BIO-RAD

Bio-Plex Pro Human Cytokine Assays

Quick Guide

For Use with	Instruction Manual #
Bio-Plex Pro Cytokine, Chemokine, and Growth Factor Assays	10000111560

This guide can be used to prepare and run a full 1 x 96-well assay plate. New users can download the complete manual, which includes detailed instructions and a list of kit components, at **bio-rad.com/bio-plex**.

Initial Preparation

1. Plan the plate layout.
2. Start up/warm up the Bio-Plex Multiplex Immunoassay System (**30 min**).
   - Bring diluents, including wash buffer, assay buffer, standard diluent HB, detection antibody diluent HB, and sample diluent HB, to room temperature (RT). Keep the other items on ice until needed
     - Mix by inversion to ensure all salts are in solution
     - Prepare 1x wash buffer: dilute **1 part** 10x wash buffer (60 ml) with **9 parts** distilled water (540 ml)
   - Begin to thaw frozen samples
3. Prepare the sample dilution according to the guidelines provided in the following table. It is important to centrifuge serum or plasma samples at **1,000 x g** for **15 min** at **4°C** to remove particulates from all samples prior to use.

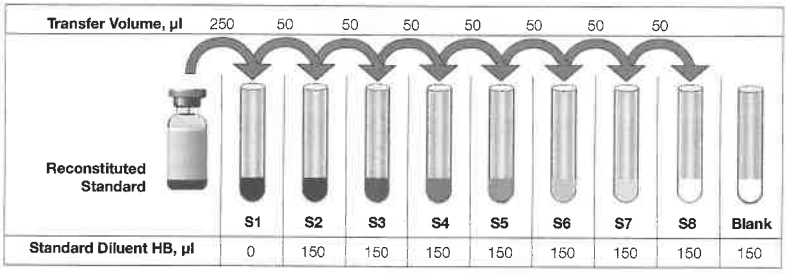
Sample Type	Recommended Sample Dilution	Diluent
Serum and plasma	1:4	Sample diluent
Culture media and fluids	User defined	Diluent + 0.5% bovine serum albumin (BSA) (w/v)

Note: ICAM-1 and VCAM-1 require higher dilution for serum and plasma (recommended 100-fold). Refer to the Bio-Plex Pro Cytokine, Chemokine, and Growth Factor Assays Instruction Manual (#10000111560) for detailed sample preparation recommendations.

4. Calibrate the Bio-Plex System within Bio-Plex Manager Software.
5. Reconstitute the standards and control by adding 250 µl of standard diluent HB to each. **Vortex** at medium speed for **5 sec** and incubate all vials on ice for precisely **30 min**.

6. Prepare a fourfold standard dilution series and blank as shown. **Vortex** at medium speed for **5 sec** between liquid transfers.

   Note: Standards are at S1 concentration after reconstitution and the controls are ready to use after reconstitution. Controls are included with the fixed panel only.



7. **Vortex** the coupled beads at medium speed for **30 sec** and **dilute to 1x** in Bio-Plex Assay Buffer as shown. Protect from light.

Premixed Panels

Number of Wells	10x Beads, µl	Assay Buffer, µl	Total Volume, µl
96	570	5,130	5,700

Singleplex Assays

Number of Wells	Singleplex #1 20x Beads, µl	Singleplex #2 20x Beads, µl	Assay Buffer, µl	Total Volume, µl
96	285	285	5,130	5,700

Note: 20x singleplex beads allow multiplexing up to 20 analytes.

Running the Assay

1. **Vortex** the diluted (1x) beads. **Add 50 µl** to each well of the assay plate.
2. **Wash the plate two times** with **100 µl** Bio-Plex Wash Buffer.
3. **Vortex** the samples, standards, blank, and control. **Add 50 µl** to each well.
4. Cover the plate with sealing tape. Incubate on shaker at **850 ± 50 rpm** at RT for **30 min**.
5. With 10 min left in the incubation, **vortex** the detection antibodies for **5 sec** and quick-spin to collect liquid. **Dilute to 1x** as shown.

Premixed Panels

Number of Wells	10x Detection Antibodies, µl	10x Detection Antibody Diluent HB, µl	Total Volume, µl
96	300	2,700	3,000

Singleplex Assays

Number of Wells	Singleplex #1 20x Detection Antibodies, µl	Singleplex #2 20x Detection Antibodies, µl	Detection Antibody Diluent HB, µl	Total Volume, µl
96	150	150	2,700	3,000

Note: 20x singleplex beads allow multiplexing up to 20 analytes.

6. **Wash the plate three times** with **100 µl** wash buffer.
7. **Vortex** the diluted (1x) detection antibodies. **Add 25 µl** to each well.
8. Cover the plate with sealing tape and incubate at **850 ± 50 rpm** for **30 min** at RT. Meanwhile, prepare the Bio-Plex Manager Software protocol; enter standard S1 values and units provided in the assay kit.
9. With 10 min left in the incubation, **vortex** 100x streptavidin-phycoerythrin (SA-PE) for **5 sec** and quick-spin to collect liquid. **Dilute to 1x** as shown and protect from light.

Number of Wells	100x SA-PE, µl	Assay Buffer, µl	Total Volume, µl
96	60	5,940	6,000

10. **Wash the plate three times** with **100 µl** wash buffer.
11. **Vortex** the diluted (1x) SA-PE. **Add 50 µl** to each well.
12. Cover the plate with sealing tape and incubate at **850 ± 50 rpm** for **10 min** at RT.
13. **Wash the plate three times** with **100 µl** wash buffer.
14. Resuspend the beads in **125 µl** assay buffer. Cover and shake at **850 ± 50 rpm** for **30 sec**.
15. Remove the sealing tape and **read plate** using the following settings:

Instrument	RP1 (PMT)	DD Gates	Bead Events
Bio-Plex 3D*	Standard	Select MagPlex Beads	50
Bio-Plex 100, 200*	Low	5,000 (low); 25,000 (high)	50
Bio-Plex MAGPIX	N/A, use default instrument settings		

* Or similar Luminex System.

Bio-Plex Pro Human Cytokine Assays Quick Guide

The observed concentration ranges of the control apply only when standards and controls are prepared using the provided Bio-Plex Standard Diluent HB.

Assay Workflow

Add 50 µl 1x beads to wells

↓

Wash buffer: 2 x 100 µl

↓

Add 50 µl standards, samples, and controls; incubate on shaker at 850 rpm for 30 min at RT

↓

Wash buffer: 3 x 100 µl

↓

Add 25 µl 1x detection antibody; incubate on shaker at 850 rpm for 30 min at RT

↓

Wash buffer: 3 x 100 µl

↓

Add 50 µl 1x SA-PE; incubate on shaker at 850 rpm for 10 min at RT

↓

Wash buffer: 3 x 100 µl

↓

Resuspend in 125 µl assay buffer; shake at 850 rpm for 30 sec

↓

Acquire data on Bio-Plex System

BIO-RAD

Bio-Rad Laboratories, Inc.

