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Effect of long-term Mediterranean versus low-fat diet on neutrophil count, and type 2 diabetes mellitus remission in patients with coronary heart disease: results from the CORDIOPREV study

Hatim Boughanem [1,2,3,4,8], Francisco M. Gutierrez-Mariscal [1,2,3,4,8], Antonio Pablo Arenas-de Larriva [1,2,3,4], José D. Torres-Peña [1,2,3,4], Juan L. Romero-Cabrera [1,2,3,4], Oriol Alberto Rangel-Zuñiga [1,2,3,4], Helena García-Fernández [1,2,3,4], Alicia Podadera-Herreros [1,2,3,4], Fernando Rodríguez-Cantalejo [5], Oliver Soehnlein [6], Manuel Macias-Gonzalez [4,7], Francisco J. Tinahones [4,7], Elena M. Yubero Serrano [1,2,3,4], Pablo Perez-Martinez [1,2,3,4], Javier Delgado-Lista [1,2,3,4,9] and José López-Miranda [1,2,3,4,9] ✉

BACKGROUND: Recent evidence links diet and physical activity with type 2 diabetes mellitus (T2DM) remission, but emerging findings suggest that immune system dysregulation may play a crucial role. This study aimed to investigate the associations between neutrophils and T2DM remission.

METHODS: We conducted a comprehensive analysis of newly-diagnosed T2DM patients ($N = 183$) from the CORDIOPREV study, without glucose-lowering treatment, and were randomized to follow either a Mediterranean or low-fat diet. Patients were classified into two groups: Responders, who achieved T2DM remission ($n = 73$), and Non-Responders, who did not achieve remission during the 5-year dietary intervention ($n = 110$). Neutrophil count and their related-ratio (NER, NBR, NLR and NHR, normalized with erythrocytes, basophils, lymphocytes, and HDL respectively) were measured at the baseline and 5 years of follow-up.

RESULTS: The lowest baseline tertile of neutrophil count was associated with an increased likelihood of T2DM remission among patients following a Mediterranean diet (but not for low-fat diet) when compared with the highest tertile [adjusted HR of 4.23 (95% CI: 1.53–11.69)], in which similar results were observed for NER and NHR. When considering clinical and neutrophil variables, the predictive capacity of this model yielded an AUC of 0.783 (95% CI: 0.680–0.822). Furthermore, after 5-years, Responders exhibited lower neutrophil count compared to Non-responders ($p = 0.006$) and a significant decrease in neutrophil count ($p = 0.001$) compared to baseline. This decrease in neutrophil count in Responders who consumed a Mediterranean diet exhibited a significant increase in Insulin Sensitivity and Disposition Index ($p = 0.011$ and $p = 0.018$) after the follow-up period.

CONCLUSION: These findings suggest that neutrophil count can help in identifying patients that are more likely to achieve T2DM remission following a Mediterranean diet, suggesting a role on insulin sensitivity and β-cell function. Further research holds promise for providing valuable insights into the pathophysiology of T2DM.

CLINICAL TRIAL REGISTRATION: ID: NCT00924937; URL Clinical trial: https://clinicaltrials.gov/study/NCT00924937?cond=NCT00924937&rank=1.

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) represents a significant public health challenge. Globally, an estimated 537 million adults are currently living with T2DM [International Diabetes Federation (IDF), 2021]. With a global prevalence on the rise, IDF projections suggest that by 2045, approximately 783 million adults, will be affected by diabetes, marking a 46% increase [1]. Mismanaged T2DM can lead to serious complications, particularly in microvascular and macrovascular damage. Furthermore, immune dysfunction is another major complication that develops from the direct effects of hyperglycemia on cellular immunity, including reduced neutrophil function and disorders of humoral immunity [2]. Solid research indicates a dysregulation of neutrophil function, metabolism and abundance in T2DM conditions. Regarding

[1]Lipids and Atherosclerosis Unit, Department of Internal Medicine, Hospital Universitario Reina Sofía, Cordoba, Spain. [2]Department of Medical and Surgical Sciences, Universidad de Cordoba, Cordoba, Spain. [3]Maimonides Institute for Biomedical Research in Cordoba (IMIBIC), Cordoba, Spain. [4]CIBER Fisiopatologia Obesidad y Nutricion (CIBEROBN), Instituto de Salud Carlos III, Madrid, Spain. [5]Biochemical Laboratory, Reina Sofia University Hospital, Córdoba, Spain. [6]Institute of Experimental Pathology (ExPat), Center of Molecular Biology of Inflammation (ZMBE), University of Münster, Münster, Germany. [7]Department of Endocrinology and Nutrition, Virgen de la Victoria University Hospital, Malaga, Spain. Institute of Biomedical Research in Malaga (IBIMA)-Bionand Platform, University of Malaga, Malaga, Spain. [8]These authors contributed equally: Hatim Boughanem, Francisco M. Gutierrez-Mariscal. [9]These authors jointly supervised this work: Javier Delgado-Lista, José López-Miranda. ✉email: jlopezmir@uco.es

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neutrophil function, hyperglycemic states may lead to reduced mobilization of neutrophils, decreased phagocytic activity, and an impairment of NETosis (the release of neutrophil extracellular traps), ultimately resulting in a reduced immune response in T2DM [3, 4]. In the context of neutrophil abundance, several studies have shown that neutrophil-related ratios (considered as indicator of subclinical inflammation) are promising predictive markers for insulin resistance, prediabetes, and T2DM [5, 6]. However, more studies are needed to address conflictive findings.

