCI-11-384-C-8 and covering the 5′ half of the gene was isolated from the CITB human genomic BAC library (Research Genetics, Inc., Huntsville, AL) by PCR-based screening using the same primers that were used for RH mapping. Exon-intron boundaries were defined by directly sequencing these two BAC clones using the Sequiterm EXCEL II DNA sequencing kit (Epicentre, Madison, WI) with ^{32}P-deoxy-ATP.

Mutation screening

The coding sequence of *IKBKB* was screened for sequence differences by dideoxyfingerprinting (ddF) (9), the promoter region by direct sequencing. DNA fragments (170–600 bp) covering the exons, exon-intron boundaries, and 1,600 bp of 5′ flanking region were amplified by PCR from the DNA of one affected member from each family for which linkage could not be excluded (LOD score > -2.0). The primers and annealing temperatures are available from the authors. PCR products were purified and subjected to Sanger's dideoxy chain termination reaction using dideoxy-GTP in a 10-μl reaction as described by Sarkar *et al.* (9). To increase the sensitivity, reactions were performed twice, with the forward and the reverse primer. After adding 20 μl of stop/denaturing solution (7 M urea, 50% formamide, bromophenol blue, and xylene cyanol) and heating the samples at 95 C for 5 min, 4 μl were electrophoresed overnight in a nondenaturing 0.75× mutation detection enhancement gel in 0.5× Tris-borate EDTA on a sequencing apparatus at a constant power of 6 W at room temperature. Dried gels were autoradiographed overnight. Samples with changes in band patterns were sequenced by the Big-Dye Terminator Cycle Sequencing Kit version 2.0 (PE Applied Biosystems, Foster City, CA) using an ABI 310 Genetic Analyzer. DNA fragments from the promoter region were directly sequenced without prior ddF screening. Allele frequencies were determined in the 32 original family probands, 171 patients with type 2 diabetes, and 182 nondiabetic controls by PCR, dot-blotting and allele specific hybridization. The −1775del/insC polymorphism was genotyped by fragment length analysis on an ABI 310 Genetic Analyzer. Subjects with type 2 diabetes were randomly selected from a sample of Joslin Clinic patients aged 40–64 yr who met the following criteria: 1) having diabetes that was diagnosed after age 35; and 2) being treated with diet or oral agents for at least two years after diagnosis of diabetes. Nondiabetic controls consisted of nondiabetic spouses of family members. All these subjects were of Caucasian origin.

Results

To screen the *IKBKB* locus for major gene effects, we examined its linkage with diabetes in 32 families with a transmission of early-onset type 2 diabetes consistent with an autosomal dominant mode of inheritance. Consistent with its previous mapping by FISH (10), the *IKBKB* gene was mapped by radiation hybrid screening to chromosome 8p at 7 centirays (about 200 kb) from marker SHGC-9715. The LOD score for this location was 17.1. The closest polymorphic marker on the version 2.0 SHGC G3 map was identified as Genethon marker AFM156xa3 (*D8S268*) located 18 centirays (about 500 kb) centromeric to SHGC-9715. Based on this mapping information, four markers (*D8S1821, D8S532, D8S1460,* and *D8S538*) spanning about 5 centimorgans in this region were chosen for linkage analysis. Strong evidence against linkage was observed at each marker position. Results are reported in Table 1 for the two markers flanking the gene on the centromeric and telomeric sides (*D8S532* and *D8S1460*). In a parametric analysis using GENEHUNTER, both markers produced strongly negative total LOD scores (-54.9 and -46.2 for *D8S532* and *D8S1460*, respectively). Results were similarly negative when the six non-Caucasian families where considered separately. A multipoint LOD score smaller than -2.0, excluding linkage, was observed at either marker position in 15 families. Of the remaining pedigrees, four had a positive LOD score, reaching in one case (family 29) a value of 1.6. All seven affected members of this family shared the same *D8S532-D8S1460* haplotype, which was present in only one of the five nondiabetic members who were examined. However, the evidence of linkage heterogeneity was not significant, with a maximum heterogeneity LOD score of $+0.05$. Nonparametric analysis, which considers only affected individuals, also failed to detect significant evidence of linkage at either marker position. The smallest *P* value (0.02) was observed in family 29.

All 17 families for which linkage with the *IKBKB* locus could not be excluded were screened for sequence differences in the *IKBKB* coding sequence and promoter. The *IKBKB* coding sequence consists of 2271 bp. After isolating and sequencing two BAC clones (RPCI-11-384-C-8 and CITB 137-M-4) containing the entire cDNA, the gene was found to include 22 exons ranging in size from 50–194 bp (Fig. 1). The gene organization and exon-intron boundaries were subsequently confirmed by the draft genome sequence published by the Human Genome Project (contig no. NT_017505). The sequence information was used to amplify all coding exons, exon-intron boundaries, and about 1,600 bp of the 5′ flanking region from one proband for each family. After screening these fragments by ddF and/or direct sequencing, we found a germline variant in the 5′ flanking region (−1775del/insC) in one family, but this did not segregate with diabetes. Polymorphisms were identified in the 5′ flanking region (−1547T > A), exon 11 (c.1083A > G, L361L), and intron 12 (IVS12+14t > a) (Fig. 1). Two candidate single nucleotide polymorphisms (SNPs) were reported in exon 19 in the public SNP database (SNP nos. 1057763 and 1057764), but these could not be confirmed when the exon was directly sequenced in the family probands. All polymorphisms had similar allele frequencies in the 32 family probands, 171 individuals with later-onset type 2 diabetes, and 182 nondiabetic controls, indicating that these sequence differences did not contribute to the common form of type 2 diabetes in this population (Table 2). Results were similarly negative when study subjects were stratified by body mass index or age at diagnosis, or when all polymorphisms were considered together as haplotypes (data not shown).

TABLE 1. Results of multipoint analysis for linkage between type 2 diabetes and *IKBKB* locus

Family	D8S532			D8S1460		
	LOD	Z_{all}	*P*	LOD	Z_{all}	*P*
1	**−1.59**	**+0.43**	**0.22**	**−1.63**	**+0.43**	**0.22**
2	−2.13	−0.10	0.29	−1.52	−0.07	0.26
3	**−1.60**	**−0.04**	**0.18**	**−1.69**	**−0.03**	**0.17**
4	−2.39	−0.24	0.49	−2.02	−0.23	0.46
5	**−1.73**	**−0.05**	**0.27**	**−1.94**	**−0.03**	**0.27**
6	−2.67	−0.70	0.92	−2.67	−0.70	0.92
7	−2.80	−0.48	0.77	−2.80	−0.48	0.77
8	**−1.88**	**−0.11**	**0.31**	**−1.61**	**−0.09**	**0.29**
9	−2.34	−0.45	0.80	−1.60	−0.29	0.53
10	−2.74	−0.37	0.75	−2.74	−0.37	0.76
11	−2.87	−0.41	0.65	−2.87	−0.41	0.65
12	**−1.57**	**−0.12**	**0.30**	**+0.57**	**+0.36**	**0.08**
13	−3.64	−0.39	0.79	−3.50	−0.38	0.79
14	**−1.69**	**+0.09**	**0.12**	**−1.69**	**+0.09**	**0.12**
15	**−0.25**	**+1.05**	**0.05**	**−0.25**	**+1.03**	**0.05**
16	**−0.78**	**−0.07**	**0.32**	**−0.39**	**−0.14**	**0.40**
17	−2.38	−0.53	0.77	−2.13	−0.49	0.69
18	−3.36	−0.37	0.89	−3.96	−0.36	0.88
19	−1.97	−0.27	0.54	−1.96	−0.27	0.53
20	−2.01	−0.24	0.48	−0.78	−0.04	0.26
21	−4.90	−0.17	0.43	−1.56	−0.14	0.34
22	−2.16	−0.27	0.52	−2.26	−0.27	0.53
23	**−0.41**	**+0.27**	**0.20**	**−0.40**	**+0.28**	**0.19**
24	**−1.21**	**−0.86**	**0.87**	**−0.90**	**−0.27**	**0.37**
25	**−0.56**	**−0.21**	**0.40**	**−0.31**	**+0.01**	**0.29**
26	**+0.27**	**+1.10**	**0.02**	**+0.26**	**+1.06**	**0.02**
27	**+1.16**	**+1.27**	**0.12**	**+1.13**	**+1.20**	**0.12**
28	**−0.12**	**−0.56**	**0.63**	**−0.10**	**−0.54**	**0.62**
29	**+1.65**	**+3.32**	**0.02**	**+1.64**	**+3.26**	**0.02**
30	**−1.57**	**+0.07**	**0.22**	**−1.65**	**−0.25**	**0.44**
31	**−0.61**	**−0.14**	**0.34**	**−0.78**	**−0.16**	**0.39**
32	−4.00	−0.16	0.84	−4.03	−0.16	0.82
Total	−54.9	+0.05	0.40	−46.2	+0.27	0.31

Families for which linkage could not be excluded in the parametric analysis are indicated in *bold*.

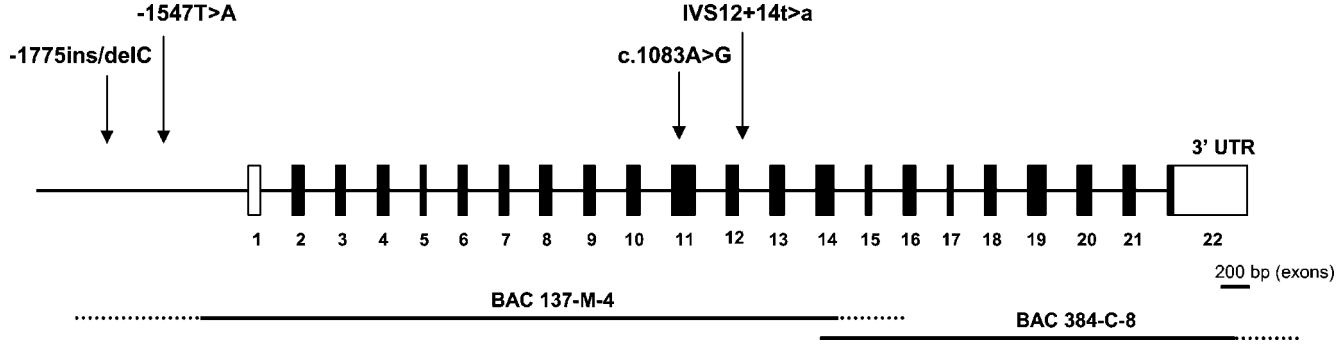

FIG. 1. Exon-intron structure and polymorphism location in the *IKBKB* gene. Exons are indicated as *black* (translated portions) and *white* (untranslated portions) *boxes*. BAC clones containing different portions of the gene are indicated below the exons. Introns are not in scale.