Life Science Group

Web site bio-rad.com **USA** 1 800 424 6723 **Australia** 61 2 9914 2800
Austria 43 01 877 89019 **Belgium** 32 03 710 53 00 **Brazil** 55 11 3065 7550
Canada 1 905 364 3435 **China** 86 21 6169 8500 **Czech Republic** 36 01 459 6192
Denmark 45 04 452 10 00 **Finland** 35 08 980 422 00 **France** 33 01 479 593 00
Germany 49 089 3188 4393 **Hong Kong** 852 2789 3300 **Hungary** 36 01 459 6190
India 91 124 4029300 **Israel** 972 03 963 6050 **Italy** 39 02 49486600 **Japan** 81 3 6361 7000
Korea 82 2 3473 4460 **Mexico** 52 555 488 7670 **The Netherlands** 310 318 540 666
New Zealand 64 9 415 2280 **Norway** 47 0 233 841 30 **Poland** 36 01 459 6191
Portugal 351 21 4727717 **Russia** 7 495 721 14 04 **Singapore** 65 6415 3188
South Africa 36 01 459 6193 **Spain** 34 091 49 06 580 **Sweden** 46 08 555 127 00
Switzerland 41 0617 17 9555 **Taiwan** 886 2 2578 7189 **Thailand** 66 2 651 8311
United Arab Emirates 971 4 8187300 **United Kingdom** 44 01923 47 1301

10000076326 Ver C (10024985) US/EG

19-0840 1119 Sig 0119

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29.04.20

Dosati 95 campioni del FMS - Campioni letti sulla curva del 28-04-20

n° lotto reagenti pag. 147 book 16

04.05.20

Dosati altri 95 campioni del FMS.

08.05.20

Dosati 95 campioni del FMS

11.05.20

Terminati di dosare i campioni del FMS. Nella stessa piastra, essendoci spazio, ho dosato alcuni campioni dello studio transpenitico (nello specifico i PR mandati a Monaco ad analizzare e di cui in passato ho dosato la resistina).

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DATE DATA 29.04.20 al 11.05.20

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TITLE/TITOLO

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19.04.2021

Sono stati ordinati 5 Kit di citochine 27 plex per dosare i 385 PR spediti a Monaco - Si chiede di fare la curva solo nella prima piastra e di dosare i campioni in singolo.

BIO-RAD

Bio-Plex Pro Human Cytokine Panel Product Data Sheet

This product data sheet is for use with the Bio-Plex Pro Human Cytokine 27-Plex Panel (catalog #M500KCAF0Y), and individual singleplex set created from this panel.

Standard Lot #: 64301206
Control Lot #: 64347577
Storage: 4°C; do not freeze.
Stability: Kit components are guaranteed stable for a minimum of 6 months from date of purchase.

BIO-RAD

Bio-Plex Pro Human Cytokine Panel Product Data Sheet

Standard and Control Values

Enter the standard values and units into Bio-Plex Manager Software. The appearance of the standards or controls may vary from a white pellet to clear crystals.

Table 2. Lot-specific normalized standard (S1) values, expected control ranges, and bead regions.

Analyte	S1 Value, pg/ml	Control Range, pg/ml	Bead Region
Basic FGF	59,965	379-884	44
Eotaxin	1,488	7-17	43
G-CSF	71,162	550-1283	57
GM-CSF	7,265	49-113	34
IFN-γ	9,820	153-357	21
IL-1β	4,467	27-64	39
IL-1ra	176,363	1168-2726	25
IL-2	27,811	183-428	38
IL-4	3,422	16-36	52
IL-5	87,491	622-1452	33
IL-6	6,918	46-107	19
IL-7	42,680	304-709	74
IL-8	13,259	53-123	54
IL-9	20,125	126-294	77

Analyte	S1 Value, pg/ml	Control Range, pg/ml	Bead Region
IL-10	13,284	83-195	56
IL-12 (p70)	30,667	237-552	75
IL-13	5,599	32-75	51
IL-15	295,216	2155-5027	73
IL-17	43,802	239-557	76
IP-10	23,159	114-266	48
MCP-1	9,569	49-114	53
MIP-1α	1,461	9-21	55
MIP-1β	5,906	43-101	18
PDGF-BB	40,349	342-798	47
RANTES	22,379	203-475	37
TNF-α	54,925	281-656	36
VEGF	69,212	282-658	45

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BOOK PAGE

TITLE/TITOLO **PROJECT/PROGETTTO**

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BIO-RAD
Bio-Plex Pro Assays 10x Wash Buffer
60 ml
Cat. #171304040
Batch 64305532
Exp 2023-09-10
Store at 4°C
For research use only.
Warning
Bio-Rad Laboratories, Inc. www.bio-rad.com
Made in USA
See SDS for Product Safety Information.
10000047358 B

BIO-RAD
Control 64339338
Exp 2022-03-10
Store at 4°C
Bio-Plex Pro™ Hu Cyto Grp I 27-Plex
Coupled Magnetic Beads
(10x)
Warning
10000045161 C
Made in USA

BIO-RAD
Bio-Plex Sample Diluent HB
30 ml
Batch 64320434
Exp 2023-10-04
Store at 4°C
Part #10041561
For 1 x 96-well assay.
(01)03610520427064
(17)231004
(10)64320434
Bio-Rad Laboratories, Inc. www.bio-rad.com
Made in USA
10000045374 B

BIO-RAD
Control 64313984
Exp 2023-11-10
Store at 4°C
Streptavidin-PE (100x)
1 x 96-well
L005380 A
Made in USA

BIO-RAD
Control 64313984
Exp 2023-11-10
Store at 4°C
Streptavidin-PE (100x)
1 x 96-well
L005380 A
Made in USA

BIO-RAD
Bio-Plex Assay Buffer, 1x
50 ml
For 1 x 96-well multiplex assay
Batch 64322727
Exp 2023-12-16
Store at 2–8°C.
For research use only.
Part #10014822
(01)03610520411986
(17)231216
(10)64322727
Bio-Rad Laboratories, Inc. www.bio-rad.com
Made in USA
L000933 B

BIO-RAD
Bio-Plex Detection Antibody Diluent HB
3.5 ml
Batch 64329793
Exp 2023-11-11
Store at 4°C
Part #10032400
For 1 x 96-well assay.
(01)03610520424711
(17)231111
(10)64329793
Bio-Rad Laboratories, Inc. www.bio-rad.com
Made in USA
10000045449 B

BIO-RAD
Control 64339338
Exp 2022-06-08
Store at 4°C
Bio-Plex Pro™ Hu Cyto Grp I 27-Plex
Detection Antibodies (10x)
Warning

BIO-RAD
Control 64347577
Exp 2022-10-10
Store at 4°C
Bio-Plex Pro™ Hu Cytokine Screening Panel 50-Plex Control
Bio-Rad Laboratories, Inc.
Made in USA
10000092352 A
www.bio-rad.com

BIO-RAD
Bio-Plex Standard Diluent HB
10 ml
Batch 64330544
Exp 2023-11-12
Store at 4°C
Part #10022627
For 1 x 96-well assay
Bio-Rad Laboratories, Inc. www.bio-rad.com
Made in USA
10000045237 B

BIO-RAD
Control 64301206
Exp 2023-10-10
Store at 4°C
Bio-Plex Pro™ Hu Cytokine Screening Panel 50-Plex Standards
Bio-Rad Laboratories, Inc.
Made in USA
10000092097 A
www.bio-rad.com

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03.06.2023

Si ordinano 9 Kit delle citochine 27 plex per terminare la caratterizzazione dei GMS e dei PR.

Inizio col dosare i campioni del GMS (DT2)

Bio-Plex Pro Human Cytokine Panel Product Data Sheet

This product data sheet is for use with the Bio-Plex Pro Human Cytokine 27-Plex Panel (catalog #M500KCAF0Y), and individual singleplex set created from this panel.

Standard Lot #: 64466525
Control Lot #: 64490550
Storage: 4°C; do not freeze.
Stability: Kit components are guaranteed stable for a minimum of 6 months from date of purchase.

Bio-Plex Pro Human Cytokine Panel Product Data Sheet

Standard and Control Values

Enter the standard values and units into Bio-Plex Manager Software. The appearance of the standards or controls may vary from a white pellet to clear crystals.

