RESEARCH ARTICLE

Higher Nrf2 Level is Correlated with Metabolic Parameters in Type 2 Diabetes Mellitus

Rina Triana[1,2,*], Indriyanti Rafi Sukmawati[1,2], Mas Rizky Anggun Adipurna Syamsunarno[3], Keri Lestari[1,2]

[1]Faculty of Pharmacy, Universitas Padjadjaran, Jl. Raya Bandung Sumedang KM 21, Jatinangor, 45363, Indonesia
[2]Prodia Clinical Laboratory, Jl. Kramat Raya No. 150, Jakarta, 10430, Indonesia
[3]Department of Biomedical Sciences, Faculty of Medicine, Universitas Padjadjaran, Jl. Eyckman No. 38, Bandung, Indonesia

*Corresponding author. Email: rina.triana@prodia.co.id

Abstract

BACKGROUND: Type 2 diabetes mellitus (T2DM) is the most common metabolic disorder associated with oxidative stress and chronic inflammation. Understanding the regulatory mechanisms related to the role of nuclear factor erythroid 2 (Nrf2), a master regulator of cellular antioxidant defenses, in individuals with T2DM, is essential. Therefore, in-depth investigation regarding the associations between Nrf2 levels, and metabolic parameters, such as waist circumference, fasting glucose levels, HbA1C, and serum inflammatory markers expressions is needed to elucidate the mechanisms driving T2DM and its metabolic disturbances.

METHODS: This was a cross-sectional study including 90 T2DM and 25 healthy subjects. Nrf2 level; serum inflammatory markers levels, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, interferon (IFN)-α2, IFN-γ, monocyte chemoattractant protein (MCP)-1, IL-10, IL-12p70, IL-17A, IL-18 and IL-23; as well as waist circumference; fasting glucose levels; and HbA1C were measured. Nrf2 concentrations in peripheral blood mononuclear cells were determined using enzyme-linked immunosorbent assay (ELISA), while serum inflammatory markers concentrations were quantified with flowcytometry.

RESULTS: Nrf2, TNF-α, and IL-17A levels were significantly higher in the T2DM group, while IL-1β, IFN-α 2, IL-10, IL-12p70, and IL-23 levels were elevated in healthy controls Nrf2 exhibited positive correlations with waist circumference, fasting glucose, HbA1C, and TNF-α. Conversely, an inverse correlation of Nrf2 was observed with IL-1β, IL-12p70 and IL-17A.

CONCLUSION: Correlations between Nrf2 and metabolic clinical parameters suggest its role in regulating glucose metabolism and adiposity. Elevated Nrf2 levels observed in T2DM patients may present novel therapeutic avenues for enhancing endogenous antioxidant defenses.

KEYWORDS: type 2 diabetes mellitus, Nrf2, oxidative stress, inflammation, metabolic regulation, therapeutic strategies

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Introduction

Diabetes, a chronic metabolic disorder affecting the body's energy metabolism, possesses significant health risks by damaging vital organs like the heart, blood vessels, eyes, kidneys, and nerves over time.(1) Based on International Diabetes Federation (IDF) data, Indonesia ranked fifth among the top 10 countries with the most DM cases, approximately 10.7 million cases.(2) Data from the Indonesian Basic Health Research in 2013 and 2018 show a consistent upward trend in diabetes prevalence among individuals aged 15 and older, with a significant portion of cases in those aged 45 and above.(3,4)

The elevation of blood glucose levels, or hyperglycemia, triggers the excessive production of free radicals through various biochemical pathways, leading to oxidative stress. Oxidative stress refers to an inequilibrium between the production of reactive oxygen species (ROS) and the organism's ability to neutralize them using antioxidant mechanisms. Effective management of hyperglycemia and the mitigation of ROS overproduction are pivotal in delaying the onset of diabetes. Cellular antioxidant enzymes play a crucial role in regulating ROS generation, detoxifying them, and shielding cells from ROS-induced damage, thus providing protection against oxidative stress.(5,6)

