

New insights into oxidative medicine: unraveling the complexity of oxidative stress in health and disease

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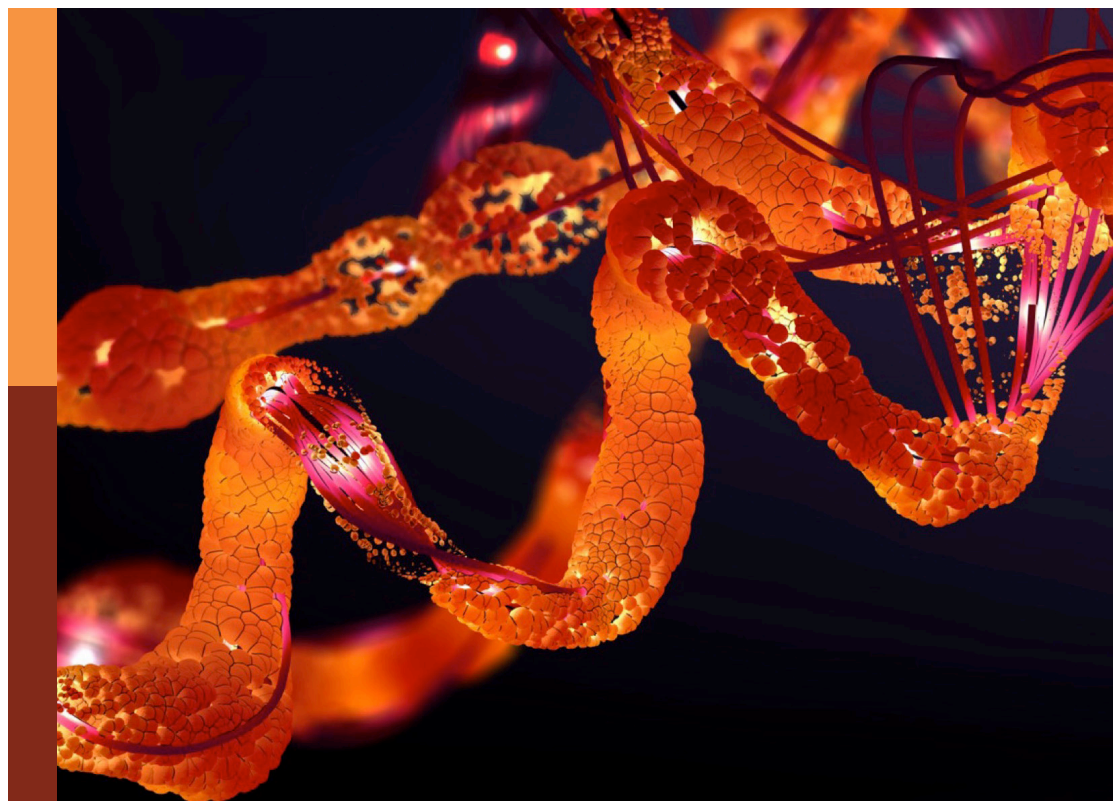
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New insights into oxidative medicine: unraveling the complexity of oxidative stress in health and disease

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Editorial: New insights into oxidative medicine: unraveling the complexity of oxidative stress in health and disease

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KEYWORDS

oxidative stress, oxidative medicine, redox balance, redox status, reactive oxygen species, lifestyle

Editorial on the Research Topic

New insights into oxidative medicine: unraveling the complexity of oxidative stress in health and disease

One of the most common molecular mechanisms affecting health and disease is oxidative stress (OS)- a condition arising from an imbalance between oxidant production and antioxidant defenses-which contributes to inflammation, metabolic alterations, tissue damage, and aging. The ten original and review articles in The Research Topic “*New Insights into Oxidative Medicine: Unraveling the Complexity of Oxidative Stress in Health and Disease*” collectively highlight the multifaceted nature of oxidative processes and their clinical implications, spanning from molecular biology to population-level epidemiology.

Several studies contained in this Research Topic explored how oxidative balance is related to lifestyle and metabolic status. Using large-scale data from the National Health and Nutrition Examination Survey (NHANES), [Zhang et al.](#) demonstrated that an increased oxidative balance score (OBS), integrating dietary and behavioral factors, inversely correlates with the risk of rheumatoid arthritis, suggesting that prevention could be achieved by modulating redox status through nutrition and lifestyle.

Similarly, in an epidemiological study, [Tao et al.](#) found a negative association between OBS and hypertension, particularly in younger adults, thus emphasizing the protective role of antioxidants against cardiovascular risk. These findings were reinforced by [Qin et al.](#), which introduced the novel *Life's Crucial 9* (LC9) score, integrating mental health as a component of cardiovascular wellbeing, and showed that systemic inflammation and oxidative stress mediate, at least in part, its association with chronic kidney disease, emphasizing the holistic importance of psychosomatic and redox homeostasis in systemic disorders.

Oxidative mechanisms were further explored, through innovative experimental models, at the molecular and cellular level, in the study by [Cao et al.](#) who investigated the effects of single-wall carbon nanohorns on nasopharyngeal

carcinoma cells and identified endoplasmic reticulum stress as the key mediator of ROS-induced apoptosis, offering valuable insights into the safety and potential biomedical applications of nanomaterials. In the study by [Wu et al.](#), a comprehensive analysis of oxidative stress-related genes in uveal melanoma and the prognostic value of *CALM1*, a crucial modulator of apoptosis and antioxidant defense are reported. These findings illustrate how dysregulated oxidative mechanisms may drive both degenerative and proliferative diseases, depending on cellular context and adaptive capacity.

Both the studies of [Zhao et al.](#) and [Luo et al.](#) clearly show how redox status and immune function are strictly linked. Mitochondrial dysfunction has been reported by [Zhao et al.](#) in systemic lupus erythematosus (SLE), highlighting the promising diagnostic value of mitochondrial mass and membrane potential in lymphocytes as biomarkers of disease activity. In parallel, [Luo et al.](#) reviewed oxidative stress markers and antioxidants in atopic dermatitis, emphasizing how reactive oxygen species have a role in skin barrier dysfunction and chronic inflammation, and describing emerging antioxidant delivery systems such as nanomaterials, hydrogels, and microneedles. These findings collectively demonstrate that ROS represent central mediators at the interface between immunity, metabolism, and tissue integrity.

Expanding the systemic view, [Nuñez-Selles et al.](#) offered a comprehensive review linking oxidative biomarkers to the progression of hypertension and diabetes mellitus, indicating OS as both an inducer and amplifier of cardiometabolic dysfunction. The authors propose the concept of “precision redox medicine” where tailored antioxidant treatment and lifestyle modification are managed by specific biomarker levels. [Zhu et al.](#), in their article, contributed to this translational perspective by developing and validating a predictive model for Meige syndrome based on redox markers, where albumin, gamma-glutamyl transferase, total bilirubin, and the urea nitrogen-to-creatinine ratio represent independent predictors. In another study, [Mao et al.](#) connect oxidative stress to immune senescence and disease severity and highlight its potential as a therapeutic target in community-acquired pneumonia in older adults.