Discussion

Several studies have emphasized the role of naturally occurring inhibitors of insulin action such as TNF-α, α-HS glycoprotein, membrane glycoprotein PC-1, ras associated with diabetes, and resistin in the etiology of insulin-resistance and type 2 diabetes (11–15). An amino acid polymorphism in one of these molecules (PC-1 K121Q) has been recently found to be associated with insulin resistance, raising the hypothesis that sequence variants in natural inhibitors of insulin action may influence susceptibility to type 2 diabetes (16). Our results indicate that genetic variability in Ikkβ—another important mediator of insulin resistance—is unlikely to contribute to the development of early-onset, autosomal dominant type 2 diabetes. The similarity of allele frequencies in unrelated cases with later-onset diabetes and nondiabetic controls also argues against a major role in common type 2 diabetes. In fact, the *IKBKB* gene sequence appears to be remarkably well conserved among individuals. Based on a genome-wide average of one common (>5%) SNP every 450 bp (16), one would expect at least four or five sequence differences in the *IKBKB* cDNA. In contrast, we identified only one SNP, which did not affect the amino acid

TABLE 2. DNA polymorphisms in the *IKBKB* gene among Caucasian individuals

Location	Nucleotide position	Substitution	Amino acid change	Allele frequencies			*P* value
				C	DM	FP	
Promoter region	−1775	ins/delC		0.000	0.000	0.016	
Promoter region	−1547	T>A		0.060	0.048	0.031	0.56
Exon 11	+1083	A>G	L361L	0.017	0.009	0.016	0.62
Intron 12	+14	C>A		0.069	0.079	0.094	0.74

For polymorphisms in the promoter regions, nucleotide positions are relative to the A of the ATG (+1) in the genomic sequence. For the polymorphism in exon 11, the numbers refer to the position relative to the A of the ATG in the published cDNA sequence (AF080158). For the polymorphism in intron 12, the position is relative to the splice donor (+). C = 182 nondiabetic controls, DM = 171 type 2 diabetic patients, FP = 32 diabetic family probands.

sequence. The reasons for this low variability are unclear, but an important factor may be the negative selection of polymorphisms because of Ikkβ importance in regulating inflammation and apoptosis (2). It is possible that sequence differences were missed by our mutation screening, but this seems unlikely, especially for frequent polymorphisms. First, ddF is a robust mutation screening technique having a sensitivity of almost 100% if both forward and reverse primers are used for the screening as was done in our study (9). Second, the number of individuals who were screened (17 subjects, corresponding to 34 chromosomes) provides more than 90% power to detect alleles having frequencies as low as 0.05 (17). Third, no suggestive evidence of undetected polymorphisms could be found *in silico* by aligning cDNA sequences or expressed sequence tags from different sources.

Negative results for Ikkβ are not completely unexpected because efforts to link type 2 diabetes with genetic variation in other important mediators of insulin action (*e.g.* IRS-2) have similarly failed (1, 18, 19). At the same time, it must be emphasized that our efforts do not completely exclude the *IKBKB* locus as a gene for type 2 diabetes. Our screening was limited to the coding exons and to the 1.6 kb immediately 5′ of the transcription start site. It is still possible that sequence differences in other regulatory regions that are not in linkage disequilibrium with the polymorphisms found in this study may affect susceptibility to diabetes. Moreover, the recent findings with the *NIDDM2*/calpain 10 gene suggest that intronic SNPs might be important in regulating susceptibility to common disorders (20). One must also consider that Ikkβ is part of a complex signaling network (2), and therefore the site of variability, if any, may be in upstream or downstream proteins rather than Ikkβ itself. Efforts are in progress to identify proteins that activate or are activated by Ikkβ, so that the impact of genetic variability in this new insulin-resistance pathway can be fully investigated.

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Clinical Research Article

Inflammation and Prediction of Death in Type 2 Diabetes. Evidence of an Intertwined Link With Tryptophan Metabolism

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Abstract

Context: The role of inflammation in shaping death risk in diabetes is still unclear.

Objective: To study whether inflammation is associated with and helps predict mortality risk in patients with type 2 diabetes. To explore the intertwined link between inflammation and tryptophan metabolism on death risk.

Methods: There were 2 prospective cohorts: the aggregate Gargano Mortality Study (1731 individuals; 872 all-cause deaths) as the discovery sample, and the Foggia Mortality Study (490 individuals; 256 deaths) as validation sample. Twenty-seven inflammatory markers were measured. Causal mediation analysis and in vitro studies were carried out to explore the link between inflammatory markers and the kynurenine to tryptophan ratio (KTR) in shaping mortality risk.

Results: Using multivariable stepwise Cox regression analysis, interleukin (IL)-4, IL-6, IL-8, IL-13, RANTES, and interferon gamma–induced protein-10 (IP-10) were independently associated with death. An inflammation score (I score) comprising these 6 molecules is strongly associated with death in both the discovery and the validation cohorts HR (95% CI) 2.13 (1.91-2.37) and 2.20 (1.79-2.72), respectively. The I score improved discrimination and reclassification measures (all $P < .01$) of 2 mortality prediction models based on clinical variables. The causal mediation analysis showed that 28% of the KTR effect on mortality was mediated by IP-10. Studies in cultured endothelial cells showed that 5-methoxy-tryptophan, an anti-inflammatory metabolite derived from tryptophan, reduces the expression of IP-10, thus providing a functional basis for the observed causal mediation.

Conclusion: Adding the I score to clinical prediction models may help identify individuals who are at greater risk of death. Deeply addressing the intertwined relationship between low-grade inflammation and imbalanced tryptophan metabolism in shaping mortality risk may help discover new therapies targeting patients characterized by these abnormalities.

Key Words: inflammation risk score, IP-10, tryptophan pathway, prediction models, death risk

Abbreviations: 5-MTP, 5-methoxy-DL-tryptophan; aGMS, aggregate Gargano Mortality Study; BMI, body mass index; cNRI, category-free net reclassification improvement; CV, coefficient of variation; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; FMS, Foggia Mortality Study; HbA1c, glycated hemoglobin; hs-CRP, high-sensitivity C-reactive protein; IL, interleukin; IP-10, interferon gamma–induced protein 10; I score, inflammation score; KTR, kynurenine to tryptophan ratio; RANTES, Regulated on Activation, Normal T cell Expressed and Secreted; rIDI, relative integrated discrimination improvement.

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Type 2 diabetes is a leading cause of mortality (1, 2) and the source of a heavy personal and social load. As the prevalence of the disease is exploding (3), this burden will worsen and must therefore be faced with no hesitation.

The simultaneous and aggressive treatment of several risk factors reduces the rate of death in patients with type 2 diabetes (4). Attending diabetes clinics has also been reported to reduce the risk of mortality as a likely consequence of better and timely follow-up and treatment (5). Unfortunately, such an approach is expensive and laborious and therefore cannot be implemented in all individuals with diabetes. This makes it necessary to identify patients at high risk of death who would benefit most from optimal and multifaceted management.

The discovery of new disease markers is a way to improve the prediction of clinical events (6); furthermore, as an added value, the identification of novel markers can also reveal new pathogenic pathways that open the way to new therapies targeted at specific subgroups of patients (7).

Low-grade inflammation is known to shape the risk of total mortality in the general population (8) and in people with diabetes as well (9-12). Unfortunately, studies conducted on type 2 diabetes have investigated only a few markers (9-12), thus leaving the role of most inflammatory molecules unknown. Low-grade inflammation is a complex phenomenon that involves different paths that are often interconnected. We focused on some relevant paths, involving (1) adaptive immune cytokines related to T and or B lymphocytes; (2) chemokines that control cell migration on the inflammation site; and (3) growth factors, secreted after stimulation of cytokines and capable of regulating cell proliferation and differentiation. Accordingly, we investigated the role of several inflammatory molecules belonging to these 3 different paths (listed in Table S1, along with their principal sources and main functions (13)) on mortality in people with type 2 diabetes. Total mortality was used since the information on specific causes of death was not available. We then used the more robustly associated molecules with mortality to investigate whether a parsimonious index of their overall inflammatory activity improves prediction models of death in type 2 diabetes.

Given some previous evidence about the intertwined link between inflammation and tryptophan metabolism in several complex chronic diseases and survival probability (14-17), as a secondary aim, we also investigated this interdependence in relation to the risk of mortality in type 2 diabetes.

Materials and Methods

Participants

Two prospective cohorts of patients with type 2 diabetes (diagnosed according to American Diabetes Association 2003 criteria) (18) from Apulia, central-southern Italy, were investigated.

The aggregate Gargano Mortality Study—discovery sample

The aggregate Gargano Mortality Study (aGMS) consists of 2140 people with type 2 diabetes, recruited at the Endocrine Unit of IRCCS "Casa Sollievo della Sofferenza," San Giovanni Rotondo, according to the same design and procedures from 2000 to 2008 (first period) and from 2008 to 2011 (second period) and prospectively followed until February 2023.

Serum inflammatory biomarkers were assessed in 1731 participants (80.9%), constituting the eligible sample for this analysis. Any analysis using data from aGMS was adjusted for the "recruitment period" (ie, first period = 0, second period = 1).

The Foggia Mortality Study—replication sample

The Foggia Mortality Study (FMS) comprises 1115 patients recruited consecutively at the Endocrine Unit of the University of Foggia from 2002 to 2008 and prospectively followed until March 2023. Serum inflammatory markers were assessed in 490 participants (43.9%), fully representative of the entire cohort, whose serum samples were available for this analysis.

For both cohorts, the only inclusion criterion was the presence of type 2 diabetes according to American Diabetes Association 2003 criteria (18) and the only exclusion criterion was the presence of poor life expectancy for nondiabetes-related diseases. The vital status of all participants was verified by interrogating the Italian Health Card Database upon data anonymization (https://sistemats1.sanita.finanze.it/wps/portal/).

Both prospective studies and the relative informed consent procedures were approved by the local Institutional Ethic Committees of IRCCS "Casa Sollievo della Sofferenza" and University of Foggia, respectively. All participants gave written informed consent.

Measurement of Circulating Inflammatory Markers

All circulating biomarkers were collected at baseline and immediately stored at −80° until measurements. This procedure gave stable analyte concentrations (19, 20).

Circulating levels of 27 molecules were measured simultaneously in duplicate, using a multiplex detection 27-plex kit (Bio-Plex Pro Human Cytokine 27-plex assay, Bio-Rad Cat# M500KCAF0Y, RRID:AB_2893118) (Table S1 (13)). Three quality control samples (2 sex-mixed human serum samples from blood donors and 1 control serum provided by the manufacturer) were included in each plate. The median coefficient of variation (CV) was <15% for all analyzed markers, with the only exception of interferon-γ and vascular endothelial growth factor with CVs = 18.4% and 18.5%, respectively, and tumor necrosis factor-α = 24.2%. No values were out of the limit of detection. Data analyses were performed using Bio-Plex Manager software version 6.1 (Bio-Rad). Molecule concentrations were interpolated from an appropriate standard curve.

Tryptophan, kynurenine, and their ratio (KTR) were quantified using baseline fasting serum samples and the Absolute*IDQ* p180 Kit (BIOCRATES Life Sciences), as previously described (16, 17, 21). Sample handling was performed by a Hamilton Microlab STARTM robot (Hamilton Bonaduz AG, Bonaduz, Switzerland) and a Ultravap nitrogen evaporator (Porvair Sciences, Leatherhead, UK) and standard laboratory equipment. Mass spectrometric analyses were done on an API 4000 triple quadrupole system (SCIEX Deutschland GmbH, Darmstadt, Germany) equipped with a 1260 Series HPLC (Agilent Technologies Deutschland GmbH, Böblingen, Germany) and a HTC-xc PAL autosampler (CTC Analytics, Zwingen, Switzerland) controlled by the software Analyst 1.6.2. For the liquid chromatography, compounds were identified and quantified based on scheduled multiple reaction monitoring measurements, for the flow injection analysis part of multiple reaction monitoring measurements. Data evaluation for quantification of metabolite

concentrations and quality assessment was performed with the software MultiQuant 3.0.1 (Sciex) and the MetIDQ software package. Metabolite concentrations were calculated using internal standards and reported in μM.