At the forefront of resistance against oxidative stress stands nuclear factor erythroid 2-related factor 2 (Nrf2), a central player in the control of cellular redox balance. This control, whether in stressed or unstressed conditions, primarily occurs at the transcriptional level, with the Nrf2/Keap1/ARE pathway serving as the principal mediator of this response.(7,8) Nrf2 acts as a transcriptional activator, activated upon binding to the antioxidant response element (ARE) within its target genes' promoter regions. Nrf2 forms a heterodimer and interacts with Keap1 via leucine-zipper groups.(9)

Under normal conditions, Nrf2 remains sequestered within the cytoplasm, maintaining low expression levels due to continuous polyubiquitination. However, exposure to various stressors induces Nrf2 expression and prevents ubiquitination. Subsequently, it translocates into the nucleus, collaborating with small Maf proteins and other coactivators to activate gene expression dependent on ARE.(10,11)

Nrf2 has emerged as a potential mediator in the development and progression of metabolic disorders.(12) This research aimed to delve deeper into Nrf2's role as a mediator in metabolic conditions, focusing on its associations with key clinical parameters, including waist circumference, fasting glucose levels, HbA1C, and a panel of inflammation markers such as: tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, interferon (IFN)-α2, IFN-γ, monocyte chemoattractant protein (MCP)-1, IL-10, IL-12p70, IL-17A, IL-18 and IL-23 expression in individuals diagnosed with type 2 diabetes mellitus (T2DM). By comprehensively understanding these associations, we can glean valuable insights into the intricate molecular pathways underpinning T2DM, potentially opening new avenues for innovative therapeutic strategies. This investigation seeks to elucidate the complex interconnections between Nrf2 and markers of metabolic disease, ultimately augmenting our knowledge to inform T2DM management and treatment.

In the context of managing T2DM, gaining further insights into the role of Nrf2 and its associations with specific metabolic parameters holds great potential for opening the doors to more effective therapies. Current treatment methods primarily focus on controlling blood glucose levels and potential metabolic complications.(12,13) However, utilizing Nrf2 as a potential therapeutic target may provide a more holistic and sustainable approach to managing T2DM. Furthermore, by having better understanding of the relationship between Nrf2, oxidative stress, inflammation, and metabolic parameters in the context of T2DM, we can identify more specific therapeutic targets, which, in turn, can enhance the efficiency of T2DM patient care.

Despite the growing understanding of the pivotal role of Nrf2 in cellular antioxidant defenses and its implications for metabolic health (12,14,15), there remains a need for in-depth investigations into its associations with specific metabolic parameters in individuals with T2DM. The intricate interplay between oxidative stress, inflammation, and metabolic disturbances in the context of T2DM is a complex puzzle that requires comprehensive examination. By unraveling the molecular connections between Nrf2 and key clinical markers, such as waist circumference, fasting glucose levels, HbA1C, and proinflammatory cytokine expression, our study seeks to shed light on potential therapeutic targets and novel approaches to manage T2DM and its associated complications. Recent studies have highlighted the intricate connections between oxidative stress, inflammation, and metabolic disturbances in T2DM. Nrf2's role in combating oxidative stress and regulating the inflammatory response in autoimmune diseases also has been emphasized before.(16) This highlights the potential of Nrf2 as a critical player in the interplay between oxidative stress and inflammation in the context of metabolic diseases such as T2DM. However, a comprehensive understanding of how Nrf2 correlates with specific clinical parameters in individuals with T2DM is still needed. This study was conducted to bridge this knowledge gap by investigating the associations between Nrf2 levels and key metabolic markers, thus contributing to a deeper comprehension of the molecular mechanisms driving T2DM.

Methods

Study Participants and Sample Collection
This was an observational cross-sectional study involving 115 subjects with inclusion criteria of men aged 30 to 50 years who have been diagnosed with T2DM for the T2DM

subjects and healthy men in the same age group for the healthy control. Subjects with T2DM were determined by interviewing subjects' duration of diabetes and by the examination of HbA1c (HbA1c ≥6.5%), meanwhile the healthy control should have no previous history of T2DM and have HbA1c ≤5.7%. Subjects with liver function impairment (observed by serum glutamic-oxaloacetic transaminase (SGOT)/serum glutamic-pyruvic transaminase (SGPT) levels exceeding 2 times the normal value), acute infections (observed by high-sensitivity C-reactive protein (CRP) levels >10 mg/L), and a history of cancer or undergoing cancer treatment were excluded from the study. The required sample size for this study was determined using the formula for quantitative variables.(17)

This study was conducted in accordance with the guidelines and regulations established by the Health Research Ethics Committee of the Faculty of Medicine, Universitas Padjadjaran, Bandung (Approval No. 620/UN6. KEP/EC/2020). Written informed consents were obtained voluntarily from all subjects, including T2DM patients and healthy control.