Table 2. Lot-specific normalized standard (S1) values, expected control ranges, and bead regions

Analyte	S1 Value, pg/ml	Control Range, pg/ml	Bead Region
Basic FGF	90,034	379-883	44
Eotaxin		3-6	43
G-CSF	92,868	856-1,998	57
GM-CSF	5,906	26-60	34
IFN-γ	21,391	164-382	21
IL-1β	5,490	24-57	39
IL-1ra	148,907	598-1,395	25
IL-2	28,914	121-283	38
IL-4	3,263	7-17	52
IL-5	124,608	531-1,238	33
IL-6	3,868	16-38	19
IL-7	39,799	153-357	74
IL-8	10,752	28-64	54
IL-9	39,604	132-308	77

Analyte	S1 Value, pg/ml	Control Range, pg/ml	Bead Region
IL-10	12,203	49-115	56
(p70)	24,916	118-275	75
IL-13	3,641	14-32	51
IL-15	386,583	10	73
IL-17	43,247	152-355	76
IP-10	23,777	63-146	48
MCP-1	7,667	26-62	53
	830	3-8	
MIP-1β	8,177	45-105	18
PDGF-BB	45,700	240-561	47
RANTES	14,383	94-218	37
TNF-α	72,954	190-444	36
VEGF	86,387	224-523	45

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TITLE/TITOLO **PROJECT/PROGETTTO**

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BIO-RAD
Bio-Plex Pro Assays 10x Wash Buffer
60 ml
Cat. #171304040
Batch 64450888
Exp 2025-09-21
Store at 4°C.
For research use only.
Bio-Rad Laboratories, Inc. www.bio-rad.com
Made in USA
10000047356 C

BIO-RAD
Bio-Plex Pro Hu Cytokine Screening Panel 50-Plex Control
Batch 64490550
Exp 2026-04-12
4°C
Bio-Rad Laboratories, Inc
Made in USA
10000092352 B
www.bio-rad.com

BIO-RAD
Bio-Plex Pro Hu Cytokine Screening Panel 50-Plex Standards
Batch 64466525
Exp 2026-04-12
4°C
Bio-Rad Laboratories, Inc.
Made in USA
10000092097 B
www.bio-rad.com

BIO-RAD
Bio-Plex Standard Diluent HB
10 ml
Bio-Rad Laboratories, Inc. www.bio-rad.com
Made in USA
10000045237 B

BIO-RAD
Bio-Plex Detection Antibody Diluent HB
Batch 64451998
Exp 2025-10-18
Store at 4°C
Part #10032400
For 1 x 96-well assay
3.5 ml
Bio-Rad Laboratories, Inc. www.bio-rad.com
Made in USA
10000045449 B

BIO-RAD
Bio-Plex Assay Buffer, 1x
Batch 64418224
Exp 2025-05-02
Store at 2–8°C.
For research use only
Part #10014822
50 ml
For 1 x 96-well multiplex assay
Bio-Rad Laboratories, Inc. www.bio-rad.com
Made in USA
10000033 B

BIO-RAD
Bio-Plex Sample Diluent HB
Batch 64467961
Exp 2026-01-20
Store at 4°C.
Part #10041561
For 1 x 96-well assay.
30 ml
Bio-Rad Laboratories, Inc. www.bio-rad.com
Made in USA
10000045374 B

BIO-RAD
Bio-Plex Pro Hu Cyto Grp I 27-Plex
Detection Antibodies (10x)
Batch 64480556
Exp 2024-03-10
Store at 4°C.
Warning
10000045170 D
Made in USA

BIO-RAD
Streptavidin-Phycoerythrin (100x)
Batch 64467928
Exp 2025-02-13
Store at 4°C.
1 x 96-well
Cat. #9703897
10000360 B
Made in USA

BIO-RAD
Bio-Plex Pro Hu Cyto Grp I 27-Plex
Coupled Magnetic Beads (10x)
Batch 64480555
Exp 2025-05-02
Store at 4°C.
Warning
10000045191 D
Made in USA

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Lucie Jolie

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INFORMAZIONI CONFIDENZIALI E RISERVATE

Bio-Rad Laboratories S.r.l.

20054 Segrate – Milano
Via Cellini 18/A
Tel. +39 02 21609.1
Fax. +39 02 21609.399

Capitale Sociale € 5.681.027 i.v.
R.E.A. Milano 807526
C.F. / P.IVA 00801720152

Soggetta ad attività di direzione e coordinamento da parte di Bio-Rad Laboratories Inc (USA)

Spett.le
IRCCS FONDAZIONE CASA SOLLIEVO SOFFERENZA
Viale Padre Pio
71013 San Giovanni Rotondo (FG)

Segrate, 02/02/2026

DICHIARAZIONE ESCLUSIVITA'

La sottoscritta **BIO-RAD LABORATORIES Srl**, con sede legale e amministrativa a Segrate (MI) in Via Cellini 18/A, tel. 02.216091, fax 02.21609486, Codice Fiscale/Partita IVA 00801720152, con la presente

DICHIARA

che il prodotto di seguito indicato è esclusivo del catalogo prodotti Bio-Rad ed è stato disegnato per l'utilizzo sul sistema Bio-Plex 200 System with HTF:

Cod M500KCAF0Y Bio-Plex Pro Human Cytokine 27-plex Assay

In fede

BIO-RAD LABORATORIES SRL
Monica De Polo
Procuratore Speciale

Circulating Metabolites Associate With and Improve the Prediction of All-Cause Mortality in Type 2 Diabetes

Maria Giovanna Scarale,[1] Mario Mastroianno,[2] Cornelia Prehn,[3] Massimiliano Copetti,[4] Lucia Salvemini,[1] Jerzy Adamski,[5,6,7] Salvatore De Cosmo,[8] Vincenzo Trischitta,[1,9] and Claudia Menzaghi[1]

Diabetes 2022;71:1363–1370 | https://doi.org/10.2337/db22-0095

Death rate is increased in type 2 diabetes. Unraveling biomarkers of novel pathogenic pathways capable to identify high-risk patients is instrumental to tackle this burden. We investigated the association between serum metabolites and all-cause mortality in type 2 diabetes and then whether the associated metabolites mediate the effect of inflammation on mortality risk and improve ENFORCE (EstimatioN oF mORtality risk in type2 diabetic patiEnts) and RECODe (Risk Equation for Complications Of type 2 Diabetes), two well-established all-cause mortality prediction models in diabetes. Two cohorts comprising 856 individuals (279 all-cause deaths) were analyzed. Serum metabolites (n = 188) and pro- and anti-inflammatory cytokines (n = 7) were measured. In the pooled analysis, hexanoylcarnitine, kynurenine, and tryptophan were significantly and independently associated with mortality (hazard ratio [HR] 1.60 [95% CI 1.43–1.80]; 1.53 [1.37–1.71]; and 0.71 [0.62–0.80] per 1 SD). The kynurenine-to-tryptophan ratio (KTR), a proxy of indoleamine-2,3-dioxygenase, which degrades tryptophan to kynurenine and contributes to a proinflammatory status, mediated 42% of the significant association between the antiatherogenic interleukin (IL) 13 and mortality. Adding the three metabolites improved discrimination and reclassification (all $P < 0.01$) of both mortality prediction models. In type 2 diabetes, hexanoylcarnitine, tryptophan, and kynurenine are associated to and improve the prediction of all-cause mortality. Further studies are needed to investigate whether interventions aimed at reducing KTR also reduce the risk of death, especially in patients with low IL-13.

In patients with type 2 diabetes, the rate of mortality is almost twice as much as that in individuals without diabetes (1). Unraveling biomarkers capable of pointing to novel pathogenic pathways and identifying high-risk patients suitable for more aggressive management is, therefore, instrumental to tackle this heavy burden.