Serum high-sensitivity C-reactive protein (hs-CRP) was measured in duplicate, using a Multiplex Detection 4-Plex kit (Bio-Plex Pro Human Acute Phase 4-Plex Panel (Bio-Rad Cat# 171A4C09M, RRID:AB_3391717), also containing serum amyloid P component, α2-macroglobulin, and haptoglobin. The median CV was 15% for all proteins. Data analyses were performed using Bio-Plex Manager software version 6.1 (Bio-Rad). Serum hs-CRP concentrations were interpolated from an appropriate standard curve (11).

Cell Culture

Human immortalized endothelial cells (TeloHAECs CRL-4052 from ATCC) were maintained at 37 °C and 5% CO_2 in Vascular Cell Basal Medium (ATCC PCS-100-030), supplemented with Vascular Endothelial Cell Growth Kit (ATCC PCS-100-041). After amplification, cells were seeded in 6-well plates (Sigma-Aldrich) at $\sim 5 \times 10^5$ cell per well. When 90% confluence was obtained, cell medium was changed with Vascular Cell Basal Medium plus 25 mM glucose for an additional 24 or 48 hours. In the last 12 hours of glucose incubation, 5-methoxy-DL-tryptophan (5-MTP) (Sigma-Aldrich) at a final concentration of 100 μM (or diluent) was added.

RNA Extraction, cDNA Synthesis, and Gene Expression Analysis

Total RNA was isolated from cells by using RNeasy Mini kit (Qiagen). cDNA was generated by reverse transcription with iScript Reverse Transcription Supermix for RT-qPCR (BioRad) and used as template for subsequent analyses.

Expression levels of target *CXCL10* (C-X-C motif chemokine ligand 10), which encodes for the chemokine interferon gamma–induced protein 10 (IP-10) and housekeeping (*NONO*, *PPIA*, and *RPL13A*) genes were assessed by means of a predesigned SYBR green assay (BioRad assay ID: qHsa CED0034161, qHsaCED0002050, qHsaCED0038620, qHsaCED0020417) on an ABI-PRISM7900 (Applied Biosystems). The above 3 housekeeping genes were chosen from a list of a total of 30 analyzed genes because they were showing the best control gene stability (ie, $M < 0.5$) across samples and different experimental conditions (22) (data not shown).

CXCL10 mRNA expression, normalized on the geometric mean of housekeeping genes, was calculated as the $2-\Delta\Delta CT$ ratio on the 5.5 mM glucose plus 19.5 mM mannitol-treated cells at 24 hours, and used as the reference.

Statistical Analysis

Patients' baseline characteristics were reported as mean ± SD and frequency and percentage for continuous and categorical variables, respectively. Because of skewed distribution and for comparability between different biomarkers, their concentrations were logarithmically transformed and then standardized (ie, Z score for log). All covariates with missing values <5% were imputed by the random forest method (23).

Correlations between biomarkers were assessed using the Pearson correlation coefficient.

In both studies, the time variable was defined as the time between the baseline examination and date of the event (ie, all-cause mortality) or, for subjects who did not experience the event, the date of the last available clinical follow-up. Incidence rate for all-cause mortality was expressed as the number of events per 100 person-years.

To assess the association between serum inflammatory markers levels and all-cause mortality in the discovery sample (ie, aGMS), Bonferroni adjustment for multiple comparisons was used to determine the significance threshold in a Cox proportional hazards model adjusted for the recruitment period (0 or 1). The proportional hazards assumptions were tested through Schoenfeld residuals for each analysis.

Inflammatory markers that were significantly associated with mortality after Bonferroni adjustment, were further evaluated in a fully adjusted model comprising age at recruitment, gender, smoking habit, body mass index (BMI), diabetes duration, glycated hemoglobin (HbA1c), estimated glomerular filtration rate (eGFR), ongoing treatments, and recruitment period. Furthermore, molecules survived at this last step entered jointly a Cox model using a forward–backward stepwise analysis in order to minimize potential multicollinearity issues. Finally, a weighted inflammation score (I score) using the markers as selected by the stepwise analysis was created. The I score was obtained from the sum of biomarker values, each weighted by the regression coefficient from the forward–backward stepwise Cox model (eg, [0.364 × Z score for log_IP10] + [−0.246 × Z score for log_IL-13] + [0.244 × Z score for log_IL-6] + [0.264 × Z score for log_RANTES] + [−0.102 × Z score for log_IL-4] + [0.089 × Z score for log_IL-8]) divided by its SD and finally expressed per 1 SD increase. In addition, via recursive partitioning for survival trees (24), we investigated exploratory ranges of I scores which identify subgroups of patients at different risk of death.

As possible modifiers of the effect of the I score on mortality rate, age at recruitment, gender, smoking habits, BMI, diabetes duration, HbA1c, and eGFR were investigated. These variables were treated as continuous and also, to help clinical interpretation, as categorical. Cox proportional hazards analyses stratified according to the variables mentioned above along with the presence of multiplicative interaction terms were used.

To examine whether the validated I score in the 2 study samples pooled together increases the accuracy of both 6- and 10-year all-cause mortality prediction in type 2 diabetes, 2 different well-established models were utilized: ENFORCE (25, 26) and RECODe (27, 28), respectively. To pursue this aim, 346 and 592 events were available at 6 and 10 years of follow-up, respectively. Discrimination was measured by survival *c* statistics (29) while improvement in discrimination was assessed by delta *c* statistics and the survival version of the relative integrated discrimination improvement (rIDI) (30). In addition, the survival version of the category-free net reclassification improvement (cNRI), which examines whether the predicted probabilities of individuals with and without events move in the right direction (upward and downward, respectively) from the base to the new model, was evaluated (31). The 95% CIs for discrimination and reclassification measures were computed by bootstrap.

To test the possible intertwined link between inflammation and tryptophan metabolism, a causal mediation analysis was performed (32). Firstly, we checked if the kynurenine to tryptophan ratio (KTR) was associated with all-cause mortality

(using a fully adjusted Weibull regression model) and with the 6 biomarkers firmly associated with all-cause mortality (using a fully adjusted linear regression model). Then, a causal mediation analysis was conducted using the "mediate" function defined in the "mediation" package in R. This function provided non-parametric estimates of the total, direct (ie, directly attributable to the exposure), and indirect effects, based on 1000 bootstrap replications, along with bias-corrected and accelerated 95% CIs. The percentage of mediated effect is defined as the ratio of the indirect to the total effect × 100, and its 2-sided empirical *P* value was defined as twice the number of times the percentages were negative out of the total number of bootstrap replications. Previous data make it possible to hypothesize a bidirectional link between inflammation and tryptophan metabolism in shaping the risk of cardiovascular disease (CVD) and survival probability. We then investigated 2 separate causal mediation models. One model included each cytokine as a mediator in the relationship between KTR and all-cause mortality, while the other included KTR as a mediator in the relationship between each cytokine and all-cause mortality.

$P < .05$ was considered to be statistically significant. All analyses were performed using SAS 9.4 (SAS Institute, Cary, NC) and R software (R Core Team, 2021).

Results

Clinical features of patients from the aGMS and the FMS are summarized in Table 1. In the aGMS (left), 842 deaths occurred during a 13.1-year mean follow-up (22 676 person-years), while in the FMS (right) 256 deaths were observed during a mean follow-up of 12 years (5880 person-years).

Association Between Inflammatory Markers and all-Cause Mortality

In the aGMS, 17 of the 27 markers analyzed were significantly associated with all-cause mortality, having a *P* value below the threshold derived from the Bonferroni correction ($.05/27 = 1.85 \times 10^{-3}$) (Table 2, left). Eleven of these molecules, all with a CV <15%, remained independently associated with all-cause mortality in a fully adjusted model including age at recruitment, gender, smoking habit, BMI, diabetes duration, HbA1c, eGFR, all ongoing treatments, and recruitment period (8 with increased and 3 with decreased risk; Table 2, right). The 11 independently associated markers showed a pairwise correlation ranging from −0.20 to 0.92 (Fig. S1 (13)) with 6 of them surviving a further stepwise (forward–backward) procedure (Fig. 1A). The I score, created by summing the standardized serum values of these 6 molecules (see Materials and Methods) was strongly and independently associated with all-cause mortality in the same fully adjusted model as above (Fig. 1A and Table 3).

Table 1. Clinical features of the 2 independent study cohorts

	aGMS, n = 1731	FMS, n = 490
Women (n) (%)	779 (45.0)	244 (49.8)
Age at recruitment (yrs)	62.5 ± 9.3	62.8 ± 11.6
Smoking habit (n) (%)	246 (14.2)	77 (15.7)
Diabetes duration (yrs)	11.6 ± 9.1	12.7 ± 9.7
BMI (kg/m^2)	31.0 ± 5.7	30.1 ± 5.7
HbA_{1c} (%)	8.4 ± 1.9	9.0 ± 2.1
eGFR (mL/minute/1.73 m^2)	80.2 ± 19.6	80.4 ± 25.5
Anti-hypertension therapy (n) (%)	1189 (68.7)	352 (71.8)
Insulin therapy (n) (%)	782 (45.2)	164 (33.5)
Statin therapy (n) (%)	890 (51.4)	150 (30.6)
Follow-up (yrs); (py)	13.1 ± 5.6 (22 676)	12.0 ± 5.5 (5880)
All-cause death (n) (%)	842 (48.6)	256 (52.2)
IR (n events per 100 py)[a] (95% CI)	3.2 (2.9-3.5)	3.7 (3.0-4.3)

Continuous variables are reported as mean ± SD and categorical variables as total frequencies and percentages.
Abbreviations: aGMS, aggregate Gargano Mortality Study; eGFR, estimated glomerular filtration rate (calculated using CKD-EPI equation); FMS, Foggia Mortality Study; HbA1c, glycated hemoglobin A1c; IR, incidence rate of all-cause death; py, person-years.
[a]Adjusted for age and sex.

The relationship between all-cause mortality with each single marker as well as the I score (weighted according to the same effect sizes of each marker in the aGMS) was validated in the FMS, a totally independent sample using the same fully adjusted model as before (Fig. 1B). When hs-CRP levels were added into the model the association between the I score and death rate did not change (HR moving from 2.20, 95% CI 1.79-2.72 to 2.47, 1.95-3.17; *P* for HR heterogeneity = .47).

In the 2 cohorts pooled together, patients still alive at the end of follow-up (N = 1123) have an I score = −0.31, while patients who were deceased (n = 1098) have an I score = 0.31 ($P = 2.9 \times 10^{-106}$). In this pooled sample, the association between the I score and all-cause mortality was significantly modified by age at recruitment, diabetes duration, HbA1c, and eGFR (*P* for interaction = 9.7×10^{-11}, 6.1×10^{-7}, 1.8×10^{-2}, and 2.6×10^{-2}, respectively). Subgroup analysis carried out to help clinical interpretability showed that the association between the I score and all-cause mortality was significantly and highly stronger in younger patients (ie, age below the median value of the entire cohort) (Fig. 2). Evidence of interaction in subgroup analysis was also observed with disease duration, HbA1c, and eGFR levels (ie, stronger effect in individuals with shorter duration, better glycemic control, and higher filtration rate, respectively) (Fig. 2).

In order to provide I score thresholds with clinical utility, patient subgroups at different risks for mortality have been identified through a survival tree analysis (Fig. S2 (13)). The subjects with I scores <−0.729 and ≥0.708 had the lowest (0.80 per 100 person-years, 95% CI 0.57-1.13) and the highest (11.02 per 100 person-years, 95% CI 9.74-12.47) incidence rate of mortality, respectively. Additional score thresholds that identify groups with intermediate risk of death are shown elsewhere (Fig. S2 (13)).