Measurement of Anthropometric Characteristics, Blood Pressure, Blood Glucose, HbA1c, SGOT, and SGPT

Anthropometric measurements, including height, weight, and waist circumference (WC) of subjects, as well as systolic and diastolic blood pressure (SBP and DBP), were collected following standardized protocols.

Serum bloods were withdrawn from all subjects, and fasting blood glucose levels were measured using an Architect hexokinase assay (Abbott, Chicago, IL, USA), while HbA1c levels were determined by high-performance liquid chromatography (Variant II Turbo, BioRad, Herculer, CA, USA). SGOT and SGPT were measured using a transaminase NADH without Pyridoxal-5-phosphate methods with Architect hexokinase assay (Abbott). All initial laboratory analyses were conducted at the Prodia Clinical Laboratory, Jakarta.

Quantification of Inflammatory Markers and Nrf-2

Concentrations of serum inflammatory markers (TNF-α, IL-1β, IL-6, IFN-α2, IFN-γ, MCP-1, IL-10, IL-12p70, IL-17A, IL-18 and IL-23) were quantified using flowcytometry (LEGENDplex™ Human Inflammation Panel, BioLegend, San Diego, CA, USA), following the manufacturer's instructions.(18) The panel targeted two distinct bead sets: Bead A, which included TNF-α, IL-1β, IL-6, IFN-α2, IFN-γ; and MCP-1, and Bead B, which encompassed IL-10, IL-12p70, IL-17A, IL-18, and IL-23.

The examination of Nrf2 was conducted using lysate samples of peripheral blood mononuclear cells (PBMC). Mononuclear cell isolation was performed using Ficoll (Ficoll-Paque Premium, GE Healthcare Bio-sciences, Uppsala, Sweden). A total of 2 mL of EDTA blood was combined with 2 mL of PBS. The EDTA blood and PBS were then introduced to 3 mL of Ficoll-Paque Premium in a 10 mL tube through the tube wall and subsequently, centrifuged at 400 g for 30 minutes at a temperature of 10°C. Nrf-2 concentrations in PBMC were later determined by enzyme-linked immunosorbent assay (ELISA) (TransAM Nrf2, Active Motif, Carlsbad, Ca, USA), following the manufacturer's instructions. Both inflammatory markers and Nrf-2 laboratory analyses were performed at the Prodia Clinical Laboratory.

Statistical Analysis

Statistical analyses were conducted using IBM SPSS for Windows version 24.00 (IBM Corporation, Armonk, NY, USA). The significance level for all statistical tests was set at 5%. Differences in clinical characteristics between the T2DM and healthy control groups were assessed using the Mann–Whitney U test. The correlation between variables was evaluated using Spearman rank correlation coefficients.

Results

Characteristics of Subjects

A total of 115 subjects met the selection criteria (T2DM group, n=90; healthy control group, n=25). The age range of the subjects was between 30 and 50 years. The results revealed significant differences between the T2DM and healthy control groups in various parameters. Specifically, the T2DM group exhibited markedly elevated fasting blood glucose levels (161.69±8.01 mg/dL *vs.* 84.8±1.93 mg/dL, *p*<0.001) and HbA1c levels (8.42±0.23% *vs.* 5.37±0.06%, *p*<0.001) when compared to the healthy control group. Additionally, Nrf2 levels, TNF-α levels, and IL-17A levels were significantly higher in the T2DM group than in the healthy control group (0.33±0.09 μg/uL *vs.* 0.18±0.08 μg/μL, *p*=0.002; 49.53±4.29 pg/mL *vs.* 15.65±4.89 pg/mL, *p*=0.000; 17.14±14.92 pg/mL *vs.* 4.07±0.51 pg/mL, *p*=0.000; respectively). Conversely, IL-1β levels, IFN-α2 levels, IL-10 levels, IL-12p70 levels, and IL-23 levels were significantly higher in the healthy control group compared to the T2DM group (11.66±2.15 pg/mL *vs.* 10.94±5.76 pg/mL, *p*=0.000; 9.13±1.50 pg/mL *vs.* 4.58±0.34 pg/mL, *p*=0.008; 12.79±2.13 pg/mL *vs.* 7.19±0.69 pg/mL, *p*=0.002;