Recent evidence suggests that T2DM is reversible, according to the new consensus report from the international endocrine and diabetes societies [7], in which they defined criteria for T2DM remission. Overall, lifestyle interventions are strongly associated with significant effects on T2DM remission, specially focusing on diet and physical activity [8]. Within the CORDIOPREV (CORonary Diet Intervention with Olive oil and cardiovascular PREVention) study, it has been previously demonstrated that T2DM remission, in newly-diagnosed T2DM patients with coronary heart disease (CHD), is associated with significant metabolic changes. These changes include alterations in gut diversity, epigenetic modifications, and shifts in metabolites, among other factors [9–12]. Nonetheless, the current situation underscores the necessity to explore novel mechanisms for predicting T2DM remission. To date, we remain unaware of markers that can predict which patients are more likely to experience significant improvements by adopting lifestyle changes. In this study, we analyze the absolute neutrophil count and other neutrophil-related ratios as potential predictive biomarkers for T2DM remission, (those who achieved remission from T2DM) after 5 years of dietary intervention (employing a low fat and a Mediterranean diet, without weight loss nor pharmacological treatment).

MATERIALS AND METHODS

Study design and participants

The current study was conducted as part of the CORDIOPREV study (https://clinicaltrials.gov/study/NCT00924937?cond=NCT00924937&rank=1 and the number of registrations was NCT00924937). The CORDIOPREV study is a randomized, single-blinded, controlled dietary intervention trial that involved 1002 patients with CHD, in which they were recruited between July 2009 and February 2012. The CHD patients had experienced a coronary event more than six months prior to their enrollment. Furthermore, they were assigned to follow dietary intervention for seven years, in addition to receiving conventional treatment for CHD. Dietary intervention was assigned to follow two different dietary models, consisting in a low-fat diet and a Mediterranean diet. The study's rationale, methodology, and baseline characteristics of the participants as well other findings regarding to the main outcome of this study have been previously detailed [13]. The study protocol was developed in accordance with the principles outlined in the Declaration of Helsinki and was approved by the Institutional Review Board of the Reina Sofia University Hospital in Córdoba, Spain. All participants provided written informed consent prior participating in the study.

Criteria selection for type 2 diabetes mellitus remission

Within the CORDIOPREV study, patients who had been diagnosed with T2DM at the outset of the research and were not undergoing antidiabetic treatment were included. The diabetes diagnosis adhered to the criteria established by the American Diabetes Association (ADA) [14]. These criteria involved fasting glucose levels of 126 mg/dL or higher, 2-h glucose levels during an oral glucose tolerance test (OGTT) of 200 mg/dL or higher, or an HbA1c level of 6.5% or higher [15]. T2DM remission was defined as achieving and maintaining specific blood sugar levels for at least two consecutive years without the use of diabetes medication, according to the International Experts outline of T2DM Remission criteria of ADA. These target levels included HbA1c < 6.5%, fasting plasma glucose < 126 mg/dL, and a 2-h plasma glucose level of less than 200 mg/dL following a $75 \times g$ OGTT. Patients were monitored annually starting from the first year of follow-up, and their status as either in remission or still managing diabetes was determined at the fifth year of the study [7].

Patients who had diabetes diagnosed at the beginning of the study and were not receiving glucose-lowering treatment were included in the CORDIOPREV-DIRECT study (190 out of 1002 patients). Of these, 7 patients could not be included due to their inability to perform the diagnostic test used in this work. Thus, the remaining 183 in which had T2DM at baseline and whom accomplished inclusion criteria, were included in the study. After 5-year of follow, 73 of them remitted from T2DM without the administration of diabetes medications, in which they were known as Responders. Meanwhile, Non-responders were patients who did no remitted from diabetes, during the follow-up period ($N = 110$). Data at the 5-year follow-up were unavailable for 7 patients due to their inability to undergo the diagnostic tests. Five of these patients passed away, and two abandoned the dietary intervention and did not permit clinical follow-up via electronic health records. Consequently, the analysis of follow-up included data from 176 patients.

Dietary intervention

Participants were randomly assigned to follow one of two diets within the CORDIOPREV design: a Mediterranean diet [less than 10% calories from saturated fat, 22% from monounsaturated fat (MUFA), 6% from polyunsaturated fat (PUFA) (a minimum of 35% of calories from fat), along with 15% protein, and a maximum of 50% carbohydrates], or a low-fat diet [less than 10% calories from saturated fat, 12–14% from MUFA, 6–8% from PUFA (<30% of calories from fat), along with 15% protein, and a minimum of 55% carbohydrates]. In both diets, the cholesterol content was adjusted to <300 mg/day. Both study diets included foods from all major food groups, but no total calorie restriction was set. No intervention to increase physical activity or lose weight was included.

Full details on dietary assessment and follow-up visits have been published elsewhere [16]. Briefly, participants in both intervention groups received the same intensive dietary counseling. Nutritionists carried out individual interviews at baseline and every 6 months, and quarterly group education sessions were held with up to 20 participants per session and separate sessions for each group. The present study was conducted over a follow-up period of 5 years. Details of the specific recommended diets, mean baseline values and changes in energy and nutrient intake after 5 years of intervention with both dietary patterns have been previously described. In our study, although both dietary models share common characteristics in some of the major components (i.e., high intake of vegetables, fruit, legumes, and whole grains), patients consuming the Mediterranean diet also had a high intake of oily fish, nuts, and extra virgin olive oil, together with a low intake of harmful foods such as red/processed meats and pastries/commercial bakery products.