Taken together, in the articles included in this Research Topic, oxidative stress is proposed as a unifying mechanism across diverse diseases, establishing connections among cellular signaling, metabolism, and systemic physiology. They also illustrate the transition of oxidative medicine from a descriptive field to one supported by quantitative biomarkers, predictive modeling, and mechanistic insight. The convergence of epidemiology, molecular biology, and translational research showed here highlight the growing potential of redox biology to drive preventive strategies and personalized therapies.

In conclusion, this Research Topic demonstrates that oxidative stress must not be merely considered in terms of pathological by-products but a central regulator of cellular

adaptation. Continued interdisciplinary research integrating omics technologies, computational modeling, and clinical validation will be crucial to translate redox science into targeted interventions for human health. The editors gratefully acknowledge the contribution of all authors, reviewers, and collaborators whose work has advanced our collective understanding of oxidative medicine.

Author contributions

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Oxidative balance score and the potential for suffering rheumatoid arthritis: a cross-sectional study

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Objective: Our study was conducted to explore the link between oxidative balance score (OBS) and rheumatoid arthritis (RA).

Methods: A total of 21,415 participants were included in our research from five cycles (2011–2012, 2013–2014, 2015–2016, 2017–2018, and 2017–2020) of the National Health and Nutrition Examination Survey (NHANES). Moreover, 20 elements related to diet as well as lifestyle were combined to calculate OBS. The relationship between OBS and RA was assessed by employing multivariable regression analysis, and further exploration was carried out through subgroup analysis, restricted cubic spline analysis, and sensitivity analysis. Multiple covariates were selected to adjust the model for more robust results.

Results: In our cross-sectional study, a higher OBS has a protective effect on the development of RA (OR = 0.98, 95% CI: 0.97 to 0.99). In contrast to individuals aged ≥60, the result is more prominent in the population aged 20–60 (OR = 0.97, 95% CI: 0.96 to 0.98). Marital status appears to introduce interference in the relationship between OBS and RA, and unmarried individuals exhibited different outcomes (OR = 1.02, 95% CI: 0.99 to 1.04) compared to others. The positive influence of OBS was more evident in patients with chronic kidney disease and cardiovascular disease, while it was stronger in individuals without diabetes and liver disease.

Conclusion: A higher OBS correlates with a reduced odd of RA. Further studies are needed to shoot more sights on improving dietary habits and lifestyles to gain proper OBS and explore whether OBS can be one of the measurements utilized to measure the risk of RA.

KEYWORDS

oxidative balance score, rheumatoid arthritis, NHANES, oxidative stress, nutritional status

1 Introduction

Rheumatoid arthritis (RA) is a prevalent inflammatory disorder impacting the joints chiefly. As an autoimmune disease, it can also lead to damage of multiple organ systems outside the joints, including the cardiovascular system, liver, kidney, and so on (1, 2). Today, RA impacts around one in every 200 adults of the global population, with a greater occurrence rate among women. However, the precise cause of RA still remains uncertain; both genetic and environmental factors may play a role in the development of this disease (3). A previous study proclaimed that elevated levels of reactive oxygen species (ROS) is intimately associated with RA. A variety of oxidants and antioxidants in the human body are bound to play their roles in RA, which provide some ideas for people to prevent it and measure or improve the quality of life of RA patients (4).

Many studies have investigated how adopting healthy eating habits can act as an efficient approach to lower the likelihood of RA. Research findings suggested that the addition of antioxidants could potentially serve as a beneficial complementary approach in reducing oxidative stress in individuals with RA, and the use of zinc and selenium supplementation has been employed for many years in the prevention of RA remission (5). However, according to some research, it seemed that the intake of some antioxidants does not improve RA (6). Different scientists have different results on the effects of alcohol on RA (7, 8), but the effects of smoking, overweight, and unhealthy lifestyle on RA seemed to be certain according to the available studies (9).

The oxidative balance score (OBS) is developed to comprehensively evaluate the oxidative and antioxidant condition in the human body. It integrates multiple nutrient diets and various lifestyles, and generally an elevated OBS indicates a reduced pro-oxidant burden (10). Currently, an increasing number of epidemiological research are trying to find the correlation between OBS and certain prevalent illnesses. Some studies found that higher OBS scores are associated with a reduced prevalence of cancers (11). Similar negative relationships can also be found in OBS with diabetes (12) and depression (13), and it appears to be more pronounced in women. According to a study by Wang et al., OBS can also be employed to measure all-cause mortality and CVD death (14).

Each individual has his own dietary habit and lifestyle, which can be either beneficial or harmful factors for RA. By selecting the data from the National Health and Nutrition Examination Survey (NHANES), we utilize the OBS scoring system to comprehensively evaluate these factors. We aim to conduct the first systematic assessment of their influence on RA and the potential implications involved. We hope that this can provide insights for future prevention, diagnosis, and treatment of RA.

2 Materials and methods

2.1 Data sources and study population

By collecting data on participants' health, nutrition, lifestyle, and other aspects, the NHANES uses a stratified, multistage

probability sampling way to evaluate the health and nutrition status of the American population (15). It is an important project that holds a significant value in understanding the health issues of the American population. From the study, 54,716 individuals were recruited from five cycles (2011–2012, 2013–2014, 2015–2016, 2017–2018, and 2017–2020) of the NHANES. We eliminated non-standard information involving (1) participants above 20 years old ($n = 22,867$), (2) participants who lack questionnaire information regarding RA ($n = 2,440$), (3) participants who lack dietary or laboratory information in the components of OBS ($n = 5,746$), and (4) incomplete covariates information on participants ($n = 2,248$). The elaborate flowchart outlining our work is displayed in Figure 1.

2.2 Diagnosis of RA

The evaluation of RA was established through a self-report questionnaire. The questionnaire included inquiries such as “Have you ever been diagnosed with arthritis by a doctor or other health professionals?” and “What specific type of arthritis did you have?” Respondents who answered “Yes” in the first question and identified their condition as “rheumatoid arthritis” in the second question were categorized into the RA group, while individuals who expressed a negative attitude toward the first question or choose other forms of arthritis were excluded from the non-RA group.

2.3 Computation of OBS

The computation of OBS for each individual was based on information from 16 dietary and four lifestyle elements, which can also be categorized as 15 antioxidants and five pro-oxidants (Supplementary Table S1). Specific calculation standards and methods were obtained from previous articles (16). It should be noted that during the calculation process, we need to first collect the relevant data from the physical activity questionnaire (PAQ). Then, the PAQ will be converted into metabolic equivalent (MET) based on the following formula: $PA (MET \cdot min/wk) = MET \times weekly frequency \times duration of each PA$ (17). Concrete data can all be obtained from NHANES. We use the serum cotinine concentration from laboratory data in NHANES as a substitute for smoking levels (16), and data on dietary fiber, carotene, riboflavin, niacin, calcium, magnesium, zinc, total folate, vitamins (B6, B12, C, and E), copper, selenium, total fat, iron, and alcohol are in the total nutrient intake interview. BMI can also be directly selected from examination data. There are gender differences in the scoring criteria for each indicator, and the scoring for intake of oxidants and antioxidants is also different.