Few studies have, so far, investigated the role of circulating biomarkers in predicting the risk of mortality in patients with type 2 diabetes (2–7), and even fewer have been focused on serum metabolites (8–11). These latter studies have been limited to only a few metabolites (8,10,11) and/or have not addressed the role of associated metabolites in improving preexisting prediction models

[1]Research Unit of Diabetes and Endocrine Diseases, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico "Casa Sollievo della Sofferenza," San Giovanni Rotondo, Italy
[2]Scientific Direction, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico "Casa Sollievo della Sofferenza," San Giovanni Rotondo, Italy
[3]Metabolomics and Proteomics Core (MPC), Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany
[4]Biostatistics Unit, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico "Casa Sollievo della Sofferenza," San Giovanni Rotondo, Italy
[5]Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany
[6]Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore
[7]Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia
[8]Department of Clinical Sciences, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico "Casa Sollievo Della Sofferenza," San Giovanni Rotondo, Italy
[9]Department of Experimental Medicine, "Sapienza" University, Rome, Italy

Corresponding authors: Claudia Menzaghi, c.menzaghi@operapadrepio.it, or Vincenzo Trischitta, vincenzo.trischitta@uniroma1.it

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This article contains supplementary material online at https://doi.org/10.2337/figshare.19404350.

V.T. and C.M. shared the responsibility to oversee the entire study.

M.G.S. is currently affiliated with the University Centre for Statistics in the Biomedical Sciences (CUSSB), Vita-Salute San Raffaele University, Milan, Italy.

(8–11). In details, metabolites independently associated with mortality in type 2 diabetes are mostly amino acids (8), fatty acids (10), and choline (11). In the only paper in which a larger number of metabolites were analyzed, also N2,N2-dimethylguanosine, dimethylguanidino valerate, homocitrulline, 1-methyladenosine, acylcarnitine C10:3, urobilin, and hippurate were associated with the mortality rate (9). Unfortunately, the largest metabolomic study on all-cause mortality was performed in the general population and is, therefore, not usable for deriving information in the subset of patients with type 2 diabetes (12).

In this study, we investigated the association between a large number of circulating metabolites and all-cause mortality in individuals with type 2 diabetes. After unraveling and validating some metabolites as robustly and independently associated, we explored whether they mediate the effect of inflammatory cytokines on mortality risk and improve two well-established all-cause mortality prediction models in diabetes: ENFORCE (EstimatioN oF mORtality risk in type2 diabetic patiEnts), a validated user-friendly and freely available risk calculator based on a total of nine variables (13,14), and RECODe (Risk Equation for Complications Of type 2 Diabetes), a well-performing tool, based on a total of 14 variables that have been highly validated in several distinct sets, including both population-based and trial cohorts (15,16).

RESEARCH DESIGN AND METHODS

Participants

Two cohorts of patients with type 2 diabetes (diagnosed according to American Diabetes Association 2018 criteria) from Apulia, Central-Southern Italy were analyzed.

Gargano Mortality Study 1—Discovery Sample

The Gargano Mortality Study 1 (GMS 1) includes 1,028 patients recruited from 2000 to 2005 at the Endocrine Unit of Fondazione Istituto di Ricovero e Cura a Carattere Scientifico "Casa Sollievo della Sofferenza" in San Giovanni Rotondo, followed until December 2014, and has all-cause mortality as the end point. Serum metabolites were assessed in 536 participants (52.1%), constituting the eligible sample for the present analysis.

Gargano Mortality Study 2—Replication Sample

The Gargano Mortality Study 2 (GMS 2) includes 880 patients recruited from 2008 to 2010 at the Endocrine Unit of Fondazione Istituto di Ricovero e Cura a Carattere Scientifico "Casa Sollievo della Sofferenza" in San Giovanni Rotondo, followed until December 2019, and has all-cause mortality as the end point. For this specific analysis, a sample comprising 321 patients participating in an independent substudy of the role of kidney function on mortality rate. No individuals with an estimated glomerular filtration rate (eGFR) in the range of 60 to 69 mL/min/1.73 m^2 were analyzed.

For both studies, the vital status of participants was verified by interrogating the Italian Health Card Database upon data anonymization (https://sistemats1.sanita.finanze.it/wps/portal/) (6). For all studies, the only exclusion criterion was the presence of poor life expectancy for nondiabetes-related diseases (6).

Metabolite Quantification and Normalization

Metabolite profiling was measured using baseline fasting serum samples that had been stored at −80°C since collection. Metabolite quantification was performed in the Genome Analysis Center at the Helmholtz Zentrum München. The targeted metabolomics approach was based on liquid chromatography-electrospray ionization-tandem mass spectrometry and flow injection ionization- electrospray ionization-tandem mass spectrometry measurements by Absolute*IDQ* p180 Kit (Biocrates Life Sciences AG, Innsbruck, Austria). The assay allows simultaneous quantification of 188 metabolites out of 10 μL plasma and includes free carnitine, 40 acylcarnitines (Cx:y), 21 amino acids (19 proteinogenic + citrulline + ornithine), 21 biogenic amines, hexoses (sum of hexoses − ∼90–95% glucose), 90 glycerophospholipids (14 lysophosphatidylcholines [lysoPC] and 76 phosphatidylcholines [PC], and 15 sphingolipids [SMx:y]). For a full list of all quality-controlled metabolites, see Supplementary Table 1. The procedures for sample preparation and mass spectrometric measurements, as well as the metabolite nomenclature, have been described in detail previously (17,18). Three quality control samples (sex-mixed human plasma provided by the manufacturer) and one zero sample (PBS) were included in each randomized plate.

Data evaluation for quantification of metabolite concentrations and quality assessment was performed with the software MultiQuant 3.0.1 (SCIEX) and the Met*IDQ* software package, which is an integral part of the Absolute*IDQ* Kit. Metabolite concentrations were calculated using internal standards and reported in μmol/L.

Measurement of Circulating Cytokines

Serum IL-1β, IL-2, IL-4, IL-6, IL-13, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) circulating levels were measured in duplicate, using a multiplex detection 27-plex kit from Bio-Rad. The median coefficient of variation was <25% for all analyzed cytokines. Data were analyzed as previously described (7).

Statistical Analysis

Patients' baseline characteristics are reported as mean ± SD or median and interquartile range for continuous variables and frequency and percentage for categorical variables. Values of serum metabolites below the limit of detection values have been replaced by the limit of detection itself.

Correlations between metabolites were assessed using the Spearman correlation. All covariates with missing values <5% were imputed by random forest method. Because

Table 1—Clinical features of the two independent study cohorts

	GMS 1 (n = 535)	GMS 2 (n = 321)
Male sex	250 (46.7)	183 (57.0)
Age at recruitment (years)	62.9 ± 9.8	59.7 ± 10.3
Smoking habit	40 (7.5)	55 (17.1)
Diabetes duration (years)	11.0 ± 9.0	12.6 ± 9.8
BMI (kg/m^2)	31.1 ± 5.8	31.5 ± 6.4
HbA_{1c} (%)	8.7 ± 1.9	8.2 ± 1.8
HbA_{1c} (mmol/mol)	72.0 ± 15.7	66.0 ± 14.5
eGFR (mL/min/1.73 m^2)	70.6 ± 21.1	83.7 ± 30.3
Antihypertensive therapy	340 (63.4)	251 (78.2)
Insulin therapy	226 (42.2)	137 (42.7)
Statin therapy	164 (30.7)	232 (72.3)
Follow-up (years)	10.0 ± 3.9	8.6 ± 2.6
Follow-up (py)	5,346.2	2,763.9
All-cause death	198 (37.0)	81 (25.2)
Incident rate of all-cause death events (n events per 100 py)* (95% CI)	2.8 (2.4–3.3)	2.2 (1.8–2.8)

Continuous variables are reported as mean ± SD, whereas categorical variables are reported as total frequencies and percentages. eGFR, was calculated using the CKD-EPI equation (50). *Adjusted for age and sex.

of skewed distribution and for comparability between different metabolites, their concentrations were logarithmically transformed and then standardized.