Improvement of Established Prediction Models of Mortality

In the 2 pooled cohorts, the discrimination ability (*c* statistics) for all-cause mortality was 0.77 (95% CI 0.74-0.79, 6-year prediction) and 0.77 (95% CI 0.75-0.78, 10-year prediction) for the ENFORCE (25, 26) and the RECODe (27, 28) clinical models, respectively (Table 4). The addition of the I score significantly improved the *c* statistics (delta *c* statistics) in both models (Table 4). In addition, other measures of discrimination (IDI and rIDI) and reclassification (cNRI) were ameliorated in both models (Table 4). To avoid an overoptimistic conclusion, we investigated the improvement in *c* statistics provided by the I score in the FMS alone (ie, the

Table 2. Association between inflammatory markers and all-cause mortality in the aGMS

Unadjusted model[a]				Fully adjusted model[b]		
Cytokines	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
RANTES	1.67	1.53-1.82	6.2×10^{-30}	**1.38**	**1.27-1.50**	$\mathbf{1.6 \times 10^{-14}}$
IL-6	1.33	1.26-1.41	1.5×10^{-28}	**1.28**	**1.21-1.37**	$\mathbf{7.7 \times 10^{-15}}$
IP-10	1.68	1.53-1.84	4.4×10^{-27}	**1.47**	**1.34-1.61**	$\mathbf{7.9 \times 10^{-16}}$
IL-13	0.70	0.65-0.75	1.6×10^{-23}	**0.80**	**0.74-0.86**	$\mathbf{3.7 \times 10^{-9}}$
IL-8	1.32	1.24-1.40	5.5×10^{-19}	**1.17**	**1.10-1.25**	$\mathbf{2.0 \times 10^{-6}}$
FGF basic	1.31	1.23-1.40	4.4×10^{-17}	**1.18**	**1.11-1.26**	$\mathbf{4.6 \times 10^{-7}}$
IL-1ra	1.25	1.17-1.23	8.1×10^{-12}	**1.17**	**1.10-1.25**	$\mathbf{9.6 \times 10^{-7}}$
IL-15	0.81	0.76-0.87	3.9×10^{-9}	**1.15**	**1.07-1.24**	$\mathbf{1.9 \times 10^{-4}}$
MIP-1β	1.17	1.10-1.25	7.5×10^{-7}	**1.13**	**1.06-1.20**	$\mathbf{2.1 \times 10^{-4}}$
IL-4	0.87	0.82-0.92	2.0×10^{-6}	**0.89**	**0.83-0.95**	$\mathbf{1.0 \times 10^{-3}}$
PDGF-BB	1.20	1.10-1.31	2.8×10^{-5}	**0.92**	**0.87-0.98**	$\mathbf{1.4 \times 10^{-2}}$
IFN-γ	1.14	1.06-1.23	4.0×10^{-4}	1.09	0.99-1.19	5.3×10^{-1}
Eotaxin	1.17	1.08-1.25	4.1×10^{-5}	1.06	0.99-1.14	8.3×10^{-1}
IL-5	0.88	0.82-0.95	4.3×10^{-4}	0.87	0.84-1.00	7.8×10^{-1}
MIP-1α	1.12	1.04-1.19	1.0×10^{-3}	1.05	0.98-1.12	1.5×10^{-1}
IL-7	0.90	0.84-0.96	1.0×10^{-3}	0.94	0.88-1.00	6.9×10^{-1}
G-CSF	0.89	0.84-0.95	1.0×10^{-3}	1.07	0.99-1.15	8.7×10^{-1}
IL-10	*0.90*	*0.85-0.96*	2.0×10^{-3}			
IL-2	*1.12*	*1.04-1.20*	2.0×10^{-3}			
IL-17A	*1.08*	*1.04-1.16*	2.1×10^{-2}			
TNF-α	*1.11*	*1.03-1.19*	5.0×10^{-3}			
IL-12 (p70)	*1.07*	*0.99-1.17*	5.0×10^{-2}			
IL-9	*1.04*	*0.94-1.15*	6.9×10^{-2}			
MCP-1 (MCAF)	*1.06*	*0.99-1.14*	8.7×10^{-2}			
GM-CSF	*1.06*	*098-1.14*	1.2×10^{-1}			
IL-1β	*1.05*	*0.98-1.11*	1.4×10^{-1}			
VEGF	*0.98*	*0.91-1.05*	5.2×10^{-1}			

HR 95% CI are given for SD increase in inflammatory markers levels.
In italic are molecules that are not significantly associated with all-cause mortality in univariate analyses, after Bonferroni correction for multiple comparisons (*P* threshold = 1.85 × 10-3).
In bold are molecules significantly associated with all-cause mortality in the fully adjusted model.
Abbreviations: aGMS, aggregate Gargano Mortality Study; G-CSF, Granulocyte-Colony stimulating factor; GM-CSF, Granulocyte-macrophage colony-stimulating factor; FGF basic, Fibroblast Growth Factor; IFN-γ, interferon-gamma; IL, interleukin; IL-12 (p70), Interleukin-12p70; IL-1ra, Interleukin-1 receptor agonist; IP-10, interferon gamma–induced protein 10; MCAF, monocyte chemotactic and activating factor; MCP-1, monocyte chemoattractant protein-1; MIP, macrophage, inflammatory protein; PDGF-BB, platelet-derived growth factor-BB; RANTES, Regulated on Activation, Normal T cell Expressed and Secreted; TNF-α, tumor necrosis factor-alfa; VEGF, vascular endothelial growth factor.
[a]This analysis was adjusted only for the recruitment period, as described in Materials and Methods.
[b]This analysis was fully adjusted for age at recruitment, sex, smoking habit, BMI, HbA1c, eGFR, diabetes duration, ongoing treatments, and recruitment period.

validation cohort which was not used to discover the associated markers); similarly to what was observed in the pooled analysis, the delta *c* statistic was 0.03 (95% CI 0.01-0.05, $P = 2 \times 10^{-4}$, 6-year prediction) and 0.03 (95% CI 0.02-0.05, $P = 7.5 \times 10^{-6}$, 10-year prediction), at the top of ENFORCE and RECODe, respectively.

Interestingly, very similar results were obtained on all prediction measures in both the ENFORCE and RECODe models with a parsimonious I score based only on the 4 most strongly associated inflammatory markers (ie, excluding IL-4 and IL-8) (Table S2 (13)).

In both prediction models, all improvements provided by the I score were particularly evident in younger patients (below the median age) ($P < 0.0001$ for all measures) compared with the improvements observed in older patients (see Table S3A and S3B (13)). This finding parallels the stronger association between the I score and the risk of death observed in younger subjects.

The Link Between Inflammation and Tryptophan Metabolism in Shaping the Risk of Mortality

In a subset of 828 individuals from the aGMS which is fully representative of the entire cohort (clinical features shown in Table S4 (13)) the possible link between inflammation and tryptophan metabolism, by means of serum KTR, was investigated.

Of the 6 inflammatory markers identified and validated as associated with all-cause mortality, only IL-4, IL-6, IL-13, and IP-10 were also associated with the KTR (a prerequisite to run causal mediation analysis). These 4 molecules, as well as the KTR, were associated with all-cause mortality in this

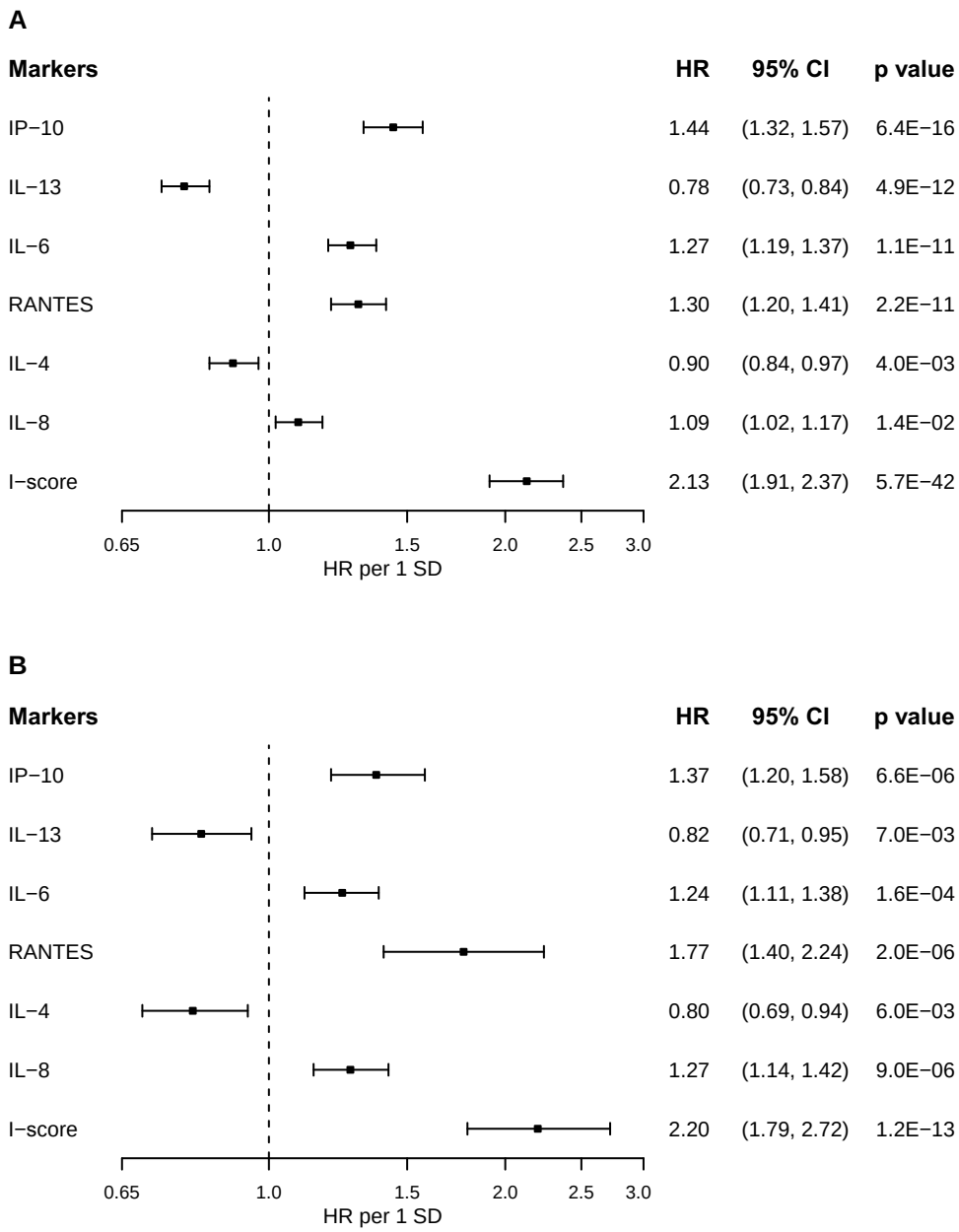

Figure 1. Associations between inflammatory markers, I score, and all-cause mortality. Hazard ratios (HRs) and 95% CIs per 1 SD increase of each inflammatory marker and the I score in aGMS (A) and FMS (B) were estimated in Cox regression models, adjusting for age at recruitment, gender, smoking habit, BMI, diabetes duration, HbA1c, eGFR, and ongoing treatments. For aGMS, the "recruitment period" was also included into the model.

subset of individuals (Table S5, panel A (13)). The association with all-cause mortality when both KTR and each of the 4 markers were considered together is shown elsewhere (Panel B of Table S5 (13)). Most of the observed associations with mortality were virtually identical in the 2 alternative models, but that of KTR after adjustment for IP-10 (29% reduction in beta value) and those of IP-10 and IL-13 after adjustment for KTR (16% and 37% reduction in beta value, respectively).