5.43±0.60 pg/mL *vs.* 4.24±0.50 pg/mL, *p*=0.002; 68.96±4.11 pg/mL *vs.* 51.03±2.59 pg/mL, *p*=0.001; respectively). Detailed characteristics of T2DM and control groups were presented in Table 1. Examples of IL-1β and TNF-a results using flowcytometry were shown in Figure 1 and Figure 2.

Correlation between Nrf-2 and Other Parameters

To investigate the relationships between Nrf2 and other parameters, we performed Spearman's correlation analysis (Table 2). The results demonstrated that Nrf2 exhibited positive correlations with waist circumference, fasting glucose, HbA1C, and TNF-α (r=0.205, *p*=0.028; r=0.195, *p*=0.037; r=0.256, *p*=0.006; r=0.281, *p*=0.002, respectively). Conversely, an inverse correlation of Nrf2 was observed with IL-1β, IL-12p70 and IL-17A (r=-0.251, *p*=0.007; r=-0.189, *p*=0.043; r=-0.333, *p*=0.000, respectively).

Discussion

Significant differences in patient characteristics between the T2DM and healthy control groups were observed in our study. The T2DM group exhibited significantly elevated fasting blood glucose and HbA1c levels, consistent with diabetes clinical features. These results confirm the validity of our participant selection and group categorization. Furthermore, our results show a significant increase in Nrf2 levels in the T2DM group. The recent findings, as demonstrated in the study, are consistent with previous studies with have shown that Nrf2 protein expression exhibited a slight increase in the hearts of mice with two months of hyperglycemia but a significant decrease in the hearts of mice with five months of hyperglycemia.[20] When combined with earlier study [16], where Nrf2 downstream genes were upregulated in the hearts of diabetic mice two weeks after streptozotocin (STZ)-induced hyperglycemia, it was shown that Nrf2 is adaptively striving to maintain its function in order to combat diabetic damage during the early stages of diabetes. However, as diabetes progresses to its later stages, the antioxidant function of the heart becomes further compromised, resulting in a decrease in cardiac Nrf2 expression. This unexpected finding prompts questions about potential mechanisms behind the upregulation of Nrf2 in T2DM. One interpretation may be that this increase

Table 1. Characteristics of the participants.

| Parameters	Mean±SEM		*p*-value
	Healthy Control (n=25)	T2DM (n=90)	
Age (years)	37.4±0.96	42.6±0.52	0.000*
Diabetes duration (years)	-	5.03±0.42	-
WC (cm)	83.04±1.11	98.56±1.24	0.000*
Fasting blood glucose (mg/dL)	84.80±1.93	161.69±8.01	0.000*
HbA1C (%)	5.37±0.06	8.42±0.23	0.000*
hsCRP (mg/L)	1.06±0.13	2.69±0.22	0.000*
Systolic (mmHg)	110.40±1.68	128.40±1.31	0.000*
Diastolic (mmHg)	72.80±1.36	82.27±0.84	0.000*
TNF-α (pg/mL)	15.65±4.89	49.53±4.29	0.000*
IL-1β (pg/mL)	11.66±2.15	10.94±5.76	0.000*
IL-6 (pg/mL)	25.88±6.08	25.52±3.17	0.592
IFN-α2 (pg/mL)	9.13±1.50	4.58±0.34	0.008*
IFN-γ (pg/mL)	16.39±3.52	12.65±1.63	0.292
MCP-1 (pg/mL)	376.60±37.21	387.14±19.83	0.959
IL-10 (pg/mL)	12.79±2.13	7.19±0.69	0.002*
IL-12 p70 (pg/mL)	5.43±0.60	4.24±0.50	0.002*
IL-17A (pg/mL)	4.07±0.51	17.14±14.92	0.000*
IL-18 (pg/mL)	290.71±63.00	263.11±24.60	0.919
IL-23 (pg/mL)	68.96±4.11	51.03±2.59	0.001*
Nrf-2 (μg/μL)	0.18±0.08	0.33±0.09	0.002*