Laboratory measurements

Anthropometric parameters were obtained using BF511 body composition analyzer/scale, (OMROM, Japan) and a wall-mounted stadiometer (Seca 242, HealthCheck Systems, Brooklyn, NY). Blood samples were obtained from all the participants after overnight fasting. Serum samples were extracted from blood samples by centrifugation at $7.000 \times g$ for 15 min at 4 °C. We measured fasting glucose, total cholesterol, triglycerides, and high-density lipoprotein (HDL)-cholesterol levels in serum using the Dimension Autoanalyzer (Dade Behring Inc., Deerfield, IL, USA). Calculations were made for low-density lipoprotein (LDL)-cholesterol using the Friedewald formula [15]. Fasting Insulin levels in plasma were assessed using a chemiluminescent (i-2000 Abbott Architect® analyzer). Plasma concentrations of high-sensitivity C-reactive protein (hs-CRP) were determined using a high-sensitivity ELISA (BioCheck, Inc., Foster City, CA, USA). Glycosylated hemoglobin (HbA1c) was measured was determined in fresh samples, using G8 (Tosoh Corporation, Shiba-Mianto-ku, Tokyo, Japan).

Absolute count of basophils, lymphocytes, monocytes and neutrophils were measured by flow cytometry using an automated analyzer. Neutrophil-to-basophil Ratio (NBR) was calculated as absolute count of neutrophils/absolute count of basophils (This marker indicates alterations in immune function and inflammatory processes, and useful in evaluating conditions, such as autoimmune diseases). Neutrophil-to-erythrocyte Ratio (NER) = absolute count of neutrophils/absolute count of erythrocyte (this biomarker indicates alterations in the immune system and inflammatory processes, and useful in evaluating various health conditions). Neutrophil-to-HDL Ratio (NHR) = absolute count of neutrophils/absolute count of HDL (biomarker for systemic inflammation and cardiovascular status, as neutrophils are associated with inflammation, while HDL is considered a cardioprotective component). Neutrophil-to-lymphocyte ratio (NLR) = was calculated as absolute count of neutrophils/absolute count of lymphocytes (biomarker for inflammation and immune response. A higher NLR indicates an increased inflammatory response).

Oral glucose tolerance test

The patients underwent an OGTT at the beginning of the study and subsequently once a year throughout the dietary intervention. After overnight fasting, participants consumed a 75-gram flavored glucose load (Trutol 75; Custom Laboratories, Baltimore, MD, USA). After this, blood samples were collected at time intervals of 0, 30, 60, 90, and 120 min to measure glucose and insulin concentrations [17]. We used this information to calculate the homeostatic model of insulin resistance (HOMA-IR), insulin sensitivity index (ISI) and the disposition index (DI) as previously described [12]. Additionally, data for the ISI from 65 patients and the DI from 102 patients were missing because they did not complete the OGTT test at the specified time intervals.

Statistical analysis

The results are presented as mean ± standard deviation (SD) for continuous variables and as numbers (percentages) for categorical variables. A Student *t*-test or Mann–Whitney test was applied according to the normality of the variables. For multiple comparison, we used Bonferroni–Hochberg to adjust the final *p*-value. Kaplan–Meier curves were used for overall survival analyses. Hazard ratio (HR) was performed using multivariate Cox proportional hazards regression for variables, using age, sex, body mass index, diet, HDL-c, triglycerides and statin treatment for the adjustment model. Interaction model was calculated by Cox regression using the adjusted variables. ROC curves of the model including the clinical and neutrophil variables. Model 1: Hb1Ac as reference. Model 2 includes age, sex, body mass index, diet, HDL, triglycerides and statin treatment, as clinical variables. Model 3 includes Model 1 and Model 2. Model 4 includes all models, plus neutrophil variables, including neutrophils, NLR, NHR, NER and NBR. Analyses and graphic representation were pointed out, performed using R v.3.5.1 software (Integrated Development for R. Rstudio, PBC, Boston, MA, USA), and the significance *p* value was set at $p < 0.05$ [18].

RESULTS

Characterization of patients with type 2 diabetes mellitus at baseline

The baseline characteristics of participants, stratified by tertiles of neutrophil count, are summarized in Table 1. We observed significant differences between the three groups in terms of sex, weight, and waist circumference ($p = 0.007$, $p = 0.020$, and $p = 0.015$, respectively). Additionally, the highest tertile (Tertile 3, high neutrophils) exhibited significant increased levels of neutrophil count, monocyte count, NER, NHR, and NLR compared to both the middle (Tertile 2, intermediate neutrophil count) and lowest tertile (Tertile 1, low neutrophil count) (all with $p < 0.001$). Furthermore, hsCRP levels were increased in the highest tertile compared to both the middle and lowest tertiles ($p = 0.001$).