The causal mediation analysis confirmed that more than one-quarter of the effect of KTR on mortality was mediated by IP-10 (28%, $P = 8 \times 10^{-6}$) and that conversely 16% ($P = 6 \times 10^{-4}$) of the effect of IP-10 is mediated by KTR. Furthermore, 39% of the effect of IL-13 on mortality was mediated by KTR ($P = 2 \times 10^{-2}$).

Cell Culture Studies to get Insight on how IP-10 Mediates the Association Between KTR and Mortality

We tested in TeloHAECs whether 5-MTP, a 5-OH-tryptophan–derived metabolite with anti-inflammatory activity (33, 34), influences the expression of IP-10 under different experimental conditions combining short- and long-term incubation at low- and high-glucose concentrations. Indeed, compared with that in cells after a 24-hour incubation at 5 mM glucose, taken as the control condition, the expression of *CXCL10*, which encodes for IP-10, was slightly but significantly increased by a 25 mM glucose concentration at 24 hours ($P = 1.8 \times 10^{-2}$; Fig. 3, left half, white bars) and almost doubled by a 48-hour incubation ($P = 4.5 \times 10^{-4}$; Fig. 3 right half, grey bars). At this time point, no further stimulatory

Table 3. Multivariable associations with all-cause mortality in the aGMS (n = 1731)

	HR	95% CI		*P*
I score	2.13	1.91	2.37	5.7×10^{-42}
Males vs females	1.39	1.19	1.61	1.9×10^{-5}
Age at recruitment (per 10 years)	2.04	1.88	2.26	9.4×10^{-44}
Smoking habit (yes vs no)	1.72	1.39	2.12	5.7×10^{-7}
BMI (per 1 unit)	1.02	1.00	1.03	1.60×10^{-2}
HbA1c (per 1 unit)	1.02	1.03	1.11	1.00×10^{-2}
Disease duration (per 10 years)	1.02	0.94	1.13	5.6×10^{-1}
eGFR (per 10 mL/min/m^2)	1.12	1.07	1.17	5.2×10^{-7}
Antihypertension therapy (yes vs no)	1.29	1.08	1.54	4.0×10^{-3}
Insulin therapy (yes vs no)	1.48	1.25	1.74	4.0×10^{-6}
Statins therapy (yes vs no)	0.92	0.79	1.06	2.5×10^{-1}

HR (95% CI) for Inflammation score is given for 1 SD increase.
Analyses were adjusted for study cohort.
Abbreviations: aGMS, aggregate Gargano Mortality Study; BMI, body mass index; eGFR, estimated glomerular filtration rate (calculated using the CKD-EPI equation); HbA1c, glycated hemoglobin A1c; I score, inflammation score.

effect of a 25 mM glucose concentration was observed. Coincubation with 100 µM 5-MTP abrogated the stimulation of IP-10 expression exerted by a high glucose concentration at 24 hours (Fig. 3, left) as well as by prolonged incubation at 48 hours, thus restoring the stimulatory effect of high glucose concentration ($P = 4E^{-2}$) (Fig. 3, right).

Discussion

We investigated comprehensively the role of low-grade inflammation on all-cause mortality in 2221 individuals with type 2 diabetes, from 2 different cohorts. Indeed, 27 inflammatory markers were measured, with 6 of them (IL-4, IL-6, IL-8, IL-13, RANTES, and IP-10) independently associated in the first discovery sample and then confirmed in the second validation sample. Of note, the I score, comprising these 6 markers, was associated with all-cause death in both the discovery and the validation sample with an effect similar in magnitude to that of being 10 years older and much stronger than that of gender, kidney function, essential hypertension, and the severity of diabetes, as indicated by insulin treatment. Notably, the association with mortality rate of the I score was independent of hs-CRP (11), suggesting that the 2 inflammatory markers provide information on different pathways related to premature death in type 2 diabetes.

The association between I score and all-cause mortality was much stronger in younger individuals and in those with shorter duration of diabetes, meaning that the deleterious effect of chronic inflammation on the probability of survival is more evident when other risk factors such as age and long disease duration are less prevalent. This suggests the need to prevent or treat inflammation in the early stages of type 2 diabetes, especially in relatively young people in whom diabetes has the greatest negative effect on life expectancy (1) and in whom intervention strategies have more time to improve the trajectory of the disease's fearsome consequences.

Importantly from a clinical point of view, the I score improved discrimination measures of ENFORCE and RECODe, 2 well-established prediction models of all-cause death in type 2 diabetes which are based on clinical variables (25-28). Although statistically significant, the improvement of the *c* statistics is rather small, but it is worth noticing that in already well-performing models, as are those we used here, this index lacks sensitivity in detecting further discrimination improvements (35). In both models, the percentage rIDI, an alternative discrimination index, is higher than the threshold requested by international guidelines for adding new biomarkers on top of established prediction models (36). In addition, adding the I score to both models correctly reclassified a significant proportion of individuals, especially nonevents, thus reducing the risk of overestimation. Finally, in both models, the improvement of discrimination and reclassification measures provided by the I score was particularly evident in patients with relatively younger age. Overall, our findings advocate the use of the I score on top of ENFORCE or RECODe to improve the identification of individuals with type 2 diabetes at highest risk of death who would benefit most from aggressive, laborious, and costly management that cannot (and probably does not need to) be implemented in all individuals with diabetes.

The I score includes both proinflammatory (IL-6 and IL-8) and anti-inflammatory (IL-4 and IL-13) inflammatory cytokines as well as 2 chemokines (RANTES or CCL5 and IP-10 or CXCL10). IL-6 has previously been studied as a predictor of CVD (37) and mortality (38) also in patients with type 2 diabetes (10, 39). IL-6 mediates humoral and cellular responses (40) and is closely related to IL-8, a neutrophil chemotactic proinflammatory cytokine also associated with (CVD) and involved in mitogenesis, angiogenesis inhibition, and leukocyte activation (41).

The anti-inflammatory IL-4 and IL-13 cytokines share many biological and immunoregulatory functions on B lymphocytes, monocytes, dendritic cells, and fibroblasts (42). Generally, IL-4/-13 signaling to peripheral tissues promotes cell proliferation, which mediates beneficial responses (43). IL-4 circulating levels are decreased in elderly people (44) and low levels of IL-13 are associated with increased mortality risk in diabetes (17).

Finally, chemokines RANTES and IP-10, which act on endothelial and vascular smooth muscle cells, are postulated to be involved in the development and manifestation of atherosclerosis and CVD (45, 46).

In type 2 diabetes, tryptophan metabolism is associated with all-cause mortality (17). In detail, serum tryptophan entails a high survival rate while, in contrast, serum kynurenine and the KTR are positively related to the risk of death (17).

Indeed, KTR is a marker of tryptophan downstream metabolism that recognizes 2 alternative pathways: one that through kynurenine is proinflammatory (47) and another that synthesizes 5-MTP, an anti-inflammatory metabolite derived from 5-OH-tryptophan (33, 34). Causal mediation analysis showed that IP-10 mediates part of the deleterious effect of KTR on the risk of death. A possible mechanism underlying this effect is, at least partially, indicated by our studies on cultured endothelial cells showing that under several experimental conditions IP-10 expression is controlled by 5-MTP, thus making this chemokine central for the anti-inflammatory effect of tryptophan metabolism. Conversely, KTR mediates part of the effect on all-cause mortality of IP-10, suggesting a binary mediation between the 2 risk factors. KTR also mediates part of the effect of IL-13, confirming our previous observation obtained in a smaller sample (17). As a whole, our data indicate the existence of a pathway that through an intertwined

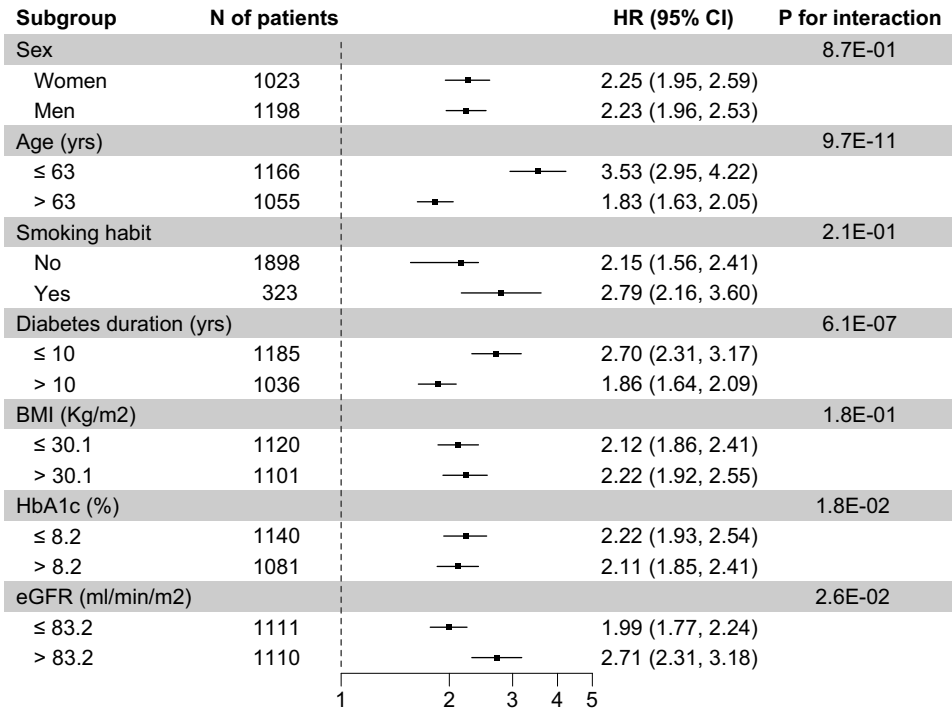

Figure 2. Associations between the I score and all-cause mortality in the combined sample (aGMS + FMS) by subgroups of demographical and clinical features. Hazard ratios (HRs) and 95% CI per 1 SD of I score increase were estimated by Cox regression models, adjusting for age at recruitment, gender, smoking habit, BMI, diabetes duration, HbA1c, eGFR, ongoing treatments, and study sample. The *P* value for interaction is shown for each subgroup.

Table 4. Prediction of all-cause mortality by I score and by ENFORCE and RECODe (without and with the I score)

	Prediction models	Discrimination				Reclassification		
		c statistics (95% CI)	Δ*c* statistics (95% CI) *P* value	IDI *P* value	%rIDI *P* value	% ½ cNRI *P* value	% Events *P* value	% Nonevents *P* value
6-yr mortality	I score	0.71 (0.69-0.74)						
	ENFORCE	0.77 (0.74-0.79)						
	ENFORCE + I score	0.80 (0.78-0.82)	0.04 (0.02-0.05) 3×10^{-8}	0.051 6×10^{-10}	33.6 4×10^{-9}	18.2 3×10^{-11}	8.7 9×10^{-2}	27.7 $<2 \times 10^{-14}$
10-yr mortality	I score	0.70 (0.68, 0.72)						
	RECODe	0.77 (0.75-0.78)						
	RECODe + I score	0.80 (0.78-0.81)	0.03 (0.02-0.04) 2×10^{-13}	0.056 $<2 \times 10^{-14}$	27.2 $<2 \times 10^{-14}$	20.5 $<2 \times 10^{-14}$	11.5 4×10^{-3}	29.5 $<2 \times 10^{-14}$

All *P values* are referred to comparisons vs the same base model (ie, with no I-score). Time horizon of 6 years for ENFORCE (n events = 346) and 10 years for RECODe (n events = 592).
Abbreviations: I score, inflammation score; cNRI, category-free net reclassification improvement; rIDI, relative integrated discrimination improvement.

relationship between low-grade inflammation and tryptophan metabolism shapes the risk of death in people with diabetes, thus opening new research routes to discover tailor-made treatments for "inflamed" patients with imbalanced tryptophan metabolism.