*Significant if *p*<0.05, tested with Mann–Whitney.
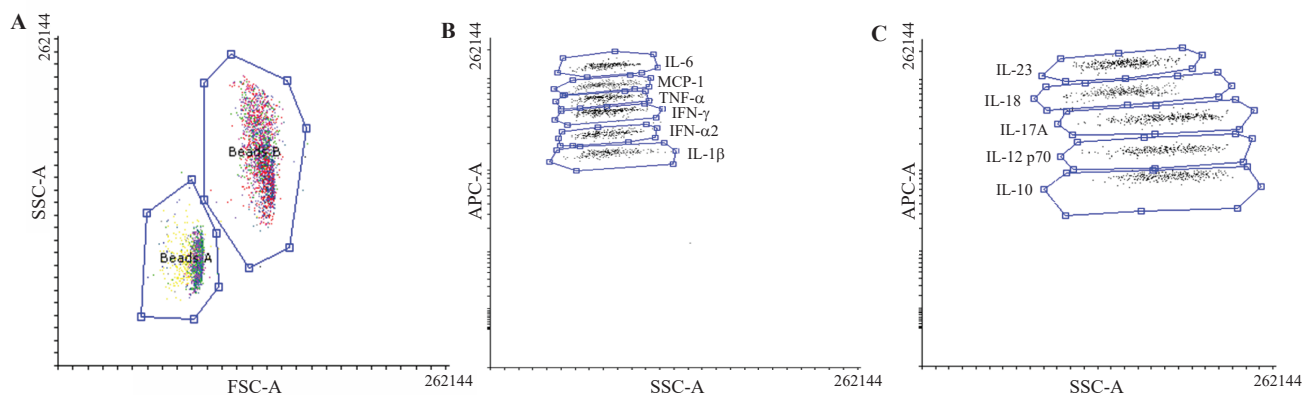
Figure 1. Flowcytometry result gating for healthy controls. A: Dot plot SSC-A and FSC-A; B: Intensity plot of Beads A in the healthy control group; C: Intensity plot of Beads B in the healthy control group. Allophycocyanins (APC) was used for the detecting biotinylated antibodies.

reflects an adaptive response to the heightened oxidative stress commonly associated with the disease.(19) T2DM is characterized by chronic hyperglycemia, which can lead to the overproduction of ROS through various biochemical pathways.(20) These ROS can cause cellular damage and contribute to the development of diabetes-related complications.(21) In an attempt to mitigate this oxidative stress, cells may activate Nrf2 as a defense mechanism to enhance the expression of antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase.(22)

Studies have shown that Nrf2 activation can ameliorate oxidative damage and protect against diabetic complications in animal models.(23,24) Furthermore, dysregulation of the Nrf2 pathway has been implicated in the pathogenesis of various metabolic disorders, including T2DM.(25)The exact mechanisms driving Nrf2 upregulation in T2DM and its functional consequences remain areas of active research. While Nrf2 activation may initially serve as a protective response, sustained or excessive Nrf2 activity could potentially have adverse effects, such as altered insulin

signaling or inflammation.(26) It is important to consider that Nrf2 is a key regulator of cellular antioxidant defenses, and its upregulation in T2DM may signify an attempt to counteract the increased oxidative stress associated with this condition.