Cox proportional hazard models to assess the probability of type 2 diabetes mellitus remission

To understand the effect of neutrophils on T2DM remission, we used a Kaplan–Meier curve and Cox proportional hazard models to assess the probability of T2DM remission at 5 years of follow-up based on baseline tertiles of absolute neutrophil count. As observed, in the overall population, we did not detect significant differences between the lowest tertile (T1) and the highest tertile (T3, as reference), whether using unadjusted neutrophil count or adjusting for BMI, sex, age, HDL-c, triglycerides, and statin use. Similarly, there were no significant differences observed when comparing the middle tertile (T2) to the highest tertile, whether using unadjusted or adjusted models (Fig. 1A), although diet interaction was significant, when compared the lowest vs highest tertiles ($p = 0.009$) and, the middle vs the highest tertile ($p = 0.046$). Therefore, we stratified our population by the type of diet. Patients under the lowest tertile who consumed a Mediterranean diet exhibited a higher probability of T2DM remission when compared with patients under the highest tertile, with a hazard ratio (HR) of 3.76 (95% CI: 1.39–10.23) in the unadjusted model and an HR of 4.23 (95% CI: 1.53–11.69) in the adjusted model ($p = 0.009$ and $p = 0.005$, respectively) (Fig. 1B). In the low-fat diet group, no significant differences were observed between the lowest and the highest tertiles, nor between the middle and the highest tertile, in any of the models (Fig. 1C).

In the same way, we analyze other neutrophil-related ratios, at baseline, to evaluate their association with T2DM remission at 5 years of follow-up. Regarding to NER, when we stratified our population by diet type, we observed that patients following the Mediterranean diet had a higher likelihood of T2DM remission in the lowest tertile compared to the highest tertile. The HR was 3.06 (95% CI: 1.19–7.83) in the unadjusted model and 3.20 (95% CI: 1.23–8.30) in the adjusted model ($p = 0.020$ and $p = 0.017$, respectively) (Supplementary Fig. 1E). Similar outcomes were noted for NHR, where patients on the Mediterranean diet had a higher likelihood of T2DM remission in the lowest compared to the highest tertile. The hazard ratio (HR) was 3.70 (95% CI: 1.35–10.122 in the unadjusted model and 4.23 (95% CI: 1.40–12.74) in the adjusted model ($p = 0.011$ and $p = 0.010$, respectively) (Supplementary Fig. 1K). We did not find association between other neutrophil-related ratios (NLR) and T2DM remission (Supplementary Fig. 1).

Generalized linear models and receiving operating characteristics for predicting type 2 diabetes mellitus remission

Next, we conducted a ROC curve analysis to assess the probability of T2DM remission in patients with T2DM. When considering only the HbA1c, the AUC of the HbA1c reference model was AUC = 0.623, (95% CI: 0.586–0.741) (Fig. 2). We conducted an additional ROC analysis based on clinical variables (which include age, sex, body mass index, diet, HDL-cholesterol, triglycerides and statin treatment). This model yielded an AUC of 0.666 (95% CI: 0.586–0.745) (Model 1 vs Model 2: $p = 0.545$). When we combined HbA1c and clinical variables, this model yielded an AUC of 0.742 (95% CI: 0.669–0.815) (Model 1 vs Model 3: $p = 0.012$). Finally, when considering all variables in the model, which include HbA1c, clinical variables and neutrophil variables (neutrophils, NLR, NHR, NER and NBR), we obtained an AUC of 0.783 (95% CI: 0.680–0.822). This model was significantly different from the reference model 1 ($p = 0.009$) and the model 2 ($p = 0.006$) (Table 2).

Decrease in neutrophils, observed in Responders, is associated with an improvement in glucose homeostasis after 5 years of follow-up

To assess changes in neutrophil count after a 5-year follow-up period, we compared absolute neutrophil count at baseline and after the 5-year period of dietary intervention. At baseline, we did not observe significant differences between Responders and Non-responders ($p = 0.574$). However, after the 5-year period, we found that Responders exhibited lower neutrophil count compared to Non-responders ($p = 0.006$). Additionally, Responders showed a significant decrease in neutrophil count after the 5-year dietary intervention when compared to baseline ($p = 0.001$) while, in Non-responders, there were no significant differences noted when comparing baseline and the neutrophil count after the 5-year follow-up period ($p = 0.508$) (Fig. 3A). Supplementary Fig. 2 shows this analysis divided by diet, in which responders decreased their neutrophil levels only by following the Mediterranean diet [$p = 0.058$ when compared responders and non-responders at baseline, and $p = 0.007$ at 5-years of follow-up. As for low-fat diet, there was non-significant differences between responders and non-responders at baseline ($p = 0.566$), and at 5-years of follow-up ($p = 0.181$)]. Overall, Responders exhibited a significantly decrease in neutrophil counts, NER, NHR, and NLR after a 5-year follow-up period, when compared with Non-responders ($p = 0.024$, $p = 0.034$, $p < 0.001$ and $p = 0.035$, respectively) (Supplementary Table 1 and Supplementary Table 2 summarizes the baseline characteristics between Responders and Non-Responders). To

Table 1. Baseline characteristics of the participants divided by Tertiles of absolute neutrophil counts.