Time variable was defined as the time between the baseline examination and the date of the event (i.e., all-cause mortality) or the date of the last available clinical follow-up for subjects who did not experience the event. The incidence rate for all-cause mortality is expressed as the number of events per 100 person-years (py).

To assess the association between the detected serum metabolites levels and all-cause mortality in the discovery sample (i.e., GMS 1), Bonferroni adjustment for multiple comparisons was used to determine the significance threshold in an unadjusted Cox proportional hazard model. Because of the potential correlation between metabolites, we next evaluated the independent associations of Bonferroni-survived metabolites using a forward-backward stepwise analysis (19) in a fully adjusted model comprising age at recruitment, sex, smoking habit, BMI, glycated hemoglobin A_{1c} (HbA_{1c}), eGFR, diabetes duration, and ongoing treatments.

Associations were then validated in an independent cohort (i.e., GMS 2), considering the fully adjusted model.

When analyses were run in the pooled sample, comprising both GMS 1 and GMS 2, they were also adjusted for study cohort factor considered as a random effect to have more robust estimates. Risks are reported as hazard ratios (HRs) along with their 95% CIs per 1 SD increase of each single metabolite.

Mediation analysis allowing for exposure-mediator interactions, and causal interpretation was carried out as previously described (20). The 95% CI of the mediation effect was computed by bootstrap based on 1,000 resamplings with replacement.

To examine whether the validated associated metabolites increase the accuracy of all-cause mortality prediction models in type 2 diabetes, two different well-established tools were used: ENFORCE (14) and RECODe (15). Discrimination was measured by survival c statistics (21) while improvement in discrimination was assessed by the Δ c statistics and survival version of the relative integrated discrimination improvement (rIDI) (22). 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 directions (upward and downward, respectively) from the base to the new model, was evaluated (23). The 95% CIs for discrimination and reclassification measures were computed by bootstrap. A P value <0.05 was considered significant. All analyses were performed using SAS 9.4 software (SAS Institute, Cary, NC) and R software packages survival and coxme (R Core Team, 2021).

Data and Resource Availability

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

RESULTS

Clinical features of patients from GMS 1 and GMS 2 as well as duration of follow-up and number of events are summarized in Table 1. In GMS 1, 198 deaths occurred during follow-up (10.0 ± 3.9 years; 5,346.2 py). In GMS 2,

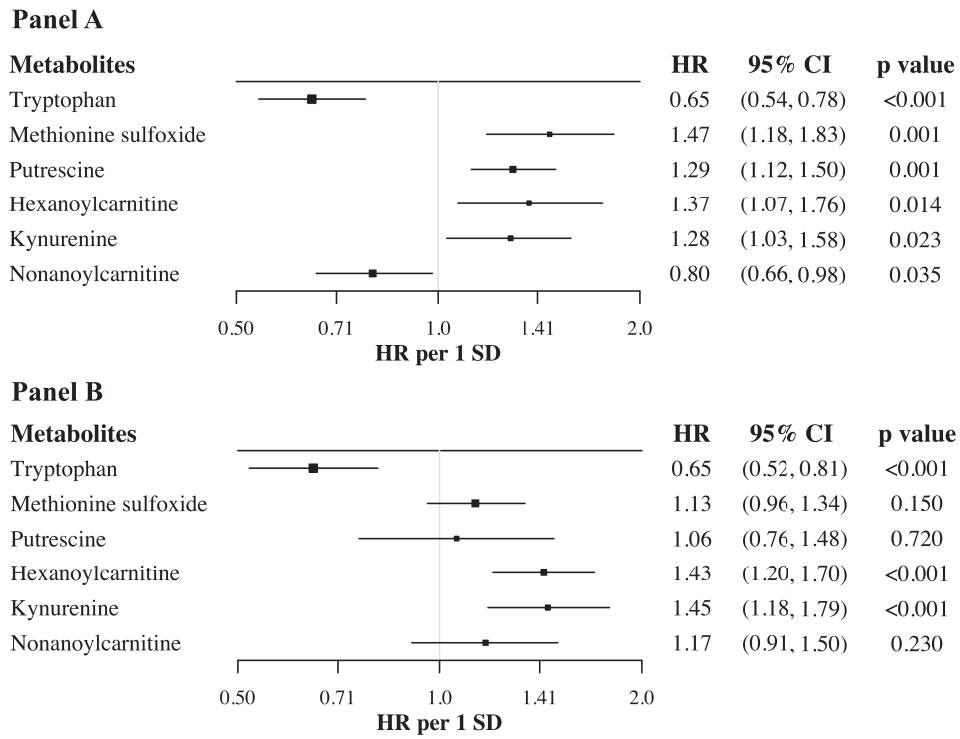

Figure 1— Independent associations between metabolites and all-cause mortality. HRs and 95% CIs for independent associations between metabolites and mortality in GMS 1 (*A*) and GMS 2 (*B*). HRs (per 1 SD increase in each metabolite concentration) were estimated in Cox regression models, adjusting for age at recruitment, sex, smoking habit, BMI, HbA_{1c}, eGFR, diabetes duration, and ongoing treatments.

81 deaths occurred during follow-up (8.6 ± 2.6 years; 2,763.9 py).

Of the 188 metabolites we measured, 5 (i.e., carnosine, L-3,4-dihydroxyphenylalanine, dopamine, nitrotyrosine, and *cis*-4-hydroxyproline) were excluded from the analyses because their value was below the detection limit in >80% samples. Also, creatinine data from the metabolomic assay (Supplementary Table 1) were not analyzed because serum creatinine values from standard baseline clinical chemistry measurements were available and used to compute eGFR.

In the GMS 1, 49 of the 182 metabolites analyzed were significantly associated with all-cause mortality after Bonferroni correction (threshold *P* value being $0.05/182 = 2.7 \times 10^{-4}$) (Supplementary Table 2).

Among these metabolites, the pairwise correlation ranged from –0.20 to 0.92 (Supplementary Fig. 1). After a stepwise (forward-backward) procedure, six metabolites remained independently associated in a fully adjusted model including age at recruitment, sex, smoking habit, BMI, HbA_{1c}, diabetes duration, eGFR, and ongoing treatments (four with increased and two with decreased risk of all-cause mortality) (Fig. 1*A*). Three of them, belonging to amino acid, biogenic amines, and acylcarnitine superfamilies, were validated in the totally independent GMS 2 cohort (Fig. 1*B*).

When data from the two independent cohorts, comprising 856 individuals and 279 events, were meta-analyzed, the three validated associations, hexanoylcarnitine (HR 1.60, 95% CI 1.43–1.80), kynurenine (HR 1.53, 95% CI 1.37–1.71), and tryptophan (HR 0.71, 95% CI 0.62–0.80) were highly significantly associated with all-cause mortality (all $P < 0.001$, per 1 SD increase), with no difference between male and female participants (*P* of sex heterogeneity = 0.17, 0.67, and 0.9, respectively). Also the kynurenine-to-tryptophan ratio (KTR), which has been previously associated with metabolic syndrome (24,25), cardiovascular disease (26,27), and mortality (28,29), was associated with all-cause death (HR 1.41, 95% CI 1.21–1.64 per 1 SD increase).