In our study, we made the significant observation that TNF-α levels and IL-17A levels were markedly elevated in individuals with T2DM when compared to the healthy control group. TNF-α and IL-17A, both well-established proinflammatory cytokine, have long been recognized for its involvement in the pathophysiology of T2DM. The literature consistently links elevated TNF-α levels and IL-17A to insulin resistance, a central component of T2DM development.(28-30) Furthermore, IL-17A has been shown to induce TNF-α production in different tissues.(30) TNF-α contributes to insulin resistance by interfering with insulin signaling pathways at the cellular level, ultimately impairing glucose uptake and utilization.(31)

In contrast, the levels of IL-1β, IFN-α2, IL-10, IL-12p70, and IL-23 were significantly higher in the healthy
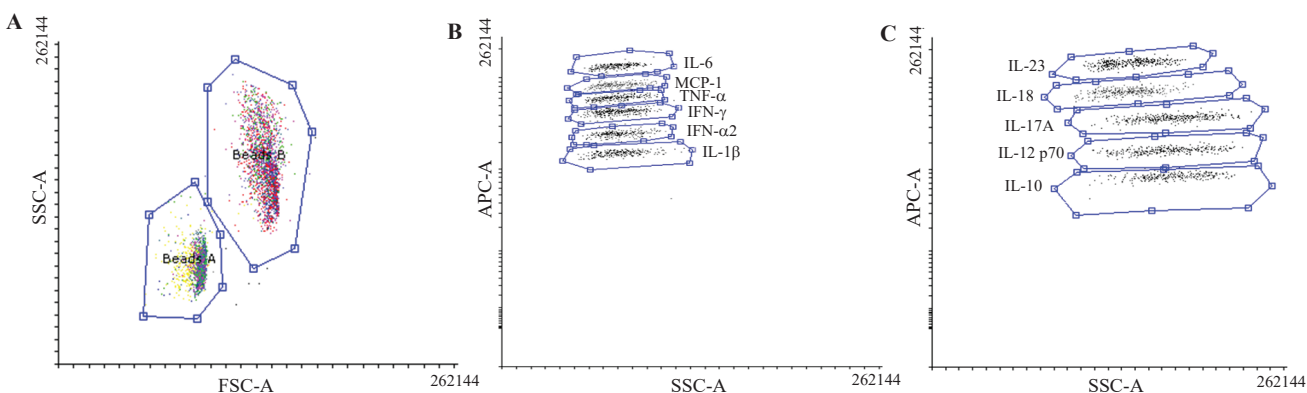

Figure 2. Flowcytometry result gating for T2DM group. A: Dot plot SSC-A and FSC-A; B: Intensity plot of Beads A in the T2DM group; C: Intensity plot of Beads B in the T2DM group. Allophycocyanins (APC) was used for the detecting biotinylated antibodies.

Table 2. Correlation between Nrf-2 and other parameters.

Parameter	Spearman's Correlation Parameters	
	r	p
Waist Circumference	0.205	0.028*
Fasting Glucose	0.195	0.037*
HbA1C	0.256	0.006**
TNF-α	0.281	0.002**
IL-1β	-0.251	0.007**
IL-6	0.068	0.468
IFN-α2	-0.039	0.680
IFN-γ	0.062	0.512
MCP-1	-0.028	0.765
IL-10	-0.182	0.051
IL-12p70	-0.189	0.043*
IL-17A	-0.333	0.000**
IL-18	-0.146	0.121
IL-23	0.013	0.891

*Correlation is significant at $p=0.05$; **Correlation is significant at $p=0.01$.

control group compared to the T2DM group. This finding aligns with existing literature, as chronic low-grade inflammation is a well-established component of T2DM pathophysiology. The observation of higher level of IL-1β, IFN-α2, IL-12p70 and IL-23 in the healthy control group, in contrast to the T2DM group, sheds light on the intricate relationship between inflammation and metabolic disorders, particularly T2DM. Chronic low-grade inflammation, often referred to as meta-inflammation, is recognized as a hallmark of T2DM pathophysiology.[32] This phenomenon is characterized by the persistent activation of proinflammatory pathways in various tissues, including adipose tissue and the liver.[31] The higher IL-10 levels in the healthy control group indicate a potentially superior capacity for inflammation regulation in this group. IL-10 is a well-known anti-inflammatory cytokine that can modulate inflammatory responses.[33]