	Tertile 1 N = 62	Tertile 2 N = 62	Tertile 3 N = 59	p value
Age, years	60.2 (9.1)	60.8 (9.9)	58.8 (8.5)	0.483
Sex, (Males/females):				0.007**
Male	44 (71.0%)[a]	56 (90.3%)	52 (88.1%)	
Female	18 (29.0%)	6 (9.7%)	7 (11.9%)	
Weight, kg	82.0 (11.9)[a]	88.8 (12.9)	84.6 (15.9)	0.020*
Waist circumference, cm	102.0 (11.0)[a]	107.5 (10.0)	106.0 (10.8)	0.015*
Body-mass index, kg/m²	30.8 (4.1)	31.8 (4.0)	30.9 (4.9)	0.346
Diet (Low-fat vs Med), n (%):				0.298
Low-fat	30 (48.4%)	38 (61.3%)	35 (59.3%)	
Med	32 (51.6%)	24 (38.7%)	24 (40.7%)	
T2DM remission, n (%):				0.342
Non-responders	33 (53.2%)	38 (61.3%)	39 (66.1%)	
Responders	29 (46.8%)	24 (38.7%)	20 (33.9%)	
Total cholesterol, mg/dL	170 (30.2)	165 (36.2)	157 (25.5)	0.080
HDL cholesterol, mg/dL	42 (8.4)	42 (10.5)	41 (9.5)	0.728
LDL cholesterol, mg/dL	96 (20.6)	92 (30.6)	87 (24.5)	0.169
Triglycerides, mg/dL	142 (66.9)	145 (72.3)	140 (75.0)	0.932
Fasting glucose plasma, mg/dL	108.5 (25.0)	114.4 (24.7)	108.0 (21.5)	0.260
Fasting insulin plasma, nmol/mL	75.3 (96.4)	83.5 (55.5)	86.8 (53.4)	0.658
HOMA-IR	4.9 (4.8)	4.0 (2.0)	4.1 (2.3)	0.424
HbA1c, %	6.6 (1.0)	6.6 (0.7)	6.8 (0.7)	0.331
Leukocytes, 10³/μL	5.7 (0.9)[a,b]	6.9 (0.8)[c]	9.1 (1.4)	<0.001***
Neutrophils, 10³/μL	3.1 (0.4)[a,b]	4.1 (0.3)[c]	6.0 (1.1)	<0.001***
Monocytes, 10³/μL	0.5 (0.4)[b]	0.5 (0.2)[c]	0.60 (0.2)	0.003**
Lymphocytes, 10³/μL	2.1 (0.6)	2.1 (0.6)	2.2 (0.6)	0.387
hs-CRP, mg/L	2.3 (2.1)[b]	3.5 (3.2)[c]	5.3 (6.1)	0.001**
NBR,	151.7 (101.5)	160.3 (122.2)	164.0 (136.6)	0.890
NER,	0.6 (0.1)[a,b]	0.9 (0.1)[c]	1.2 (0.3)	<0.001***
NHR,	0.1 (0.0)[a,b]	0.1 (0.0)[c]	0.2 (0.0)	<0.001***
NLR,	1.6 (0.4)[a,b]	2.1 (0.7)[c]	2.9 (1.1)	<0.001**
Statins, n (%):				0.191
No	11 (17.7%)	8 (12.9%)	4 (6.8%)	
Yes	51 (82.3%)	54 (87.1%)	55 (93.2%)	

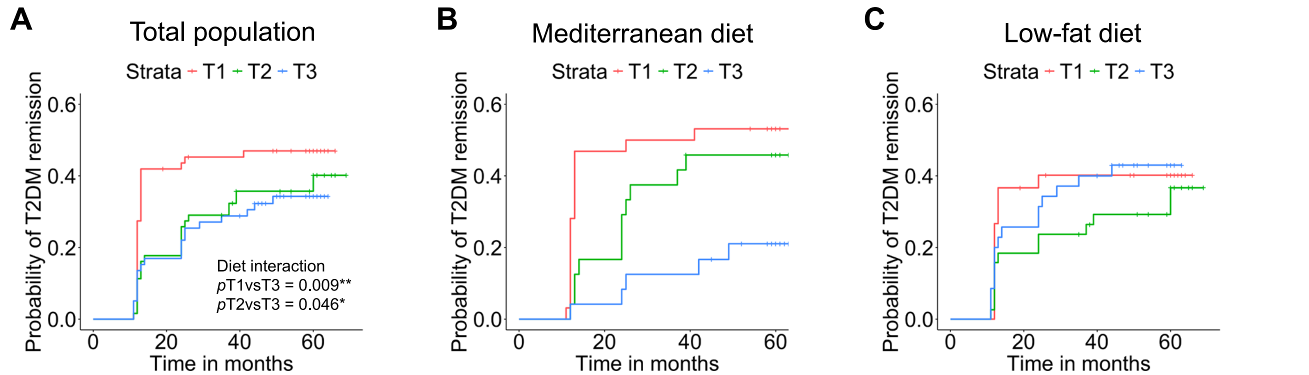
Data are represented as mean (SD) or *n* (%). Data are presented as variables under baseline tertiles of neutrophils. Asterisk indicates significant difference between groups according to the ANOVA test and Chi squared test was used for variables expressed as percentage (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$). Post-hoc analysis is indicated as follow; [a]p value = Tertile 1 vs Tertile 2; [b]p value = Tertile 1 vs Tertile 3; [c]p value = Tertile 2 vs Tertile 3.
hsCRP High-sensitive C reactive protein, *NBR* Neutrophil-to-basophil Ratio, *NER* Neutrophil-to-erythrocyte Ratio, *NHR* Neutrophil-to-HDL Ratio, *NLR* Neutrophil-to-lymphocyte ratio, *LDL* light density lipoprotein, *T2DM* Type 2 diabetes mellitus.

understand the effect of decrease in neutrophil count and dietary intervention on insulin sensitivity, specifically in Responders, we found that Responders following Mediterranean diet had an increase in ISI ($R = -0.57$, $p = 0.011$) and DI ($R = -0.58$, $p = 0.018$), when neutrophil count were decreased after a 5-years of follow-up (Fig. 3B and Supplementary Fig. 3A). In contrast, this effect was not observed in Non-responders group (Supplementary Fig. 3B and Supplementary Fig. 3C). Correlation between changes in Hb1Ac and neutrophil count are summarized in Supplementary Fig. 4.