Inflammatory Cytokines, KTR, and All-Cause Mortality

Previous findings suggest that the indoleamine 2,3-dioxygenase (IDO) enzymatic activity and its proxy KTR are stimulated by (30) and are likely to mediate the role of inflammatory cytokines on all-cause death (29). We then measured and investigated the association between several cytokines related to low-grade inflammation and both KTR and all-cause mortality. Five of those we tested (Supplementary Table 3), including IL-4, IL-6, IL-13, INF-γ, and TNF-α, were in fact associated with KTR (see Supplementary Table 4). Of these, four (but not IL-4) were also associated with all-cause mortality in our fully adjusted model (Table 2, left side). Interestingly, when KTR was also added into the model, the associations with

Table 2—Univariable associations between cytokines and all-cause mortality in the pooled sample (n = 810; 256 events)

	Associations with all-cause mortality		Associations with all-cause mortality adjusted also for KTR	
Cytokines	HR (95% CI)	*P*	HR (95% CI)	*P*
IL-6	1.41 (1.26–1.59)	<0.0001	1.44 (1.28–1.62)	<0.0001
IL-13	0.86 (0.74–0.99)	0.036	0.91 (0.79–1.05)	0.21
IFN-γ	1.53 (1.26–1.87)	<0.0001	1.63 (1.33–2.00)	<0.0001
TNF-α	1.41 (1.19–1.67)	<0.0001	1.43 (1.20–1.70)	<0.0001

HRs were estimated in Cox regression models, adjusting for age at recruitment, sex, smoking habit, BMI, HbA_{1c}, eGFR, diabetes duration, ongoing treatments, and study cohort. HRs reflect the risk per 1 SD increase in each cytokine concentration.

increased risk of death of IL-6, IFN-γ, and TNF-α, were virtually identical, while the protective effect of IL-13 was attenuated at the point of being no longer significant (Table 2, right side). Further, mediation analysis showed that a significant and nontrivial proportion (i.e., 42% [95% CI 14–199]) of the association between IL-13 and all-cause mortality went through KTR (Fig. 2).

Adding Metabolites to the ENFORCE and RECODe Mortality Prediction Models

In the pooled sample, discrimination ability (*c* statistic) of hexanoylcarnitine, kynurenine, and tryptophan considered together was 0.71 (95% CI 0.55–0.86) (Table 3). We then tested the effect of adding these three metabolites on top of ENFORCE, a well-performing, validated, and freely available (https://www.operapadrepio.it/enforce/enforce.php) prediction model for 6-year all-cause mortality in patients with type 2 diabetes. To this purpose, GMS 1 and GMS 2, comprising a total of 856 patients and 140 deaths (at 6 years) in which our ENFORCE model was applicable, were used. The addition of the three metabolites on top of ENFORCE resulted in a significant improvement of both *c* statistic and rIDI (Table 3). In addition, cNRI values showed a significant improvement in reclassification, mainly due to nonevents correctly reclassified (Table 3).

The ability of the three metabolites in improving the prediction of all-cause death was also tested in RECODe, a well-performing and validated model for 10-year mortality in patients with type 2 diabetes. To this purpose, a total of 856 patients and 230 deaths (at 10 years) from both cohorts were available. Also in this case, a significant improvement was observed both in discrimination (*c* statistic and rIDI) and reclassification (cNRI) measures (Table 3).

In all, these data consistently show that serum levels of hexanoylcarnitine, kynurenine, and tryptophan improve well-established prediction models of all-cause mortality in patients with type 2 diabetes in terms of both discrimination and reclassification.

DISCUSSION

This study used a discovery and replication design to rigorously evaluate the association between 182 metabolites measured through targeted metabolomics and all-cause mortality in 856 people with type 2 diabetes. Three biologically plausible metabolites (i.e., hexanoylcarnitine, tryptophan, and kynurenine) were independently and consistently associated

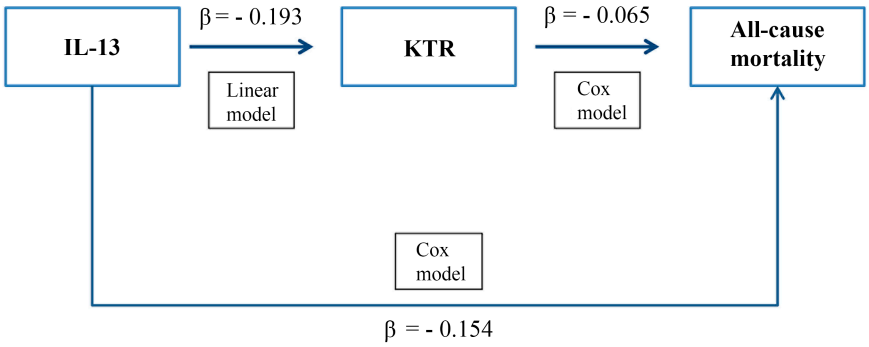

Figure 2—Mediation model showing the role of KTR on the association between IL-13 and all-cause mortality in the pooled sample. Mediation analysis was performed in a fully adjusted model, comprising study cohort, age at recruitment, sex, smoking habit, BMI, HbA_{1c}, diabetes duration, eGFR, and ongoing treatments. β, standardized coefficient of regression. The total effect of IL-13 (β = −0.154) on the outcome partly passes through KTR (β of the KTR-mediated effect of IL-13 = −0.065). The proportion explained by the KTR is equal to 42% (95% CI 14-199) (i.e., 0.065/0.154).

Table 3—Prediction of all-cause mortality by metabolites and by ENFORCE and RECODe (without and with metabolites)

	Discrimination			Reclassification		
Prediction models	*c* statistics (95% CI)	Δ *c* statistics (*P* value)	% rIDI (*P* value)	%1/2 cNRI (*P* value)	% Events (*P* value)	% Nonevents (*P* value)
Hexanoylcarnitine, tryptophan, kynurenine	0.71 (0.55–0.86)					
ENFORCE	0.77 (0.74–0.80)					
ENFORCE + hexanoylcarnitine, tryptophan, kynurenine	0.79 (0.75–0.82)	0.02 (0.01)	14.9 (0.001)	18 (<0.001)	6 (0.18)	30 (<0.001)
RECODe	0.75 (0.72–0.78)					
RECODe + hexanoylcarnitine, tryptophan, kynurenine	0.77 (0.75–0.80)	0.02 (0.005)	18.6 (0.001)	14 (<0.001)	11 (0.001)	18 (<0.001)

All *P* values are referred to comparisons vs. the same base model (i.e., with no metabolites).

with a higher risk of mortality in two independent cohorts of patients with type 2 diabetes. Of note, taking into account eGFR, a strong predictor of mortality (31), did not diminish the relationship between baseline metabolites and death. This indicates that the three associations are independent of renal function, a key point to be addressed when measuring metabolites whose serum concentration is controlled also by renal clearance (32). The same was noticed when BMI was taken into account, thus suggesting that adiposity does not play a major role on the observed associations. Given the strong relationships between the three metabolites and all-cause mortality, it is not surprising that when considered together these markers show a good discrimination ability in predicting the risk of death. More importantly, our data showing that the three metabolites together improve both discrimination and reclassification of two well-established prediction models of all-cause death in type 2 diabetes (14,15) may be of clinical impact.

Increased levels of hexanoylcarnitine, a medium-chain acylcarnitine, which is, along with other members of the acylcarnitines superfamily, a cardiometabolic risk factor in type 2 diabetes (33), has been previously associated with all-cause mortality and cancer-related mortality in men who smoke (34). Accumulation of medium-chain acylcarnitines may be indicative of inefficient β-oxidation of fatty acid as a consequence of altered mitochondrial metabolism, which is known to contribute to both insulin resistance and vascular inflammation (35,36). Tryptophan, an essential amino acid important for protein synthesis (37), has been previously associated with decreased risk of mortality in patients with type 2 diabetes, although with a weaker effect compared with ours (9), while its breakdown product, 5 methoxy-tryptophan along the serotonin pathway, is an anti-inflammatory agent with favorable effects on arterial vessels and renal function (38,39). Kynurenine, a product of tryptophan degradation along the kynurenine pathway, has been associated with risk of cardiovascular events in the general population and in several additional clinical settings (27,40). This pathway, primarily directed toward the production of NAD+ for energy metabolism (41), plays crucial roles in inflammation (41) and when dysregulated is linked to several diseases and disorders (42).