2.4 Covariates data

In addition to common demographic covariates involving age, sex, race, educational, marital, and family poverty-income ratio

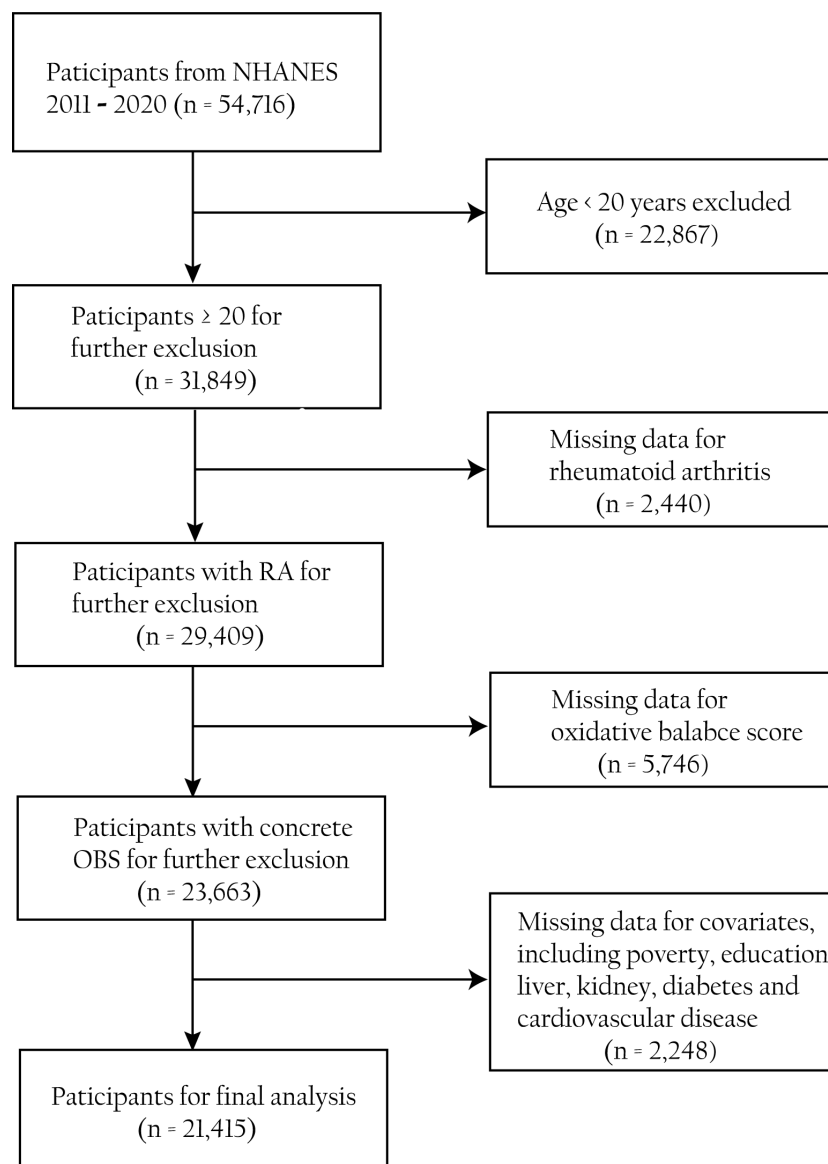


FIGURE 1
Flow diagram of the study selection process.

(PIR), we also obtained information on kidney, diabetes, cardiovascular disease (CVD), and liver disease via standardized questionnaires. These possible variables may also cause potential interference to our result. Age was classified into two groups (20–60 and ≥ 60), and PIR was segmented into three groups (≤ 1.3 , 1.3–3.5, and > 3.5), which means low, medium, and high level, respectively (18). Chronic kidney disease was explained as urine albumin/creatinine ratio > 30 mg/g or estimated eGFR ≤ 60 mL/min/1.73 m^2 (19). The diagnostic criteria for diabetes included self-reported diagnosis by a doctor and ongoing oral treatment with diabetes medication. CVD was composed of five independent questions, and any positive answer from the participants was confirmed as CVD. Liver disease was based on the self-reported questionnaire from NHANES. More detailed covariate information can be viewed in [Supplementary Table S2](#).

2.5 Statistical analysis

Every analysis was conducted using R (version 4.3.2), and statistical significance was defined as two-tailed $p < 0.05$. Categorical or continuous variables were separately demonstrated by mean $\pm$ standard deviation (SD) and percentage (%), and t -test or chi-square test was utilized to analyze them as well. Based on the characteristics of the participants, OBS was categorized into quantiles: quartile 1 (Q1): [3, 12], quartile 2 (Q2): (12, 18], quartile 3 (Q3): (18, 24], and quartile 4 (Q4): (24, 37], and quartile 1 was chosen as the reference. Four multivariate logistic regression models were developed to assess the association between OBS and the occurrence of RA. Model 1 was a basic model for no adjustments. Model 2 was adjusted for age and sex. Model 3 included adjustments for age, sex, race, education, marital status, and PIR. Model 4 further adjusted for age, sex, race, education, marital status, PIR, CKD,

diabetes, CVD, and liver disease. Odds ratio (OR) and 95% confidence interval (95% CI) were used to present the results.

Subgroup analyses were performed with the presentation of forest plots, and interaction tests were chosen to examine the relevant statistical significance. Restricted cubic splines (RCS) analysis was employed to illustrate the nonlinear correlation between OBS and RA through the value of p -nonlinear. A sensitivity analysis was performed by successively omitting each of the 20 components of OBS to examine the robustness of our findings.

3 Results

3.1 Descriptive statistics

The prevalence of RA and the characteristics of the overall 21,415 participants in the 2011–2012, 2013–2014, 2015–2016, 2017–2018, and 2017–2020 cycles of the NHANES are exhibited below. A total of 1,259 individuals were diagnosed as RA (proportion, 5.9%), and 20,156 individuals were non-RA (proportion, 94.1%). The age group of 20–60 has the largest population (proportion, 68.7%), but in contrast to the non-RA group (proportion, 29.7%), RA people aged 60 and above have a higher proportion (proportion, 57.6%). In addition, the incidence rate of CKD, diabetes, CVD, and liver diseases in the RA group is obviously higher. Overall, a significant difference can be found in RA and non-RA groups of relevant characteristics mentioned in Table 1.

We also divided 21,415 participants into four groups according to OBS. The baseline traits of participants categorized by OBS quartiles are displayed in Supplementary Table S3. Younger individuals with higher levels of education and PIR were relatively with greater OBS. It was worth mentioning that the Q1 group exhibited the highest level of various diseases, while the Q4 group showed the lowest levels among the population.