DISCUSSION

In this study, we present novel findings regarding the association between neutrophils and T2DM remission in newly-diagnosed T2DM patients with CHD. Our data suggest that neutrophil count could serve as a valuable biomarker for T2DM remission. We found that decreased baseline neutrophil count and other neutrophil-related ratios were associated with an increased likelihood of T2DM remission over a 5-year of follow-up period, especially in patients who followed a Mediterranean diet, yielding a reliable AUC in the predictive model. Furthermore, this reduction in neutrophil count was associated with an improve in insulin sensitivity in Responders who followed Mediterranean diet, suggesting a role in regulating of neutrophils on insulin sensitivity and β-cell function. These findings offer a new perspective on the impact of dietary intervention on the regulation of innate immune system and T2DM remission.

Overall, lifestyle interventions are associated with significant effects on T2DM remission [8]. In fact, several studies have demonstrated that T2DM remission can be achieved through various intervention strategies, such as bariatric surgery or caloric



A

Variables	Compare	HR (95% CI)	p value
Unadjusted			
Neutrophils	T1 vs T3	1.67 (0.94 – 2.94)	0.079
	T2 vs T3	1.14 (0.63 – 2.07)	0.658
Adjusted			
Neutrophils	T1 vs T3	1.73 (0.96 – 3.12)	0.070
	T2 vs T3	1.24 (0.67 – 2.28)	0.495

B

Variables	Compare	HR (95% CI)	p value
Unadjusted			
Neutrophils	T1 vs T3	3.76 (1.39 – 10.23)	0.009**
	T2 vs T3	2.55 (0.88 – 7.33)	0.084
Adjusted			
Neutrophils	T1 vs T3	4.23 (1.53 – 11.69)	0.005**
	T2 vs T3	2.61 (0.87 – 7.84)	0.087

C

Variables	Compare	HR (95% CI)	p value
Unadjusted			
Neutrophils	T1 vs T3	0.97 (0.46 – 2.08)	0.942
	T2 vs T3	0.73 (0.35 – 1.53)	0.402
Adjusted			
Neutrophils	T1 vs T3	0.91 (0.38 – 2.17)	0.840
	T2 vs T3	0.78 (0.36 – 1.71)	0.540

Fig. 1 Probability of type 2 diabetes remission analyzed by tertile absolute neutrophil count at baseline. A The analysis was performed using a Cox regression curve by tertiles of the absolute neutrophil count at baseline as unadjusted and adjusted by BMI, sex, diet, age, HDL-C, triglycerides and statins. T1 indicates low Neutrophils ($N = 62$), T2 indicates normal levels ($N = 62$) and T3 indicates high neutrophil levels ($N = 59$) (Reference). **B** The analysis was performed using a Cox regression curve by tertiles of the absolute neutrophil count at baseline in those participants that followed Mediterranean diet as Unadjusted and adjusted by BMI, sex, age, HDL-C, triglycerides and statins. T1 indicates low Neutrophils, T2 indicates normal levels and T3 indicates high neutrophil levels. **C** The analysis was performed using a Cox regression curve by tertiles of the absolute neutrophil count at baseline in those participants that followed low-fat diet as Unadjusted and adjusted by BMI, sex, age, HDL-C, triglycerides and statins. T1 indicates low Neutrophils, T2 indicates normal levels and T3 indicates high neutrophil levels. Asterisk indicates significant difference of the model according the Cox proportional-hazards model regression (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$). CI Confidence interval, HR Hazard ratio, T2DM Type 2 diabetes mellitus, T1 tertile 1, T2 tertile 2, T3 tertile 3.

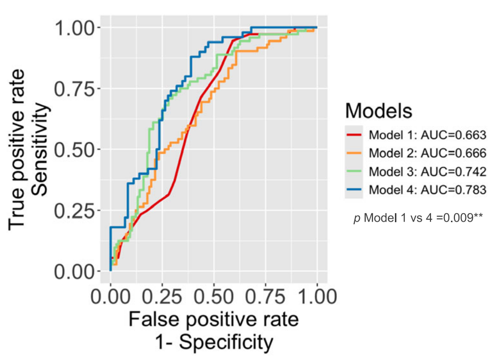


Fig. 2 Remission of type 2 diabetes mellitus assessed by ROC curve models based on clinical variables and neutrophil-related ratios. ROC curves of the model including the clinical and neutrophil-related ratios ($N = 183$). Model 1: hb1Ac as reference. Model 2: This model includes age, sex, body mass index, diet, HDL, triglycerides and statin treatment, as clinical variables. Model 3: This model includes Model 1 + Model 2. Model 4: This model includes Model 1 + Model 2 + neutrophil variables, including neutrophils, NBR, NLR, NHR and NER. # *p* value comparing Reference vs models, according the DeLong's test for two correlated ROC curves. AUC Area under curve, HbA1c Hemoglobin glycosylated, HDL high density cholesterol, NBR Neutrophil-to-basophil Ratio, NER Neutrophil-to-erythrocyte Ratio, NHR Neutrophil-to-HDL Ratio, NLR Neutrophil-to-lymphocyte ratio, ROC Receiver operating characteristic, T2DM type 2 diabetes mellitus.

restriction, as recommended by the ADA and the European Association for the Study of Diabetes [19]. In our study, we observed an association between low neutrophil count and other neutrophil-associated ratio (NER and NHR) and an increased likelihood of T2DM remission, after a 5-year follow-up, only among patients following a Mediterranean diet, suggesting their potential as predictors for T2DM remission. As reported by Bonaccio et al. (2014), in a cross-sectional analysis within a population-based cohort study that included 14,586 healthy Italian citizens, they found an inverse relationship between white blood cell count and adherence to the Mediterranean diet [20]. Indeed, previous results from the CORDIOPREV study showed great benefit following a Mediterranean diet in terms of T2DM remission. For instance, the reduction of serum levels of advanced glycation end products, after the consumption of a Mediterranean diet, were associated with an increased probability of T2DM remission [9]. Furthermore, branched-chain amino acid plasma levels, were associated with T2DM in those patients who consumed Mediterranean diet [21]. These findings highlight the potential of the Mediterranean diet in T2DM remission through different mechanisms, including by modulating the innate immune system, as proposed by this study.