The correlations observed between Nrf2 and clinical parameters shed light on potential mechanisms underlying T2DM and its metabolic disturbances. Nrf2, as a central regulator of cellular antioxidant defenses, appears to exert a multifaceted influence on the metabolic landscape.[34] The positive correlations between Nrf2 and waist circumference, fasting glucose, and HbA1c imply Nrf2's role in regulating glucose metabolism and adiposity.[25] Emerging evidence suggests that Nrf2 may impact glucose metabolism by

modulating insulin sensitivity, cellular energy balance, and lipid metabolism.[35,36]

The observed correlations between Nrf2 and inflammatory markers in T2DM are intriguing and may be attributed to Nrf2's intricate role in regulating oxidative stress and inflammation. The positive correlation with TNF-α is particularly notable, given TNF-α's well-established role in inflammation and insulin resistance in T2DM.[37] Conversely, Nrf2 can also directly modulate the expression of proinflammatory cytokines, including IL-1β, by inhibiting the pathways involved in their regulation.[38] This might explain the observed negative correlation between Nrf2 and IL-1β levels. Elevated IL-17A levels have been linked to insulin resistance and impaired glucose metabolism, suggesting a potential role in driving T2DM progression. The negative correlation between Nrf2 and IL-17A raises the intriguing possibility that Nrf2 may play a role in mitigating IL-17A-associated inflammation in T2DM. Similarly, the negative correlation between Nrf2 and IL-12p70 may suggest a regulatory role for Nrf2 in dampening the inflammatory effects of IL-12p70 in T2DM. Furthermore, the distinct roles and contexts in which TNF-α, IL-1β, IL-17A, and IL-12p70 function in inflammation could contribute to their varying correlations with Nrf2 in T2DM. It is essential to consider patient variability and environmental factors that influence these complex interactions.

Furthermore, the intriguing inverse correlation between Nrf2 and IL-1β suggests a potential anti-inflammatory role for Nrf2 in T2DM. IL-1β is a proinflammatory cytokine that has been implicated in insulin resistance and beta-cell dysfunction, both of which are central components of T2DM pathogenesis.[32] The negative correlation observed between Nrf2 and IL-1β suggests a potential anti-inflammatory role for Nrf2 in the context of T2DM.[39] This finding raises the possibility of using Nrf2 activation as a therapeutic strategy to mitigate inflammation in T2DM, aligning with the growing body of literature exploring the therapeutic potential of Nrf2 activators in metabolic disorders.[40]

The elevated Nrf2 levels in the T2DM group may offer therapeutic opportunities to enhance endogenous antioxidant defenses. Strategies activating Nrf2, such as lifestyle modifications or pharmacological agents, warrant further investigation for their potential to ameliorate oxidative stress and improve metabolic outcomes in T2DM. Targeted interventions addressing both oxidative stress and inflammation pathways may hold promise in mitigating T2DM complications.

The cross-sectional design of this study limits the ability to establish causality or determine the direction of associations. Longitudinal studies are needed to elucidate the temporal relationships between Nrf2, clinical parameters, and disease progression in T2DM. Because hyperglycemia is related to oxidative stress and inflammation, regulating the Nrf2 activity through various synthetic and natural activators may offer therapeutic benefits for the treatment and mitigation of T2DM. Further research is needed to unravel the mechanistic underpinnings of these relationships and to explore the therapeutic potential of targeting Nrf2 in T2DM management.

Conclusion

In conclusion, our study provides valuable insights into the associations between Nrf2, metabolic parameters, and inflammation in T2DM. Nrf2 exhibited positive correlations with waist circumference, fasting glucose, HbA1C, and TNF-a. Conversely, an inverse correlation of Nrf2 was observed with IL-1β, IL-12p70 and IL-17A. Correlations between Nrf2 and clinical parameters suggest its role in regulating glucose metabolism and adiposity and elevated Nrf2 levels observed in T2DM patients may present novel therapeutic avenues for enhancing endogenous antioxidant defenses. These findings contribute to our understanding of the complex pathophysiology of T2DM and may inform the development of novel treatment strategies for this prevalent metabolic disorder.

Authors Contribution

RT, IRS, MRAAS, and KL were involved in concepting and planning the research. RT and IRS performed the data acquisition/collection, calculated the experimental data and performed the analysis. All authors drafted the manuscript and interpreted the results. RT was involved in the designing of the figures. All authors took parts in giving critical revision of the manuscript.

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