3.2 Association of OBS with RA

After conducting a logistic regression analysis, the correlation between OBS and RA is exhibited in Table 2. A total of results consistently indicated a positive relationship between higher OBS scores and a reduction in RA risk. In the unadjusted model (model 1), with each OBS score increasing, a 4% lower odd of RA was displayed (OR = 0.96, 95% CI: 0.96 to 0.97). After adjusting all covariates, the association was slightly attenuated but still held positive statistical significance (OR = 0.98, 95% CI: 0.97 to 0.99). Comparing to Q3 and Q4 in model 1, we observed that the risk reduction was stronger in Q2, with a 5% lower odd of RA in each increment of OBS (OR = 0.95, 95% CI = 0.93 to 0.97). However, with the confounding factors adjusted gradually, the differences narrowed and displayed a 3% lower odd of RA in each increment of all quartiles in OBS.

3.3 Subgroup analysis

We utilized the full-adjustment model (model 4) and a conducted subgroup analysis as well as interaction tests across 10 categories,

examining different age ranges, gender, race, education, marital status, PIR, and four common types of diseases. Partial outcomes including age, gender, and four diseases are presented in Figure 2. The others are shown in Supplementary Figure S1. Contrasted with elderly individuals aged ≥ 60 (Q4: OR = 0.97, 95% CI: 0.96 to 0.98), it was evident that the impact of OBS on RA was more prominent among younger individuals aged 20–60 (Q4: OR = 0.98, 95% CI: 0.96 to 0.99) (Figure 2A). The effect of OBS on RA appeared to be less different between male and female individuals (Figure 2B). When examining diabetes (Figure 2D) and liver disease (Figure 2F), the negative correlation in individuals with each ailment exceeded that in the healthy group. However, the CKD (Figure 2C) and CVD (Figure 2E) subgroups displayed a stronger association between OBS and RA in healthy individuals. About race, the subgroup of non-Hispanic White and other race suggested a closer and negative link among all quartiles of OBS, while non-Hispanic Black exhibited a positive correlation in Q2 (OR = 1.04, 95% CI: 1.01 to 1.08) (Supplementary Figure S1A). Participants who completed education levels above high school (Supplementary Figure S1B), are married (Supplementary Figure S1C), and have PIR ranging from 1.3 to 3.5 (Supplementary Figure S1D) all exhibit a decrease in RA risk as OBS increases in all quartiles. However, we noticed that among unmarried individuals, the increase in OBS is associated with an increase in RA risk (Supplementary Figure S1C). Statistical significance was observed in the race (p for interaction = 0.034) and marital status (p for interaction = 0.032) subgroups of OBS.

3.4 Restricted cubic spline analysis

Restricted cubic spline (RCS) analysis was chosen to investigate the nonlinear association between OBS and RA. After modifying all covariates, we found that as the OBS value increases, the incidence of RA decreases (Figure 3A). This was consistent with our research findings mentioned above. Although the overall trend remains consistent, the nonlinear relationship between OBS and RA is not significant (p -nonlinear = 0.402). We also performed RCS analysis on different age groups: 20–60 (Figure 3B) and ≥ 60 (Figure 3C). We observed that the younger group (p -nonlinear = 0.315) exhibits a more pronounced nonlinear relationship compared to the older group (p -nonlinear = 0.680).

3.5 Sensitivity analysis

With the model adjusted for all covariates, sensitive analysis showed that no evident changes of outcomes between OBS and RA were observed after removing any component from the OBS. This indicated that our experimental results are robust. The concrete data could be found in Supplementary Table S4.

4 Discussion

Our study has innovatively discovered that a higher OBS has a protective effect on decreasing the odds of RA among individuals aged 20 or above. Individuals in Q2 demonstrated a higher degree of

TABLE 1 Characteristics of participants aged 20–80 by RA in the US.

Characteristics	Overall (<i>n</i> = 21,415)	RA (<i>n</i> = 1,259)	Non-RA (<i>n</i> = 20,156)	<i>p</i> -value
Age, <i>n</i> (%)	48.7 ± 17.4	60.2 ± 13.1	48.0 ± 17.4	<0.001
20–60	14,705 (68.7)	534 (42.4)	14,171 (70.3)	
≥60	6,710 (31.3)	725 (57.6)	5,985 (29.7)	
Sex, <i>n</i> (%)				<0.001
Male	10,432 (48.7)	549 (43.5)	9,883 (49.0)	
Female	10,983 (51.3)	710 (56.5)	10,273 (51.0)	
Race, <i>n</i> (%)				<0.001
Mexican American	2,760 (12.9)	150 (11.9)	2,610 (12.9)	
Other Hispanic	2,125 (9.9)	131 (10.4)	1,994 (9.9)	
Non-Hispanic White	8,413 (39.3)	445 (35.3)	7,968 (39.5)	
Non-Hispanic Black	4,729 (22.1)	421 (33.4)	4,308 (21.4)	
Other race	3,388 (15.8)	112 (9.0)	3,276 (16.3)	
Education, <i>n</i> (%)				<0.001
Below high school	3,937 (18.4)	328 (26.1)	3,609 (17.9)	
High school	4,840 (22.6)	322 (25.6)	4,518 (22.4)	
Above high school	12,638 (59.0)	609 (48.3)	12,029 (59.7)	
Marital status, <i>n</i> (%)				<0.001
Married/cohabiting	12,833 (59.9)	691 (54.9)	12,142 (60.2)	
Widowed/divorced/separated	4,446 (20.8)	445 (35.3)	4,001 (19.9)	
Never married	4,136 (19.3)	123 (9.8)	4,013 (19.9)	
PIR, <i>n</i> (%)	2.0 ± 0.8	1.8 ± 0.8	2.0 ± 0.8	<0.001
≤1.3	6,531 (30.5)	523 (41.5)	6,008 (29.8)	
1.3–3.5	8,083 (37.7)	443 (35.2)	7,640 (37.9)	
>3.5	6,801 (31.8)	293 (23.3)	6,508 (32.3)	
CKD, <i>n</i> (%)				<0.001
Yes	5,775 (27.0)	519 (41.2)	5,256 (26.1)	
No	15,640 (73.0)	740 (58.8)	14,900 (73.9)	
Diabetes, <i>n</i> (%)				<0.001
Yes	2,147 (10.0)	234 (18.6)	1,913 (9.5)	
No	19,268 (90.0)	1,025 (81.4)	18,243 (90.5)	
CVD, <i>n</i> (%)				<0.001
Yes	2,207 (10.3)	316 (25.1)	1,891 (9.4)	
No	19,208 (89.7)	943 (74.9)	18,265 (90.6)	
Liver disease, <i>n</i> (%)				<0.001
Yes	928 (4.3)	88 (7.0)	840 (4.2)	
No	20,487 (95.7)	1,171 (93.0)	19,316 (95.8)	