To evaluate the predictive utility of neutrophil count in T2DM remission, we studied the evolution of neutrophil count after dietary intervention. We observed that neutrophil count decreased only in Responders indicating a role in T2DM remission process, suggesting that changes in neutrophil count could explain some process of T2DM remission. Indeed, within the CORDIOPREV study, several predictive tools have been proposed to predict T2DM remission, such as metabolome, microbiota and miRNA profiles [10–12]. In this study, we purpose neutrophils and neutrophil-related ratios as potential biomarkers for T2DM remission. We found that these ratios, in combination with clinical variables and Hb1Ac yielded an AUC of 0.783, which has a good predictive capacity for T2DM remission after a dietary intervention with Mediterranean diet.

In line with these findings, several studies have reported the utility of neutrophil count in predicting T2DM remission. For instance, a study conducted by Bonaventura et al. (2019) found in patients who underwent bariatric surgery, that an NLR of ≤1.97 can predict T2DM remission over a 5-year period [22]. Additionally, Zubiaga et al. (2020) showed that preoperative NLR value (underwent with bariatric surgery) was correlated with 5-year

Table 2. Prediction of Type 2 diabetes mellitus remission using different models by including neutrophil variables performed by ROC curve analysis.

Model	AUC	Sensitivity	Specificity	Accuracy	Threshold
Model 1	0.623 (0.586–0.741)	0.945	0.409	0.515	0.333
Model 2	0.666 (0.586–0.745)	0.903	0.392	0.500	0.316
Model 3	0.742 (0.669–0.815)	0.708	0.720	0.630	0.437
Model 4	**0.783 (0.680–0.822)**	**0.819**	**0.607**	**0.584**	**0.364**

Receiver operating characteristic curves of the model including the clinical and neutrophil variables.
Model 1: Hb1Ac as reference.
Model 2: This model includes clinical variables, such as age, sex, body mass index, diet, HDL, triglycerides and statin treatment.
Model 3: This model includes Model 1 + Model 2.
Model 4: This model contains Model 1 + Model 2 + neutrophil variables, including neutrophils, NLR, NBR, NHR, and NER.
Bold numbers indicate the best model used to predict T2DM remission.
AUC Area under curve, *HbA1c* Hemoglobin glycosylated, *HDL* high density lipoprotein, *HOMA-IR* Homeostasis model assessment-insulin resistance, *hsCRP* High-sensitive C reactive protein, *NER* Neutrophil-to-erythrocyte Ratio, *NHR* Neutrophil-to-HDL Ratio, *NLR* Neutrophil-to-lymphocyte ratio.

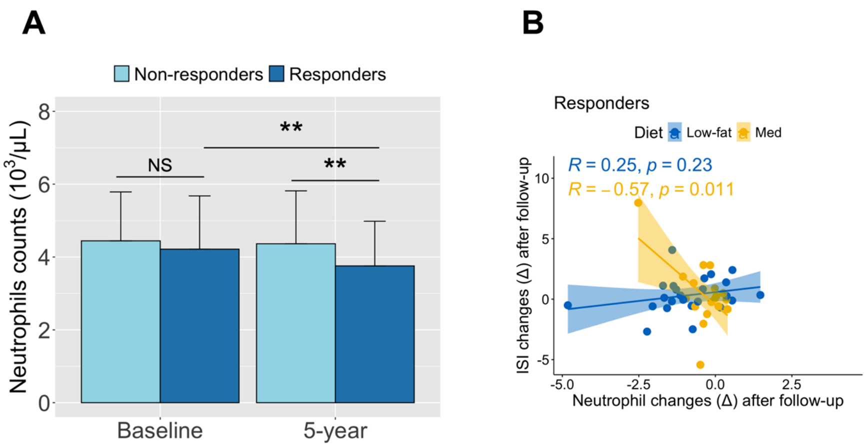


Fig. 3 Neutrophil changes over the dietary intervention and their correlation with insulin sensitivity in responders. A Comparison of absolute neutrophil count between non-responders and responders at baseline and at 5-year of follow-up ($N = 176$). Asterisks indicate significant differences between the groups according to the Mann Whitney test corrected by Bonferroni–Hochberg test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) due to the multi-comparison test. Comparison between 0- and 5-year time were used using paired Mann Whitney test corrected by Bonferroni–Hochberg test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **B** Correlation between changes in Insulin Sensitivity Index neutrophil changes after 5-year of follow-up in responders, and split by Diet: Low-fat and Mediterranean Diet, according to the Pearson Correlation ($N = 46$). ISI Insulin Sensitivity Index, Med Mediterranean Diet, NS No significant, T2DM type 2 diabetes mellitus.

post-surgery anthropometric and glycemic outcomes, as well as a complete remission of T2DM [23]. We, then, add a new marker, as neutrophil count, that could serve as a good predictor for T2DM remission after a 5-year follow-up following a Mediterranean diet; Nevertheless, further research is warranted to gain a comprehensive understanding of the molecular mechanisms involved. Understanding the relationship between neutrophils and T2DM remission could have significant clinical implications. If further validated, neutrophil and their related-ratios could serve as a biomarker for predicting T2DM remission, developing targeted prevention and treatment strategies, and identifying the underlying mechanisms linking neutrophils to T2DM remission.