Given the opposite association with mortality rate of tryptophan and kynurenine, it was not surprising that their ratio, KTR, a marker of mortality risk in the general population (29), was associated with the risk of death also in our sample.

Interestingly, KTR is a reliable marker of the IDO activity that degrades tryptophan into kynurenine (41), triggering the homonymous aforementioned deleterious pathway. Furthermore, an increased IDO activity also reduces tryptophan metabolism in the beneficial alternative serotonin pathway. Overall, a shift toward an unhealthy proinflammatory status and subsequent vascular damage is likely to be the final net result of the described alteration of tryptophan metabolism due to IDO overactivity.

IDO is under the control of pro- and anti-inflammatory cytokines, is increased in conditions of low-grade inflammation (30), and is coherently associated with metabolic syndrome (24,25), cardiovascular disease (26,27), and mortality in several clinical sets (28,29). The robust association between KTR and all-cause mortality, as well as the evidence that KTR mediates a nontrivial proportion of the IL-13 antiatherogenic protective effect (43) on mortality risk we here report, is therefore along the same line of previous findings (24–29) and supports the role of KTR (as a proxy of IDO activity) in shaping survival probability (28,29) also in type 2 diabetes. Interestingly, tryptophan supplementation directly or through lifestyle intervention (44) has been reported to prevent and treat cardiovascular disease (45), social behavior, mood and sleep disorders, and several additional chronic diseases (46), possibly by priming the beneficial serotonin pathway.

As said, the addition of hexanoylcarnitine, tryptophan, and kynurenine considered together improves the discrimination ability of both ENFORCE (14) and RECODe (15), two established prediction models of all-cause mortality in patients with type 2 diabetes. Although statistically

significant, the improvement of the *c* statistic 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 (47). It is also important noticing that in both models, the percentage rIDI, also an index of discrimination, is more than twice the threshold requested by international guidelines for adding new biomarkers on top of established prediction models (48). This important statistical and clinical improvement was further reinforced by data from reclassification measures. In fact, adding the three metabolites to ENFORCE and RECODe made it possible to correctly reclassify a consistent proportion of individuals, especially nonevents, thus reducing the risk of overestimation.

Our study has several strengths. We used a rigorous study design with discovery and replication cohorts prospectively analyzed with complete information, including standardized clinical evaluations and mortality validated by death certificates. We also used quality-controlled metabolomics profiling and correction for multiple comparisons. Previous studies of mortality and the metabolome in patients with type 2 diabetes have evaluated only few metabolites (8,10,11) and/or have not addressed the role of mortality-associated metabolites in improving preexisting and established prediction models (8–11). Our study, instead, evaluated a large number of metabolites and discovered new associated markers that improve two well-established and validated prediction models (14,15), thus making our finding implementable in the real-life clinical set.

Conversely, we have to recognize several limitations, including the relatively small size of the cohorts, the fact that they are geographically close to each other, thus limiting the generalizability of our finding, and finally, the lack of data on cause-specific mortality, which does not allow us to address the role of important shapers of life expectancy, including cardiovascular disease and cancer.

In conclusion, in patients with type 2 diabetes, hexanoylcarnitine, tryptophan, and kynurenine are reproducible risk factors for all-cause death and improve established, well-performing prediction models of mortality risk. We believe that a study like ours paves the way for different precision medicine approaches in type 2 diabetes, albeit with different timelines. On the precision prediction side (49), before our data become implementable in daily clinical work, the mortality-associated metabolites need to be enrolled in a standard clinical chemistry assay and validated in larger and less homogeneous cohorts. Conversely, on the treatment side, it is still necessary to investigate whether directing tryptophan metabolism toward the serotonin pathway reduces the risk of death in individuals with diabetes, particularly those with low IL-13 values, before a precision therapeutic approach can be implemented.

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Clinical Research Article

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

Claudia Menzaghi,[1] Antonella Marucci,[1] Mario Mastroianno,[2] Giulio Di Ciaccia,[1] Maria Pia Armillotta,[1] Cornelia Prehn,[3] Lucia Salvemini,[1] Davide Mangiacotti,[1] Jerzy Adamski,[4,5,6] Andrea Fontana,[7] Salvatore De Cosmo,[8] Olga Lamacchia,[9] Massimiliano Copetti,[7] and Vincenzo Trischitta[1,10]

[1]Research Unit of Diabetes and Endocrine Diseases, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico "Casa Sollievo della Sofferenza," 71013 San Giovanni Rotondo, Italy
[2]Scientific Direction, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico "Casa Sollievo della Sofferenza," 71013 San Giovanni Rotondo, Italy
[3]Metabolomics and Proteomics Core, Helmholtz Zentrum München, German Research Center for Environmental Health, 85764 Neuherberg, Germany
[4]Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, 85764 Neuherberg, Germany
[5]Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117597, Singapore
[6]Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, 1000 Ljubljana, Slovenia
[7]Biostatistics Unit, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico "Casa Sollievo della Sofferenza," 71013 San Giovanni Rotondo, Italy
[8]Unit of Internal Medicine, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico "Casa Sollievo della Sofferenza," 71013 San Giovanni Rotondo, Italy
[9]Endocrinology Unit, Department of Medical and Surgical Sciences, University of Foggia, 71122 Foggia, Italy
[10]Department of Experimental Medicine, Sapienza University of Rome, 00185 Rome, Italy

Correspondence: Claudia Menzaghi, PhD, Research Unit of Diabetes and Endocrine Diseases, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico "Casa Sollievo della Sofferenza", Viale Padre Pio, 71013 San Giovanni Rotondo, Italy. Email: c.menzaghi@operapadrepio.it; or Vincenzo Trischitta, MD, Research Unit of Diabetes and Endocrine Diseases, Fondazione Istituto di Ricovero e Cura aCarattere Scientifico "Casa Sollievo della Sofferenza," 71013 San Giovanni Rotondo, Italy. Email: vincenzo.trischitta@uniroma1.it.

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 ~5×10^5 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