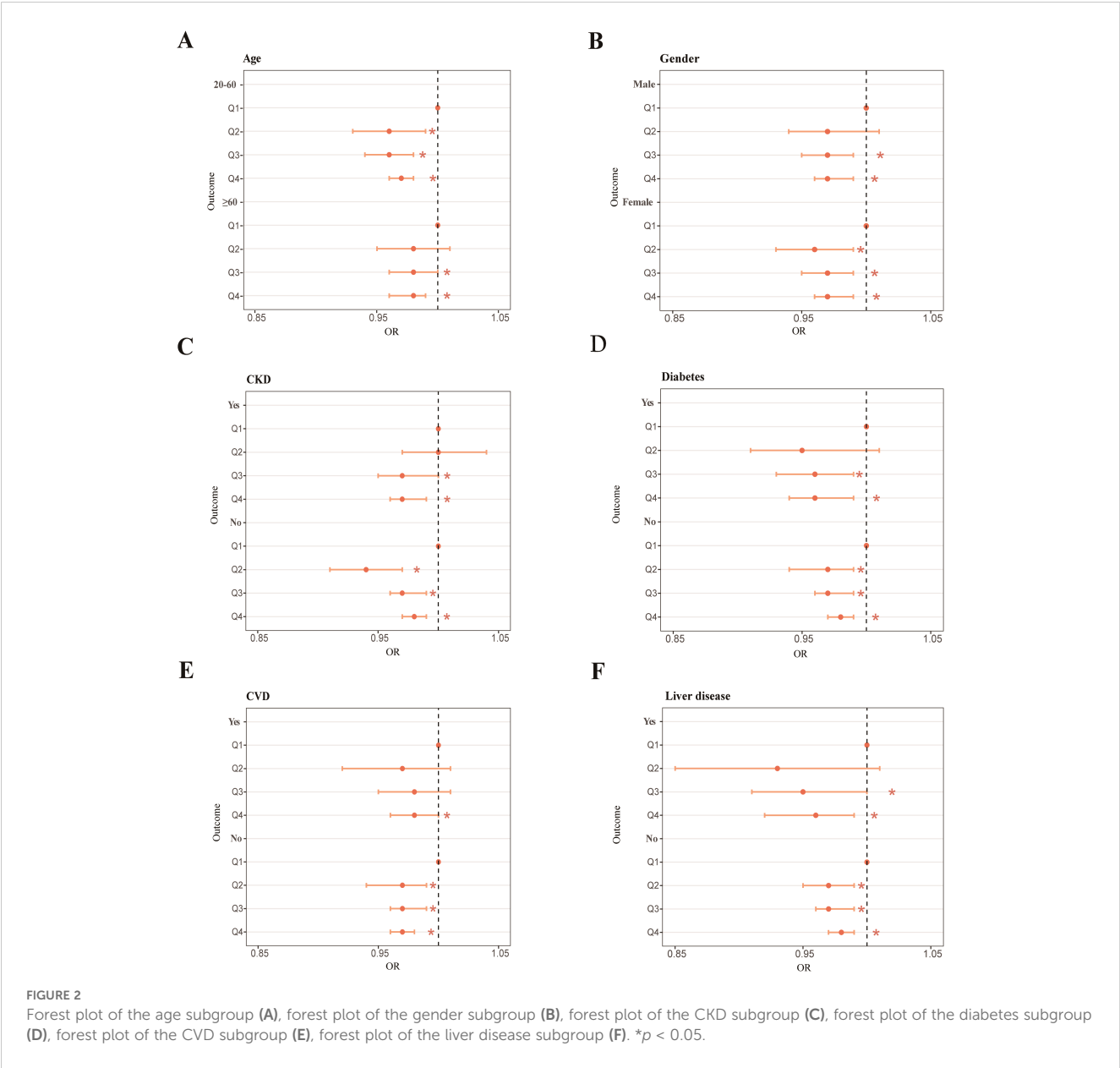
Continuous variables were presented as mean ± standard deviation (SD). Categorical variables were presented as *n* (%). RA, rheumatoid arthritis; PIR, family poverty–income ratio; CKD, chronic kidney disease; CVD, cardiovascular disease.

TABLE 2 Relationship between OBS and RA in the US.

	Model 1		Model 2		Model 3		Model 4	
	OR (95% CI)	<i>p</i> -value	OR (95% CI)	<i>p</i> -value	OR (95% CI)	<i>p</i> -value	OR (95% CI)	<i>p</i> -value
Continuous	0.96 (0.96, 0.97)	<0.001	0.97 (0.96, 0.98)	<0.001	0.98 (0.97, 0.98)	<0.001	0.98 (0.97, 0.99)	<0.001
Q1	1.00 (ref)		1.00 (ref)		1.00 (ref)		1.00 (ref)	
Q2	0.95 (0.93, 0.97)	<0.001	0.95 (0.93, 0.97)	<0.001	0.96 (0.94, 0.98)	<0.001	0.97 (0.94, 0.99)	0.003
Q3	0.96 (0.95, 0.97)	<0.001	0.97 (0.95, 0.98)	<0.001	0.97 (0.96, 0.98)	<0.001	0.97 (0.96, 0.99)	<0.001
Q4	0.96 (0.95, 0.97)	<0.001	0.97 (0.96, 0.98)	<0.001	0.97 (0.96, 0.98)	<0.001	0.97 (0.97, 0.98)	<0.001

Model 1, no adjustment for covariates; model 2, adjustments for age and gender; model 3, adjustments for age, gender, race, education, marital, and PIR; model 4, adjustments for age, gender, race, education, marital, PIR, CKD, diabetes, CVD, and liver disease.

association compared to those in Q1, and the results remained unchanged after all covariates have been adjusted. Compared to their counterparts, OBS exhibited a more pronounced association with RA in younger, highly educated, married, and non-Hispanic White participants. However, a high level of OBS was not conducive to reduce the incidence of RA among unmarried participants. Individuals who suffered CKD or CVD showed a closer linkage between OBS and RA, but those with diabetes or liver disease



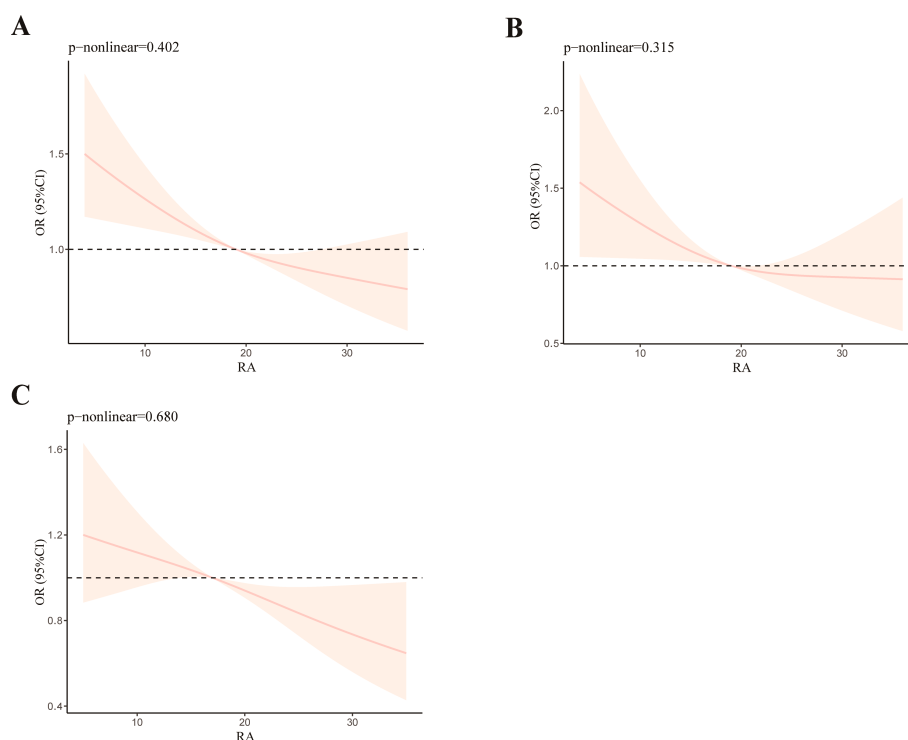


FIGURE 3
RCS analysis of OBS and RA (A), RCS analysis of OBS and RA with participants aged from 20 to 60 (B), and RCS analysis of OBS and RA with participants aged ≥ 60 (C).