One mechanistic approach to understanding the association between neutrophils and T2DM remission has been suggested by Liberale et al. (2017). The authors demonstrated that morbidly obese T2DM patients who underwent bariatric surgery exhibited reduced levels of matrix metalloproteinase (MMP)-8, a protein produced by neutrophils when they are activated. Furthermore, an inverse correlation between MMP-8 levels and HbA1c was observed at both 1- and 3-year follow-ups after surgery, which is consistent with our findings [24]. In any way, an increased impairment of neutrophils in hyperglycemia, through an increase in NETosis process, is incompletely understood mechanisms, but it also appears to be elevated in patients with T2DM who have tightly controlled glucose levels, in which can induce detrimental effects and organ damage in diabetic patients [25]. These results support the findings from our study, highlighting the relationship between neutrophils, and glucose homeostasis in T2DM remission.

Circulating neutrophil count is tightly regulated in homeostasis involving a set of cytokines and growth factors [26]. Yet, under conditions of chronic low-grade inflammation including hyperglycemia, dyslipidemia, or aging, the balance can be disturbed and as a consequence of neutrophil count in blood increase [27]. Increases in neutrophil count in conditions of metabolic disturbances has been associated with an increased risk for cardiovascular complications including myocardial infraction and stroke [28]. Indeed, neutrophil count and neutrophil-associated ratios have in the past years become powerful predictors of cardiovascular complications, as they normalize the fluctuation of neutrophil count and decrease their variability [29, 30]. However, just very recently Mendelian-randomization has proven that increased neutrophil count are not just bystanders in cardiovascular complications but are indeed causally involved. Given the tight relationship of chronic-low grade inflammation with neutrophil count in blood, it is very likely that the decrease in neutrophil count after 5 years of Mediterranean diet is indeed a consequence of lower systemic inflammation and decrease in hyperglycemia. However, cytokines and growth factors regulating neutrophil count including CXCL8, GCSF, IL17, and IL23 are yet to be quantified to get a better understanding of how the Mediterranean diet controls granulopoiesis. In addition, data on neutrophil phenotype or function are unfortunately not available from our study and given the emerging field of neutrophil diversity [31], future work will need to reveal how the appearance and the functionality of neutrophils changes with the alteration of diet.

There are several limitations that worth to mention related this study. Firstly, this research is based on a long-term and well-controlled dietary intervention, which may not accurately reflect real-world conditions in a free-living population. Following this limitation, all patients were well-monitored. Then, we assume that the effects we observed are due to the intervention. The second limitation is that T2DM remission was not the primary endpoint of the CORDIOPREV trial. As a result, our findings should be interpreted with caution and confirmed by further studies where T2DM remission is considered as the primary outcome. Finally, further molecular and mechanistic research on neutrophils and T2DM remission is needed to provide a comprehensive understanding that can explain the results observed in this study.

CONCLUSION

Our study highlights significant associations between neutrophil-related ratios and T2DM remission. Lower baseline neutrophil count was associated with an increased likelihood of T2DM remission among individuals following to a Mediterranean diet, underscoring their potential as predictive markers for T2DM remission. Moreover, a decrease in neutrophil count over a 5-year follow-up period were associated with an improvement in insulin sensitivity and β-cell function, especially in Responders following a Mediterranean diet. This suggests that monitoring neutrophil-related ratios can be a promising biomarker in identifying individuals more likely to attain T2DM remission. Further research is needed to understand their role on the pathophysiology of T2DM remission.

DATA AVAILABILITY

Collaborations with the CORDIOPREV study are available to Biomedical Institutions, contingent upon the approval of a scientific research proposal. The type of materials provided—whether electronic data, physical records, or biological samples—will depend on the nature of the collaboration. All partnerships will proceed following the establishment of a collaboration agreement. The terms of this agreement, including the scope of shared resources such as anonymized participant data, data dictionaries, biological samples, physical records, or other specific datasets, will be tailored to each individual project. Data described in the manuscript, code book, and analytic code will be made available upon request from the corresponding author.

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AUTHOR CONTRIBUTIONS

HB and FJMG contributed equally to this work. JDL and JLM equally contributing senior authors. HB wrote the draft manuscript. HB, FJMG, APAL, JDTP, JLRC, HGF, APH, and OARZ collected data and performed the classification of participants. HB, FJMG, and

FRC performed the experiments. APAL, JDTP, and JLRC performed the medical revisions of participants and clinical databases, and HB, FJMG performed the statistical analysis. EMYS, FJT, OS, PPM, JDL, and JLM interpreted the data and contributed to the discussion. JDL, PPM, FJT, OS, and JLM contributed to the writing of the manuscript and revised it critically for important intellectual content. JLM and JDL are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study protocol was developed in accordance with the principles outlined in the Declaration of Helsinki and was approved by the Institutional Review Board of the Reina Sofia University Hospital in Córdoba, Spain. All participants provided written informed consent prior participating in the study, consent to participate and consent to publish.

ADDITIONAL INFORMATION

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Correspondence and requests for materials should be addressed to José. López-Miranda.