Our study has several strengths. We used a rigorous study design with discovery and replication cohorts prospectively analyzed with a clinical phenotype thoroughly investigated. The adopted pipeline to skim the most associated serum inflammatory markers with all-cause mortality included Bonferroni adjustment, confounders adjusted model, and stepwise analysis, a conservative approach that lowers the false-positive rate.

We also carried out a comprehensive analysis on a large number of quality-controlled inflammatory biomarkers, whereas previous studies on the role of inflammation on mortality in patients with type 2 diabetes have evaluated only a few markers (9-12). In addition, our present study tested the ability of an I score, based on the most robustly associated markers, to improve 2 well-established and validated prediction models (25-28), thus making our findings implementable in the real-life clinical setting. Finally, we gained information on the intertwined relationship between the inflammation and tryptophan pathways (14) whose link is a promising field of study aimed at paving the way for new therapies to treat individuals with type 2 diabetes (and perhaps also other chronic conditions) characterized by low-grade inflammation and imbalanced tryptophan metabolism.

We have also to recognize several limitations. Firstly, the 2 cohorts we studied are geographically close, thus limiting

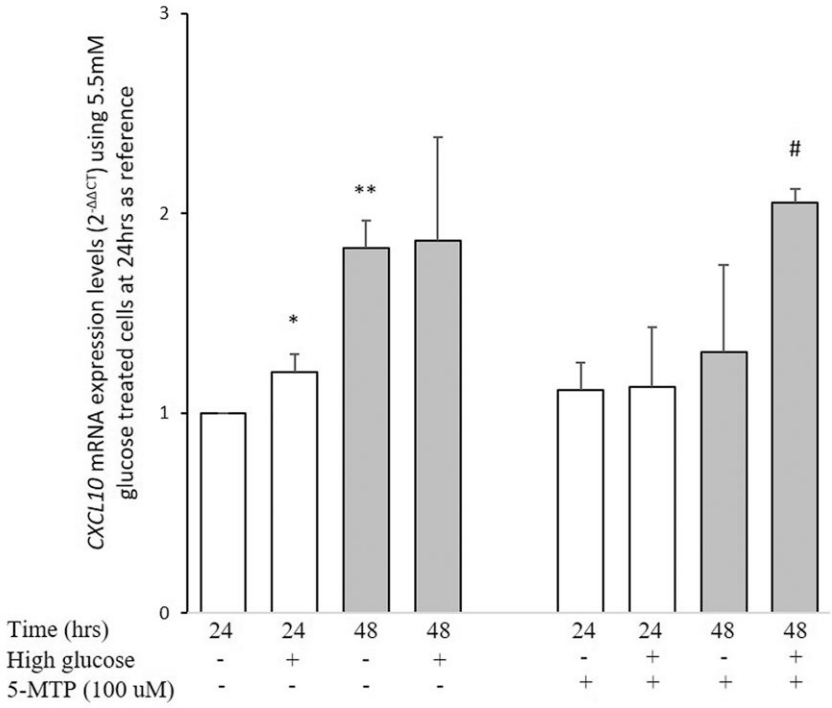

Figure 3. Glucose and 5-MTP induced C-X-C motif chemokine ligand 10 (*CXCL10)* mRNA expression. Bars represent *CXCL10* mRNA expression (which encodes for the chemokine IP-10), calculated as indicated in Materials and Methods, induced by high glucose (25 mM) concentration with or without 100 μM 5-MTP in cells incubated for 24 (white bars) and 48 (grey bars) hours. Data are means ± SD of 3 independent experiments. *Second vs first bar $P = 1.8 \times 10^{-2}$; **third vs first bar $P = 4.5 \times 10^{-4}$; #eighth vs seventh bar $P = 4.2 \times 10^{-2}$.

the generalizability of our finding. In addition, we lack data on cardiovascular mortality, thus making it impossible to look for the association between this specific cause of death and inflammation previously reported to be associated with some inflammatory markers in the general population (38). This said, it should also be considered that in individuals with diabetes cardiovascular mortality, although still important (48, 49), is no longer the leading cause of death, now representing only one-quarter of all events (50).

In conclusion, we have comprehensively investigated and characterized the association between inflammation and all-cause mortality in people with type 2 diabetes, which may have clinical implication in identifying individuals who are at greater risk of death. We also provided evidence that an intertwined relationship between inflammation and the tryptophan metabolism influences the rate of mortality. This may pave the way for new therapies targeted at subgroups of patients characterized by low-grade inflammation and imbalanced tryptophan metabolism.

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Author Contributions

C.M. and V.T. conceived the study and wrote the manuscript. C.M., A.M., G.C., M.M., A.F., M.C., and V.T. participated in data analysis and interpretation of results. M.M., M.P.A., O.L., S.D.C., and V.T. contributed to data collection. C.P. and J.A. supervised the target metabolomics analysis. L.S. performed laboratory testing. A.M. and D.M. performed cellular study. All authors critically revised the paper and approved its final version. C.M. and V.T. are the guarantors of this work and, as such, had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Disclosures

The authors have nothing to disclose.

Data Availability

The data sets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Clinical Research Article

GFR Decline Predicts Total Mortality and Mediates the Effect of Tryptophan Metabolism on Death Risk in Type 2 Diabetes

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Abstract

Context: The independent role of glomerular filtration rate (GFR) decline in shaping the risk of mortality in people with type 2 diabetes has only been partially addressed.

Objective: The objective of the study was 2-fold: (1) to investigate the association between all-cause mortality and eGFR changes over time; (2) to understand whether renal dysfunction mediates the effect of tryptophan metabolism on death risk.

Methods: Prospective study with an average follow-up of 14.8 years at a research hospital. The aggregate Gargano Mortality Study included 962 patients with type 2 diabetes who had at least 3 eGFR recordings and at least 1.5 years of follow-up. This was an observational study, with no interventions. Rate of all-cause mortality was measured.

Results: Age- and sex-adjusted annual incident rate of mortality was 2.75 events per 100 person-years. The median annual rate of decline of eGFR was 1.3 mL/min per 1.73 m^2 per year (range −3.7; 7.8). The decline of kidney function was strongly and independently associated with the risk of death. Serum kynurenine to tryptophan ratio (KTR) was associated with both eGFR decline and all-cause mortality. Causal mediation analysis showed that 24.3% of the association between KTR and mortality was mediated by eGFR decline.

Conclusion: In patients with type 2 diabetes, eGFR decline is independently associated with the risk of all-cause mortality and mediates a significant proportion of the association between tryptophan metabolism and death.

Key Words: albuminuria, causal mediation, death, kidney function, tryptophan metabolism

Abbreviations: ACR, albumin to creatinine ratio; aGMS, aggregate Gargano Mortality Study; BMI, body mass index; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; KTR, kynurenine to tryptophan ratio.

Diabetes is recognized as the fastest growing chronic disease in the world (1) associated with substantial increase of mortality. Approximately 6.7 million adults are estimated to have died from diabetes or its complications in 2021 (1), making diabetes a leading cause of death worldwide (2). The rate of mortality in the general population has decreased significantly in recent years thanks to interventions that reduce the risk of myocardial infarction, such as lipid-lowering and antihypertensive therapy, and better glycemic control over time (3-5). However, the risk of death in patients with type 2 diabetes is still high, being about double that of people without the disease (6). Several studies in high-risk people have shown a

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strong relationship between the annual rate of decline in estimated glomerular filtration rate (eGFR) and all-cause mortality (7-11). Unfortunately, such a relationship has been only sparsely addressed in people with type 2 diabetes (12, 13). Therefore, the main aim of our study was to investigate the association between changes in renal function over a long follow-up period and the risk of all-cause mortality in people with type 2 diabetes from southern Italy. Furthermore, since the serum kynurenine to tryptophan ratio (KTR) is associated with both the decline of eGFR and the rate of mortality (14-16), we also investigated whether kidney dysfunction plays the role of mediator between tryptophan metabolism and death risk in type 2 diabetes.

Materials and Methods

Study Population

The aggregate Gargano Mortality Study (aGMS) consists of 2140 people with type 2 diabetes recruited according to the same design and procedures from 2000 to 2008 (first period) and from 2008 to 2011 (second period) at the Endocrinology Unit of the IRCCS (Istituto di Ricovero e Cura a Carattere Scientifico) "Casa Sollievo della Sofferenza," San Giovanni Rotondo and followed until February 2023. The only inclusion criterion was the presence of type 2 diabetes according to the 2003 American Diabetes Association criteria. Exclusion criteria were dialysis or transplantation and poor life expectancy for nondiabetes-related diseases. Any analysis using data from aGMS data is adjusted for the "recruitment period" (see below). This specific study was carried out in those 962 patients who had at least 3 eGFR recordings and at least 1.5 years of follow-up. The study and the informed consent procedures were approved by the local Institutional Ethic Committee IRCCS "Casa Sollievo della Sofferenza." All participants gave written consent. The study was conducted and reported according to the STROBE checklist (17).

Exposure

In the present study, the exposure was the rate of eGFR decline over the time. We measured standardized serum creatinine using the modified kinetic Jaffe reaction (Hitachi 737 autoanalyzer), calibrated to be traceable by isotope dilution mass spectrometry. GFR was estimated for each patient using the Chronic Kidney Disease Epidemiology Collaboration formula (18) derived from serum creatinine values at baseline and during follow-up. Repeated-measures, longitudinal, multilevel (mixed-effects) models with follow-up time as a fixed effect were used to compute the annual change in eGFR (measured as mL/min per 1.73 m^2) for each participant.

Study Endpoint

The vital status of all participants was verified by interrogating the Italian Health Card Database upon data anonymization (https://sistemats1.sanita.finanze.it/wps/portal/).

Clinical and Biochemical Measurements

Duration of type 2 diabetes and current glucose-, blood pressure–, and lipid-lowering treatments were recorded for all patients. A physical examination, including measurement of height, weight, and blood pressure, was performed on all subjects enrolled in the study. A fasting venous blood sample was taken from all patients for the measurement of serum total cholesterol, high-density lipoprotein cholesterol, triglycerides, glycated hemoglobin (HbA1c), and creatinine.

Urinary albumin and creatinine concentration were determined by the nephelometric method (Behring Nephelometer Analyzer; Behring, Marburg, Germany) and the Jaffe reaction rate method, respectively, and were determined on an early morning first void sterile urine sample. Urinary albumin excretion was calculated and reported as urinary albumin to creatinine ratio (ACR). Increased urinary albumin excretion was diagnosed as microalbuminuria if the urinary ACR ratio was >2.5 mg/mmol in men and >3.5 mg/mmol in women and ≤30 mg/mmol in both sexes and macroalbuminuria if the ACR ratio was >30 mg/mmol in both sexes.

Tryptophan, kynurenine, and their ratio (KTR) were quantified using baseline fasting serum samples and the AbsoluteIDQ p180 kit (Biocrates Life Sciences), as previously described (14, 16, 19).