displayed a weaker connection. Through RCS, the nonlinear relationship between OBS and RA was not significant. However, after conducting the subgroup RCS based on age, it was indicated that age might be a potential factor influencing the nonlinear relationship.

The risk of RA is closely associated with oxidative stress (4, 20, 21). Diet has long been considered by scientists as a risk factor for triggering RA (5, 22), and numerous studies have confirmed that consuming foods rich in antioxidant capacity can intervene in RA through mechanisms like anti-inflammatory (23) or gutting microbiota regulation (22). However, there are still many studies that questioned this assertion (6, 24–26). According to some cohort studies, vitamins, trace elements, and some dietary supplements from daily diets could potentially prevent the occurrence of RA in the long term (27, 28). These main components were adopted into OBS so as to evaluate the comprehensive effects clearly.

There was less controversy among scientists regarding the effects of physical activity, and proper exercise greatly prevents RA or improves the quality of life of RA patients (29). However, it seemed that BMI and smoking potentially influence the risk of RA with complex interactions which needed to be taken seriously (30). The impact of alcohol consumption on RA remained fully unclear, and there might be potential relationships with the frequency of drinking and the types of alcohol consumed (31). The impact of these lifestyle factors on RA, whether positive or negative, is incorporated into the final score presented by OBS. Previous research suggested that identifying the effects of oxidative stress-related exposures on health outcomes from different people is pretty

challenging because each influential factor is often weak and interrelated (32). Therefore, as a comprehensive measurement, OBS includes both pro-oxidant and antioxidant dietary and lifestyle factors and appears more reasonable to reflect the interaction of pro- and anti-oxidant factors on oxidative stress-related health outcomes (33).

A higher OBS objectively reflects higher levels of antioxidant capacity in the body (34). Our research results are consistent with the existing mainstream findings. However, we noticed that in the models where adjustments were not fully made (model 1, model 2, and model 3), the relationship between OBS and RA appeared stronger in Q2 compared to the higher levels of OBS in Q3 and Q4. This demonstrated that the confounding disease factors, including CKD, diabetes, CVD, and liver disease, had interfered in the association between OBS and RA to some extent. According to a study by Hickson et al., individuals diagnosed with RA were more likely to suffer CKD than those without RA, and the corresponding risk of mortality also increased (35). It was widely recognized that individuals with RA bear a greater burden and faced a twofold-higher risk of CVD (36), and persistent inflammation was considered as the potential mechanism (37). Most existing studies demonstrated that patients with RA were prone to get a greater odd of liver disease, especially non-alcoholic fatty liver disease (NAFLD) (38, 39). A meta-analysis pointed out that one in every three RA patients is affected by NAFLD, which is nearly equivalent to the overall prevalence rate in the general population (39). Comparatively speaking, there were more varying opinions regarding the association between RA and diabetes (40, 41).

However, the consensus from most rigorously conducted studies still suggested a link between them (42).

Existing studies had shown a gender correlation between OBS and health outcomes like diabetes (12), depression (13), and even sleep quality (16). However, this was not well reflected in our subgroup analysis. Generally, RA is divided into early-onset (EORA) and late-onset (LORA) types, with the threshold typically set at 60 years of age (43). We found that the association between OBS and EORA was stronger compared to LORA, and a more significant nonlinear relationship was also found in EORA. These could be attributed to the higher prevalence of LORA (44). Elderly persons also tended to have higher inflammatory parameters and weaker adaptability to oxidative stress (45). The characteristics or behaviors of the younger population, like coffee consumption (46, 47) and sleep loss (48, 49), lead to a more complex relationship, too. Therefore, intervening in RA by improving diet and lifestyle seems to be more effective for younger individuals. However, there was no definitive conclusion in the scientific community regarding the prognosis of EORA and LORA (44). By introducing OBS, we offered a fresh perspective for future research to analyze the age effects on RA.

It was worth mentioning that the subgroup of marital status showed a significant impact in our analysis. In the unmarried group, the positive influence from OBS is not evident after adjusting some common confounding factors. A review by Manfredini et al. explained that married individuals exhibited significantly better health outcomes compared to those who were unmarried (50). Married individuals may exhibit healthier dietary habits (51) and higher levels of physical activity (52) than single ones. These factors were precisely components of OBS. Moreover, previous studies indicated that marriage can decrease the risk of RA and slow down the progression of the disease (53), and it was said that social support played an important role in it (54). However, we noticed that a study by Reese et al. stated that it was more significant to think about the degree of adjustment in a marriage rather than merely whether or not one is married (55). The positive psychological implications of a good marital status can be greatly helpful for RA patients (55, 56).

Currently, the treatment of RA mainly relies on anti-inflammatory drugs and immunomodulators (57, 58). There is indeed controversy in the scientific community regarding the efficacy of antioxidants in the treatment of RA (59, 60). Some studies suggest that antioxidants may help reduce inflammation and oxidative stress, potentially improving RA (61, 62). However, other research indicate that their effect on RA may be limited (60, 63). By utilizing OBS and integrating people's daily diet and lifestyle, we hope to provide a new approach for the prevention, treatment, and prognosis of RA through a series of routine measures.

Our research holds some advantages, namely: (1) we have integrated data from the NHANES database over the past decade, providing a sufficient sample size and population representativeness; (2) we utilized an integrated OBS incorporating diet and lifestyle factors for RA and found a negative correlation between them, which provided a more comprehensive and objective direction for future researchers in this field; (3) we adjusted for numerous confounding factors and employed various analytical methods to demonstrate the

reliability of our findings; and (4) we discovered that OBS has a more significant protective effect on younger, married individuals, while it exhibits an opposite effect on the unmarried population.

There still some disadvantages that exist in our study, namely: (1) limited by the cross-sectional study, we were unable to ascertain a causal correlation between OBS and RA; (2) OBS consists of diet component and lifestyle component—we did not separately analyze and discuss the potential mechanisms of each one; and (3) although there are differences among subgroups, these differences are not highly significant. Thus, further research are needed to corroborate our findings.