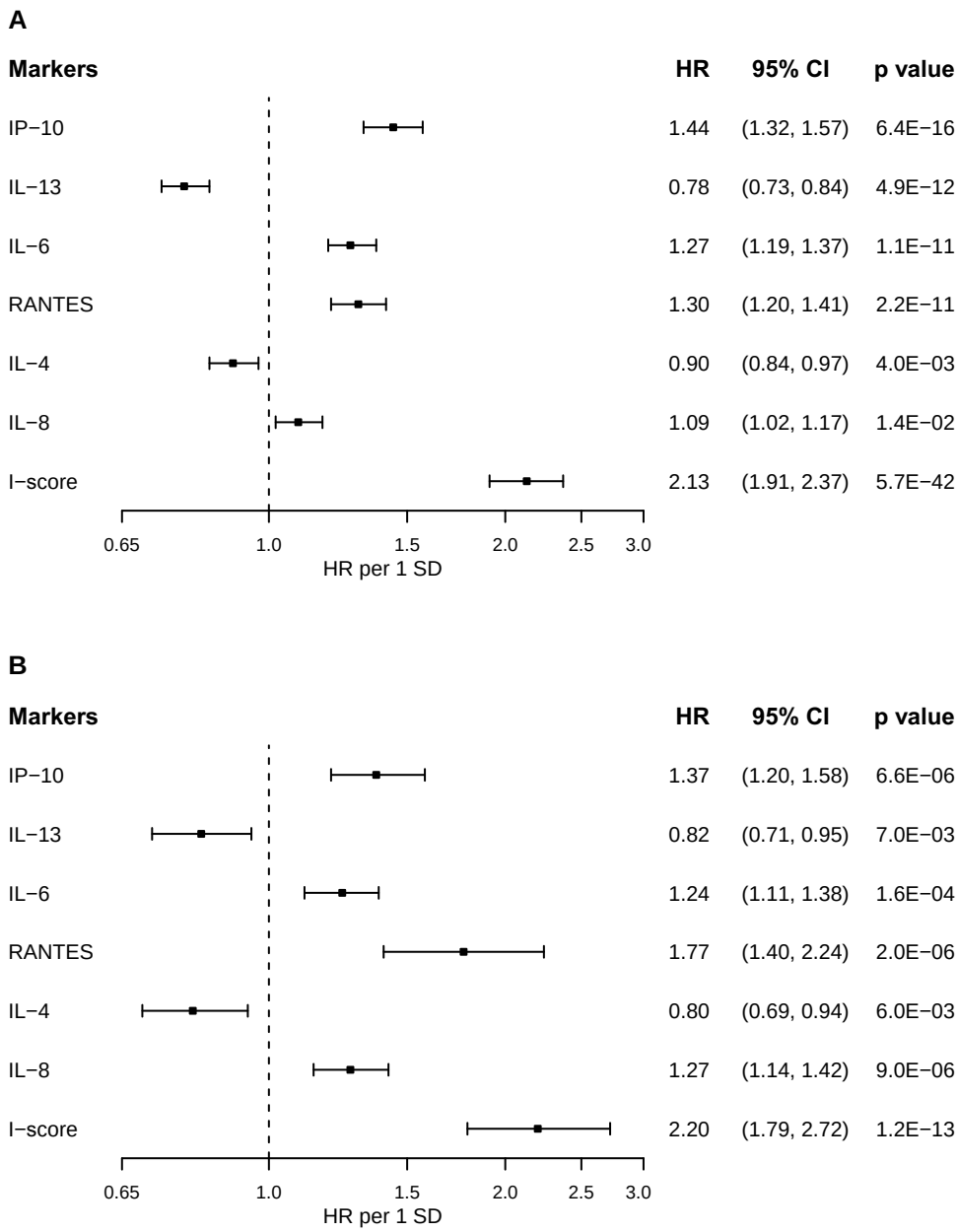

Figure 1. Associations between inflammatory markers, I score, and all-cause mortality. Hazard ratios (HRs) and 95% CIs per 1 SD increase of each inflammatory marker and the I score in aGMS (A) and FMS (B) were estimated in Cox regression models, adjusting for age at recruitment, gender, smoking habit, 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

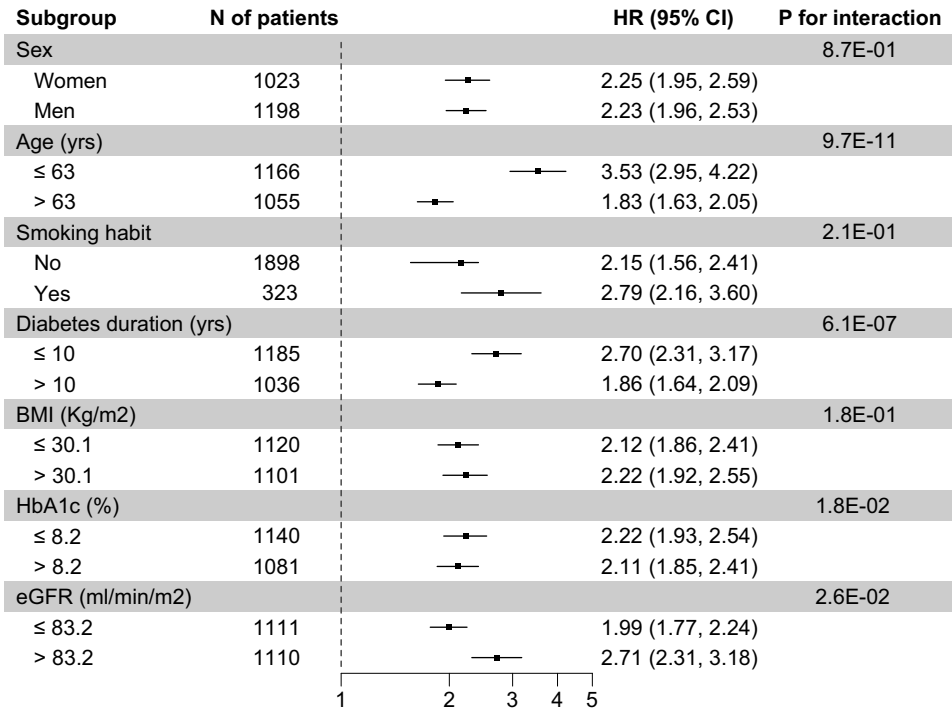

Figure 2. Associations between the I score and all-cause mortality in the combined sample (aGMS + FMS) by subgroups of demographical and clinical features. Hazard ratios (HRs) and 95% CI per 1 SD of I score increase were estimated by Cox regression models, adjusting for age at recruitment, gender, smoking 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

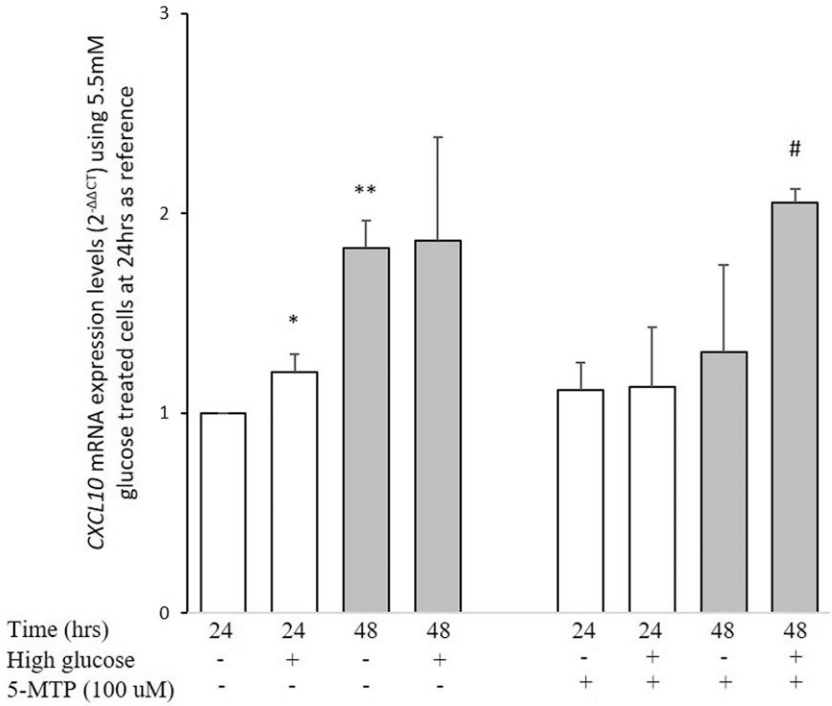

Figure 3. Glucose and 5-MTP induced C-X-C motif chemokine ligand 10 (*CXCL10)* mRNA expression. Bars represent *CXCL10* mRNA expression (which encodes for the chemokine IP-10), calculated as indicated in Materials and Methods, induced by high glucose (25 mM) concentration with or without 100 μM 5-MTP 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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