Briefly, sample handling was performed by a Hamilton Microlab STAR robot (Hamilton Bonaduz AG, Bonaduz, Switzerland) and a Ultravap nitrogen evaporator (Porvair Sciences, Leatherhead, UK), as well as standard laboratory equipment. Mass spectrometric analyses were done on an API 4000 triple quadrupole system (SCIEX Deutschland GmbH, Darmstadt, Germany) equipped with a 1260 Series HPLC (Agilent Technologies Deutschland GmbH, Böblingen, Germany) and a HTC-xc PAL auto sampler (CTC Analytics, Zwingen, Switzerland) controlled by the software Analyst 1.6.2. For the liquid chromatography part, compounds were identified and quantified based on scheduled multiple reaction monitoring measurements, for the flow injection analysis part on multiple reaction monitoring. Data evaluation for quantification of metabolite concentrations and quality assessment was performed with the software MultiQuant 3.0.1 (Sciex) and the MetIDQ software package. Metabolite concentrations were calculated using internal standards and reported in µM. KTR was calculated trough the MetIDQ RatioExplorer Module.

Statistical Analysis

Patients' baseline characteristics were reported as mean ± SD or median (range) and frequency (percentage) for continuous and categorical variables, respectively. Possible differences in case-mix across subsamples used for different analyses were investigated measuring the standardized mean differences. Missing data rates in subjects' covariates at baseline used in this study (ie, gender, age, body mass index [BMI], duration of disease, HbA1c, baseline eGFR, and medications) were, when present, always less than 5%. Missing data, therefore, were imputed using a random forest approach (20). Age- and sex-adjusted mortality rate was estimated using Poisson models and was reported as number of deaths per 100 person-years. Overall survival was defined as the time between enrollment and death; for subjects who did not experience the end point, survival time was censored at the time of the last available follow-up visit. Time to death analyses were performed using univariable and multivariable Cox proportional hazards regression models and risks were reported as hazard ratios (HRs) along with their 95% CI. To properly assess the most reliable relationship between the rate of eGFR decline and all-cause mortality rate, eGFR decline was included into Cox models as (1) a linear term only, (2) a quadratic term only, (3) both linear and quadratic terms. Goodness of fit of each Cox model was evaluated by the Akaike Information

Criterion. The model including the linear term only achieved the best fit (ie, minimum Akaike Information Criterion), a finding that is fully compatible with the linear relationship observed between eGFR decline and mortality rate. The representation of eGFR decline tertiles was also provided for ease of clinical interpretation. Two separate Cox models were performed. The first model was adjusted only for the recruitment period (0 or 1); the second model also included demographics characteristics (ie, sex and age at recruitment), diabetes-related parameters (ie, basal body mass index, HbA1c, eGFR, and disease duration), and medications (ie, glucose-, blood pressure–, and lipid-lowering treatments at recruitment).

To investigate whether the eGFR annual decline acts as a mediator of the relationship between KTR and mortality risk, a causal mediation analysis was performed (21). The KTR values were first logarithmized and then standardized. Prior to assessing causal mediation analysis, we checked whether (1) KTR was associated with all-cause mortality using a fully adjusted Weibull regression model; and (2) eGFR annual decline and KTR were correlated. Then, a causal mediation analysis was conducted using the "mediate" function defined in the "mediation" package in R. This function provided nonparametric estimates of the total, direct, and indirect effects based on 1000 bootstrap replication. $P < .05$ was considered for statistical significance. Statistical analyses and graphs were performed using R software.

Results

Patient Characteristics

The main clinical features of the 962 study individuals are summarized in Table 1. To provide some clues as to how the subsets used in this study are representative of the entire cohort, the clinical characteristics of all 2140 aGMS individuals and the 575 used for metabolomic studies are also shown in Table 1 (indeed, standardized mean differences were in the range 0.029-0.173, thus suggesting that differences across the 3 samples were negligible). Overall, the mean age of patients was 60.8 ± 9.7 years, 47.3% were women, mean duration of diabetes was 10.8 ± 9.0 years, and the mean value of HbA1c was 8.5 ± 1.9%. A total of 600 (62.4%) and 419 (43.6%) patients were on blood pressure– and lipid-lowering treatment, respectively. Mean eGFR value was 79.2 ± 21.5 mL/min/1.73 m^2. During a median follow-up of 14.8 ± 5.5 years, 452 (47%) patients died. Age- and sex-adjusted annual incident rate of mortality was 2.75 events per 100 person-years. The median annual rate of eGFR decline was 1.3 mL/min per 1.73 m^2 per year (range −3.7 to 7.8) (Table 1).

Table 1. Clinical features of the entire aGMS cohort (N = 2140), the 962 study individuals and the 575 subjects for metabolomics study

	N = 2140	N = 962	N = 575
Women n (%)	1145 (53.5)	455 (47.3)	275 (47.8)
Age at recruitment (years)	62.0 ± 9.42	60.8 ± 9.7	60.9 ± 9.8
Smokers n (%)	310 (14.5)	148 (15.4)	79 (13.7)
BMI (kg/m^2)	31.0 ± 5.8	31.2 ± 5.8	31.2 ± 6.0
Diabetes duration (years)	11.2 ± 9.0	10.8 ± 9.0	11.6 ± 9.3
HbA1c (%)	8.5 ± 1.9	8.5 ± 1.9	8.4 ± 1.9
HbA1c (mmol/mol)	69 ± 20.8	69 ± 20.8	68 ± 20.8
Systolic blood pressure (mmHg)	134.7 ± 17.5	134.2 ± 17.2	134.4 ± 17.0
Diastolic blood pressure (mmHg)	78.6 (9.21)	78.8 (9.15)	78.8 (8.90)
Total cholesterol (mg/dL)	188.9 ± 49.8	190.6 ± 55.7	189.5 ± 56.2
HDL cholesterol (mg/dL)	44.6 ± 12.7	44.5 ± 13.0	44.2 ± 12.7
Triglycerides (mg/dL)	128.0 [91.0-181.0]	128.0 [89.0-180.8]	128.0 [90.0-180.0]
Insulin (w/wo other glucose-lowering agents) n (%)	894 (41.8)	400 (41.6)	268 (46.6)
Lipid-lowering treatment, n (%)	1016 (47.5)	419 (43.6)	292 (50.8)
Blood pressure-lowering treatment (%)	1418 (66.3)	600 (62.4)	376 (65.4)
eGFR (mL/min/1.73 m^2)	80.9 ± 18.7	79.2 ± 21.5	77.4 ± 26.1
ACR (mg/mmol)[a]	2.07 (0.82-6.27)	1.38 (0.68-3.70)	1.56 (0.70-4.96)
Annual rate of eGFR decline[b]	1.3 (−3.7-7.8)	1.3 (−3.7-7.8)	1.3 (−2.2-0.6)
All-cause death (n) (%)	1067 (49.9)	452 (47.0)	227 (39.5)
Follow-up (years) (py)	13.7 ± 5.8 (29 285)	14.8 ± 5.5 (14 197)	13.3 ± 5.2 (7670)
IR (n events per 100 py) (95% CI)[c]	3.32 (3.1-3.55)	2.75 (2.5-3.1)	2.46 (2.11-2.88)

Continuous variables are reported as mean ± SD or median and categorical variables as total frequencies and percentages. Skewed continuous variables are presented as median (range).
Data above the dotted line have been collected at baseline, while those below the dotted line refer to the follow-up and incidence death, the study outcome.
Abbreviations: ACR, albumin–creatine ratio; aGMS, aggregate Gargano Mortality Study BMI, body mass index; HbA1c, glycated hemoglobin; IR, incidence rate; HDL, high-density lipoprotein; glycated hemoglobin; eGFR, estimated glomerular filtration rate, calculated using the Chronic Kidney Disease Epidemiology Collaboration equation.
[a]Information on albuminuria was available for only 1529 patients out of the total 2140-patient group, for 637 patients out of the total 962-patient group and for all subjects in the 575-patient group.
[b]In the whole cohort, comprising 2140 patients, annual rate of eGFR decline was available only in the 962 study individuals used in this analysis.
[c]IR of mortality is given ×100 person-years, after adjusting for age and gender.

Table 2. Univariable and multivariable Cox regression model for the risk of mortality—role of baseline variables

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*-value	HR (95% CI)	*P* value
Annual rate of decline in eGFR (1 mL/min per 1.73 m^2)	1.43 (1.34-1.53)	<.0001	1.31 (1.21-1.41)	<.001
Sex (male vs female)	1.14 (0.95-1.38)	.1542	1.68 (1.38-2.04)	<.0001
Age at recruitment (1 year)	1.10 (1.09-1.11)	<.0001	1.08 (1.07-1.09)	<.0001
BMI (1 kg/m^2)	1.01 (1.00-1.03)	.1506	1.02 (1.00-1.04)	.02
Diabetes duration (1 year)	1.05 (1.04-1.06)	<.0001	1.01 (1.00-1.02)	.10
HbA1c (1 unit %)	1.13 (1.08-1.18)	<.0001	1.13 (1.07-1.19)	<.0001
eGFR (1 mL/min per 1.73 m^2)	0.97 (0.97-0.97)	<.0001	0.99 (0.98-0.99)	<.0001
Insulin (w/wo other glucose-lowering agents) (yes vs no)	2.76 (2.29-3.34)	<.0001	1.79 (14.43-2.23)	<.001
Lipid-lowering treatment (yes vs no)	1.50 (1.24-1.82)	<.0001	1.01 (0.82-1.24)	.96
Blood pressure-lowering treatment (yes vs no)	2.00 (1.63-2.45)	<.0001	1.17 (0.93-1.49)	.19

HRs (per mL/min increase in the annual rate of eGFR loss) were estimated in a Cox regression model, adjusted for gender, age at recruitment, HbA1c, BMI, eGFR, diabetes duration, insulin (w/wo other glucose-lowering agents), lipid-lowering treatment, blood pressure–lowering treatment, and recruitment period.
Abbreviations: BMI, body mass index; eGFR, estimated glomerular filtration rate, calculated using the Chronic Kidney Disease Epidemiology Collaboration equation; HbA1c, glycated hemoglobin; HR, hazard ratio.

Association Between eGFR Decline and All-cause Mortality in the Whole Population

A strong association was observed between the annual rate of eGFR decline and mortality: for each mL/min increase in the annual rate of eGFR loss, there was a 31% increase in all-cause mortality, independent of all possible confounders we were able to take into account, including gender, age, BMI, duration of disease, HbA1c, baseline eGFR, glucose-, blood pressure–, and lipid-lowering treatments (adjusted HR 1.31, 1.21-1.41, $P < .001$) (Table 2). To facilitate clinical interpretation, patients were subgrouped according to tertiles of eGFR annual decline. Clinical features and incidence rate of all-cause mortality across eGFR tertiles are shown elsewhere (Table S1 (22)). Compared with those with less pronounced eGFR decline (tertile 1), patients with the most rapid decline (tertile 3) had significantly higher age, BMI, disease duration, triglycerides, systolic blood pressure, ACR, HbA1c values, and higher prevalence of blood pressure– and lipid-lowering treatments. They also had significantly lower baseline eGFR values. In the fully adjusted model, patients in tertile 2 and tertile 3 had 45% and 81% higher mortality rate than those in tertile 1 (Table 3 and Fig. 1).

Association Between eGFR Decline and All-cause Mortality in Individuals With Information on Albuminuria

Of note, in the 637 representative individuals for whom information on albuminuria was available (clinical features shown in Table S2 (22)), the association between eGFR annual decline and all-cause mortality (HR 1.31, 95% CI 1.21-1.41, $P < .001$) did not change when ACR was also considered in the fully adjusted model (HR 1.33, 95% CI 1.21-1.46, $P < .001$).