5 Conclusion

In conclusion, a protective effect was discovered between OBS and RA among participants from NHANES. This correlation was more obvious in younger as well as married individuals. In conducting a subgroup analysis of common diseases including CKD, diabetes, CVD, and liver disease, OBS presents a different extent to its impact on RA. By taking into account individual differences such as age and marital status, we hope to provide personalized approaches for the prevention of RA. As a method closer to the general public and one that is easier to calculate, OBS will be expected to offer its unique value for the diagnosis and treatment of RA in the future.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: NHANES.

Author contributions

YZ: Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. HY: Formal analysis, Writing – review & editing, Supervision, Data curation. JF: Visualization, Software, Writing – review & editing, Formal analysis. RZ: Validation, Methodology, Writing – review & editing, Software, Formal analysis. JX: Supervision, Writing – review & editing, Validation, Methodology. LL: Software, Writing – review & editing, Validation, Methodology. MD: Formal analysis, Writing – review & editing, Validation. ZL: Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2024.1454594/full#supplementary-material>

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The mechanism of endoplasmic reticulum (ER) stress in cell apoptosis and ROS (reactive oxygen species) of CNE2 cell line induced by single wall carbon nanohorn (SWCNH)

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Introduction: The interaction between the materials themselves and cancer cells are rarely explored. Therefore, the biological roles of raw single wall carbon nanohorn (SWCNH) on endoplasmic reticulum (ER) stress in the apoptosis of CNE2 were explored.

Methods: Therefore, ERS of CNE2 cells was induced by SWCNH, and 4-phenylbutyrate (4-PBA) was selected as a inhibitor of ERS. CNE2 cells were co-cultured with SWCNH, 4-PBA and SWCNH+4-PBA, respectively. Furthermore, the apoptotic status of CNE2 cells and its ROS (Reactive oxygen species) levels were determined. Moreover, the apoptotic protein expression of caspase 3 (cysteinyI aspartate specific proteinase 3), the expression levels of ER pathway protein eIF2 α , ATF4 and CHOP, or the OS (Oxidative stress)-related proteins NQO1, GCLC, HO-1, and Nrf2 was detected, respectively.

Results: CNE2 apoptotic rate, ROS levels, the caspase 3 or ER pathway proteins ATF4 and CHOP expression, even the NQO1, GCLC, HO-1, and Nrf2 levels of oxidative stress-related proteins in the groups of SWCNH and SWCNH+4-PBA were higher compared to the control group. Moreover, these indicators were higher compared to the group of SWCNH+4-PBA ($p < 0.05$).

Discussion: ER stress is the key possible mechanism of CNE2 apoptosis induced by SWCNH. After injury of ERS, SWCNH causes oxidative stress injury, which may eventually lead to apoptosis of CNE2 cells.

KEYWORDS

endoplasmic reticulum stress (ERS), apoptosis, nasopharyngeal carcinoma (NPC), oxidative stress, single-walled carbon nanohorn (SWCNH)

Introduction

The scientists have started the theories and applications of SWCNH (Single-Walled Carbon Nanohorn) (Iijima et al., 1999). Due to its large surface area and specific surface structure, especially its affinity for biomolecules, SWCNH seems to provide biomedical and pharmaceutical prospects. With its closed-tip single-walled nano-scale cavity structure, as well as the advantages of easy dispersion in solvents, uniform size, and high purity, SWCNH maybe the excellent carrier utilizing the drug delivery systems (Murakami and Tsuchida, 2008; Xu et al., 2008; Ajima et al., 2005; Matsumura et al., 2007; Murakami et al., 2008; Ajima et al., 2008).

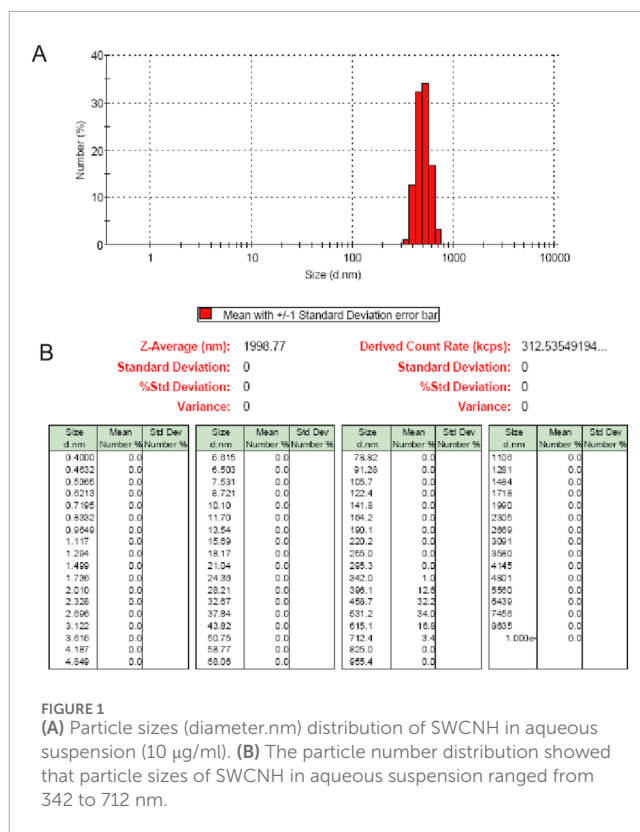
As an important subcellular structure and functional unit of human body, endoplasmic reticulum (ER) is a common place for many kinds of intracellular and extracellular signal transduction. Under pathological conditions, endoplasmic reticulum stress (ERS) occurs in ER, which produces a large number of unfolded or misfolded proteins and Ca^{2+} balance disorders. Persistent ERS can cause cell dysfunction and trigger oxidative emergency damage (Malhotra and Kaufman, 2007; Lenna et al., 2014). Onoda et al. has confirmed that ERS could be induced by carbon nanoparticles (Onoda et al., 2020). Nasopharyngeal carcinoma (NPC) is an aggressive malignant tumor, especially for patients with recurrent or metastatic NPC. Although platinum-containing induction chemotherapy and radiotherapy is used as the first-line standard treatment, the median progression-free survival of NPC patients is only about 7 months (Iijima et al., 1999). The incidence and mortality of NPC in China are higher than the global average, and the death cases account for about 40% of all patients deaths from NPC in the world (Murakami and Tsuchida, 2008; Chen et al., 2019; Siegel et al. 2021). The etiology of NPC is complex, including genetic susceptibility, Epstein-Barrvirus (EBV) infection, and other environmental risk factors such as smoking, ingestion of preserved foods (Salted fish, etc.), and occupational exposure (Niedobitek, 2000). Recurrence and metastasis are the main reasons for the failure of NPC treatment. GP (Gemcitabine combined with cisplatin) chemotherapy is currently the first-line standard treatment for NPC patients with recurrent or metastatic, but its efficacy is limited and needs to be improved urgently (Zheng et al., 2019).

To provide new treatment strategies for patients with NPC is the crucial significance for accomplishing the improved prognosis of advanced NPC. Therefore, we investigated the bio-effect and mechanism of ERS in apoptosis of CNE2 cell line induced by SWCNH in this work.