Subgroup Analyses

In subgroup analyses, the association between eGFR decline and mortality was significantly stronger in individuals relatively younger and with better glycemic control (individual age and HbA1c below the median value of the entire cohort; *P* for interaction = .01 and .02, respectively, Fig. 2).

Table 3. Risk of all-cause mortality by tertiles of eGFR decline

		HR (95% CI)	*P* value
Model 1	T1	1	<.001
	T2	2.19 (1.69-2.82)	<.001
	T3	3.14 (2.45-3.99)	
Model 2	T1	1	<.001
	T2	1.45 (1.11-1.87)	<.001
	T3	1.81 (1.40-2.34)	

eGFR decline was −3.74 to 0.79; 0.79-1.85; 1.85-7.77 in tertile 1 (T1), 2 (T2) and 3 (T3), respectively (negative values indicate improved eGFR at follow-up). The rate of death in tertile 1 is the reference (HR made equal to 1).
Model 1: adjusted only for the recruitment period.
Model 2: adjusted for gender, age at recruitment, HbA1c, BMI, eGFR, diabetes duration, insulin (w/wo other glucose-lowering agents), lipid-lowering treatment, blood pressure–lowering treatment, and the recruitment period.
Abbreviations: BMI, body mass index; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; HR, hazard ratio.

Mediation Analysis Explaining Association Between KTR and All-cause Mortality

In the subset of 575 representative patients for whom KTR data were available (clinical features described in Table 1) we found a significant association between KTR and both rate of eGFR decline ($R^2 = 0.16$, $P = .0002$) and all-cause mortality (fully adjusted HR per 1 SD increase in KTR values 1.33, 95% CI 1.09-1.63, $P = .005$). In this subset, the adjusted association between eGFR annual decline and all-cause mortality (HR 1.29, 95% CI 1.17-1.43) was slightly attenuated when also KTR was considered in the fully adjusted model (HR 1.27, 95% CI 1.14-1.40). Indeed, causal mediation analysis showed that a significant and nontrivial proportion of the association between KTR and all-cause mortality was mediated by eGFR decline (ie, 24.3%, $P = .006$).

Discussion

In this longitudinal study including 962 patients with type 2 diabetes we observed a direct, independent relationship between the annual decline in kidney function and the rate of all-cause mortality. This association is clinically significant, with

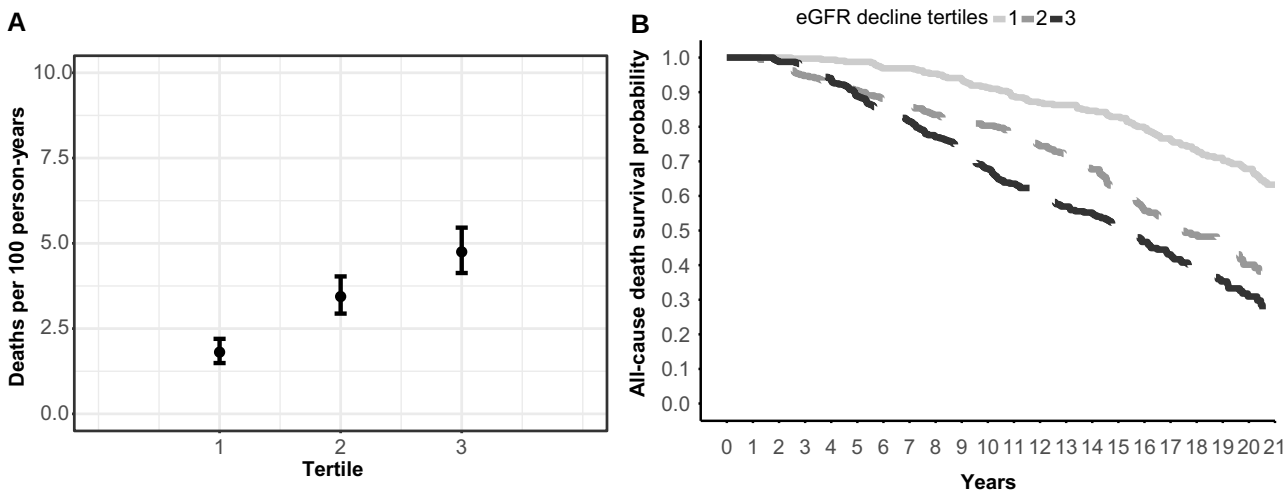

Figure 1. Mortality by tertiles of eGFR decline. (A) Incidence rate of mortality per tertile. The incidence rate is given ×100 person-years after adjustment for age and sex. (B) Kaplan–Meier survival curve: tertile 3 in black, tertile 2 in dark grey, and tertile 1 in light grey.

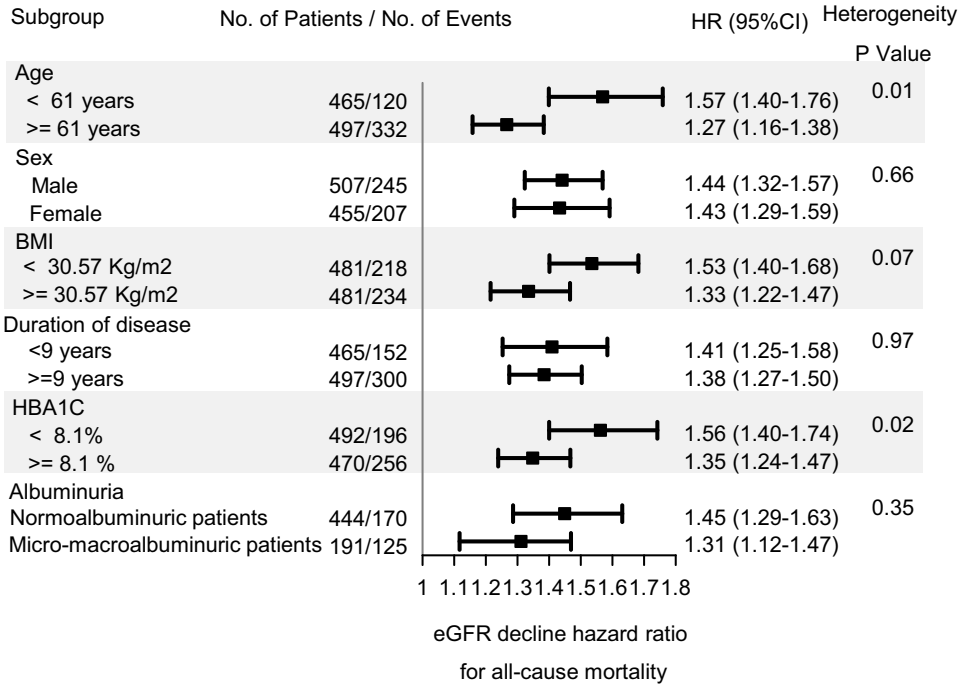

Figure 2. Association between eGFR decline and mortality in subgroups of demographical and clinical features. Hazard ratios (HR) and 95% CI per each mL/min increase in the annual rate of eGFR loss were estimated by Cox regression models. The *P* value for heterogeneity is shown for each subgroup.

each milliliter of eGFR decline increasing the risk of death by 31%. In addition, when looking at tertiles of eGFR decline, individuals with the fastest loss of renal function (ie, belonging to tertile 3) showed an 81% increased risk of mortality compared with individuals of tertile 1. Of note, the association between eGFR decline and mortality was modified by both age and HbA1c, being significantly stronger in relatively younger subjects and in those with better glycemic control meaning that the deleterious effect of kidney dysfunction on the risk of death is more evident when other risk factors such as age and poor glycemic control are less prevalent. Finally, causal mediation analysis showed that eGFR decline mediates approximately one-fourth of the association between mortality and KTR, a marker of tryptophan downstream metabolism that recognizes 2 alternative pathways: one that through kynurenine is proinflammatory and another that synthesizes 5 methoxy-tryptophan, a molecule with anti-inflammatory properties (23). Tryptophan depletion has been reported to be associated with impaired kidney function (24). The degradation of tryptophan in the kynurenine pathway is mediated by the enzyme indoleamine 2,3-dioxygenase of which KTR is a proxy reflecting its activity. It is of note that in the general population the KTR was associated with incident chronic kidney disease and GFR decline (25). This latter association has also been reported in patients with type 2 diabetes by us and others (14, 15), which makes it possible to hypothesize a

deleterious effect of kynurenine and kynurenine metabolites on mesangial cell proliferation (26) and kidney fibrosis (27) as well as reactive oxygen species production, cell damage, and apoptosis in the kidney (28).

Recently, several studies have reported a positive association between the annual rate of decline in eGFR and all-cause mortality in both the general population and in people with chronic kidney disease (7-11). Similar data have also been reported twice in people with diabetes, although a long follow-up like ours was available only in Chinese people (12) but not in people of different ancestral origin, mostly Europeans (13). In addition, eGFR decline is positively associated with major adverse cardiovascular events, including mortality, in people with type 2 diabetes from France (29). Therefore, taken altogether our and previous (12, 13, 29) studies clearly indicate that the association between eGFR decline and mortality rate is a generalizable phenomenon independent of ethnicity and environmental background. It is worth noting that novel antidiabetic drugs such as SGLT2 inhibitors and GLP1-RA, which are able to slow GFR decline, also reduce all-cause mortality, thus reinforcing the plausibility of a causal role of rapid GFR decline in shaping survival probability in people with type 2 diabetes (30). The mechanisms underlying the association between rapid decline in eGFR and increased risk of all-cause mortality are beyond the scope of this study, but several mechanisms could be hypothesized. First, patients with worsening renal function have an atherogenic risk profile that predisposes to cardiovascular events and heart failure (7, 31), both major causes of death in people with diabetes. The relationship between GFR decline and heart failure is also supported by the common effect of SGLT2 inhibitors on both kidney function decline and heart failure, suggesting common etiological factors between the 2 outcomes.

This may well be due to endothelial dysfunction, oxidative stress, and vascular damage as well activation of the renin–angiotensin system, conditions which are associated with renal impairment (32, 33). In addition, renal function loss can lead to physical decline, frailty, and other indirect causes of mortality (34).

The strengths of our study include the long duration of follow-up, the rigorous design of the study, the ability to adjust for a number of important risk factors, and the use of linear mixed models to calculate eGFR slope. Conversely, we must also recognize several limitations. These include the use of estimated rather than measured GFR, the lack of ACR values for all patients in the study, and the relatively small size of the study cohort. In addition, no information on cardiovascular and cancer mortality was available, thus impeding us to look for the association between GFR decline and these specific causes of death previously reported in type 2 diabetes (29). Finally, the lack of data on new antidiabetic drug classes (SGLT2 inhibitors and GLP-1 analogs) that are known to impact on the risk of death is also a limitation of our study.

In summary, we show that in patients with type 2 diabetes, a rapid decline in eGFR is strongly associated with future risk of all-cause mortality. Measuring the eGFR slope in the clinical setting can therefore be useful to identify those patients who are at high risk of all-cause death and who require close follow-up for the early initiation of preventive and therapeutic strategies. In addition, eGFR decline mediates approximately one-quarter of the association between KTR and mortality. This finding points to a novel pathogenic pathway linking tryptophan metabolism, kidney dysfunction, and risk of death, thus calling for further studies to identify new treatments tailored to patients whose clinical trajectory is likely to follow this path.

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Disclosures

The authors have nothing to disclose.

Data Availability

Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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