Materials and methods

SWCNH characteristics

As previously reported (Li et al., 2010), SWCNH was synthesized using method of arc discharge method, and then dried at 100°C in air. The analysis of C, H, N analysis was conducted with the elemental analyzer (Vario EL III). X-ray fluorescence spectrometer (S4-Explorer) was used to measure the other elements. The mesopore size and surface area of SWCNH were also detected with the surface area analyzer (ASAP2010 V3.02E) based on the method of B.E.T. Moreover, the AccuPyc 1330 pycnometer was used to determine the density of



SWCNH particle. Besides, SWCNH particle size was measured with Zetasizer Nano ZS at 298.3K based on the dynamic light scattering.

Characterization of dishes coated with SWCNH

The culture dishes were prepared with dispersed SWCNH, which was firstly soluted using ultrapure water. Normal PS (polystyrene dish) was 60-mm, and spotted with the dispersed SWCNH aliquot (10 µg/mL). Furthermore, these dishes were dried in air at 60°C, then sterilized utilizing UV irradiation.

Besides, PS dishes coated with SWCNH (0.85 µg/cm²) were performed SEM measurements based on SIRION field emission scanning electronic microscope.

Cell culture

The poorly differentiated human NPC cell line CNE2 (CBP60003) was obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). CNE2 was cultured with RPMI-1640 + 10% fetal bovine serum using the above dishes coated with SWCNH.

Cell synchronization and mitotic index

Mitotic events were scored by time-lapse video microscopy and DNA staining. The cells were synchronized as described above and

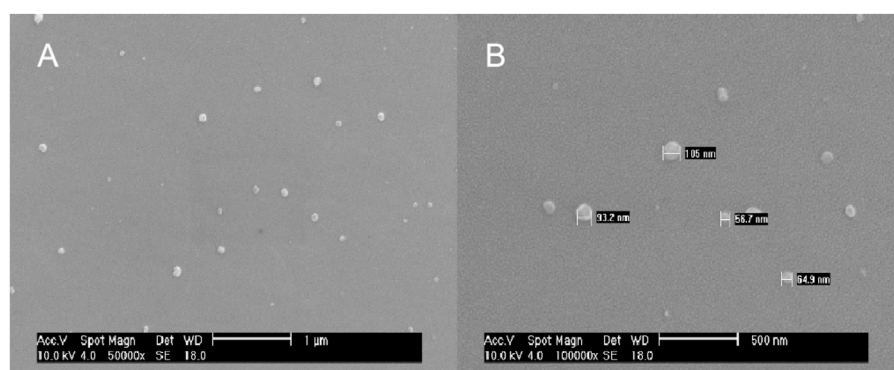


FIGURE 2

The films of SWCNH/PS observed by SEM. The SEM of films showed that SWCNH on PS surface ($0.85 \mu\text{g}/\text{cm}^2$) were individual spherical particles with diameters of 60–100 nm. (A) 500,00x, scale bar represents 1 μm . (B) 1,000,00x, scale bar represents 500 nm.

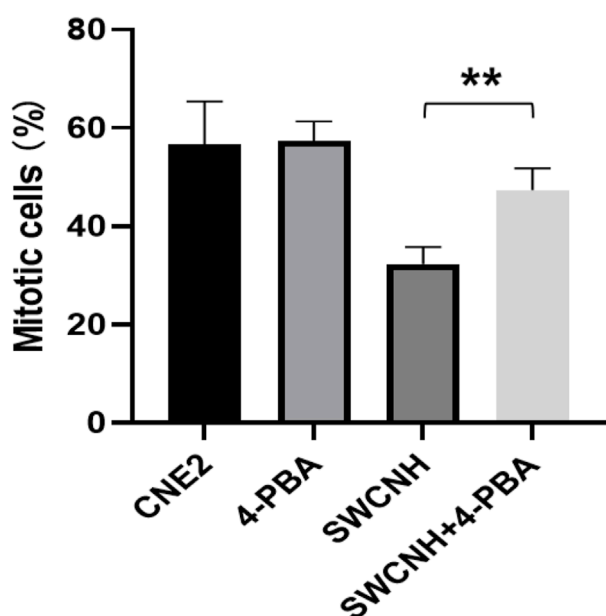


FIGURE 3

SWCNH inhibited mitotic entry of CNE2 cells. The rate of mitotic entry of CNE2 cells in SWCNH group and SWCNH +4-PBA group were lower compared to the control group. Moreover, the rate of mitotic entry of CNE2 cells in SWCNH group was also lower compared to SWCNH+4-PBA group ($p < 0.05$). But, the rate of mitotic entry of CNE2 cells in 4-PBA group was similar to the control group ($p > 0.05$). 4-phenylbutyrate (4-PBA) was selected as a inhibitor of ERS.

then cultured in SWNH-coated for 48 h. Real-time images were captured every 10 min with Openlab software (PerkinElmer Inc., Waltham, MA, USA). Mitotic events of control, cells were scored by their morphological change (from flat to round-up). For each experiment, at least 800 cells were videotaped, tracked, and analyzed. Alternatively, nocodazole (100 ng/mL) was added into the medium and after release, the cells were collected, fixed, and stained with DNA dye (Hoechst 33,258; Invitrogen, Carlsbad, CA, USA). Mitotic cells were scored by nuclear morphology and DNA condensation.

Cell apoptosis

After 48 h of co-culture, the adherent CNE2 cells were digested by trypsin and gently blown into a single cell suspension by suction tube. In each group, 1×10^6 cells were collected, and 0.1 mol/L phosphate buffer (PBS) was added. After centrifugal washing twice, the binding buffer, propidium iodide, 7-AAD and/or annexin V-fluorescein isothiocyanate were added respectively. Reaction under dark conditions for 25 min, then the apoptotic rate was determined with flow cytometry.

Caspase-3 expression

After 48 h of co-culture, the adherent CNE2 cells were digested by trypsin and gently blown into a single cell suspension by suction tube. In each group, 1×10^6 cells were collected. Reaction on ice for 40 min after adding cell lysate, then the protein supernatants were transferred into a novel tube and stored in the environment of -80°C after centrifugation for 10 min with 12,000 r/min at 4°C . BCA protein concentration detection kit was used to detect the concentration of protein samples. The samples were fully denatured in water bath at 100°C for 5 min 40 μg denatured protein sample was added into each pore, the initial electrophoretic voltage was 80V. When bromophenol blue entered the junction of separating gel and concentrating gel, the voltage was adjusted to 120 V. When bromophenol blue entered the bottom edge of separating gel, the electrophoresis was completed. The protein was transferred for 90 min at 90 V. After sealing with skimmed milk, it was incubated overnight with 1:1,000 diluted caspase three antibody, and reacted with 1:2,000 diluted second antibody at room temperature for 60 min. The expression of caspase three was analyzed by taking β -actin as internal reference after exposing.

ROS level

After 48 h of co-culture, CNE2 cells were digested by trypsin and 1×10^6 cells were collected from each group. 2', 7'-dichlorofluorescent yellow diacetate diluted with serum-free

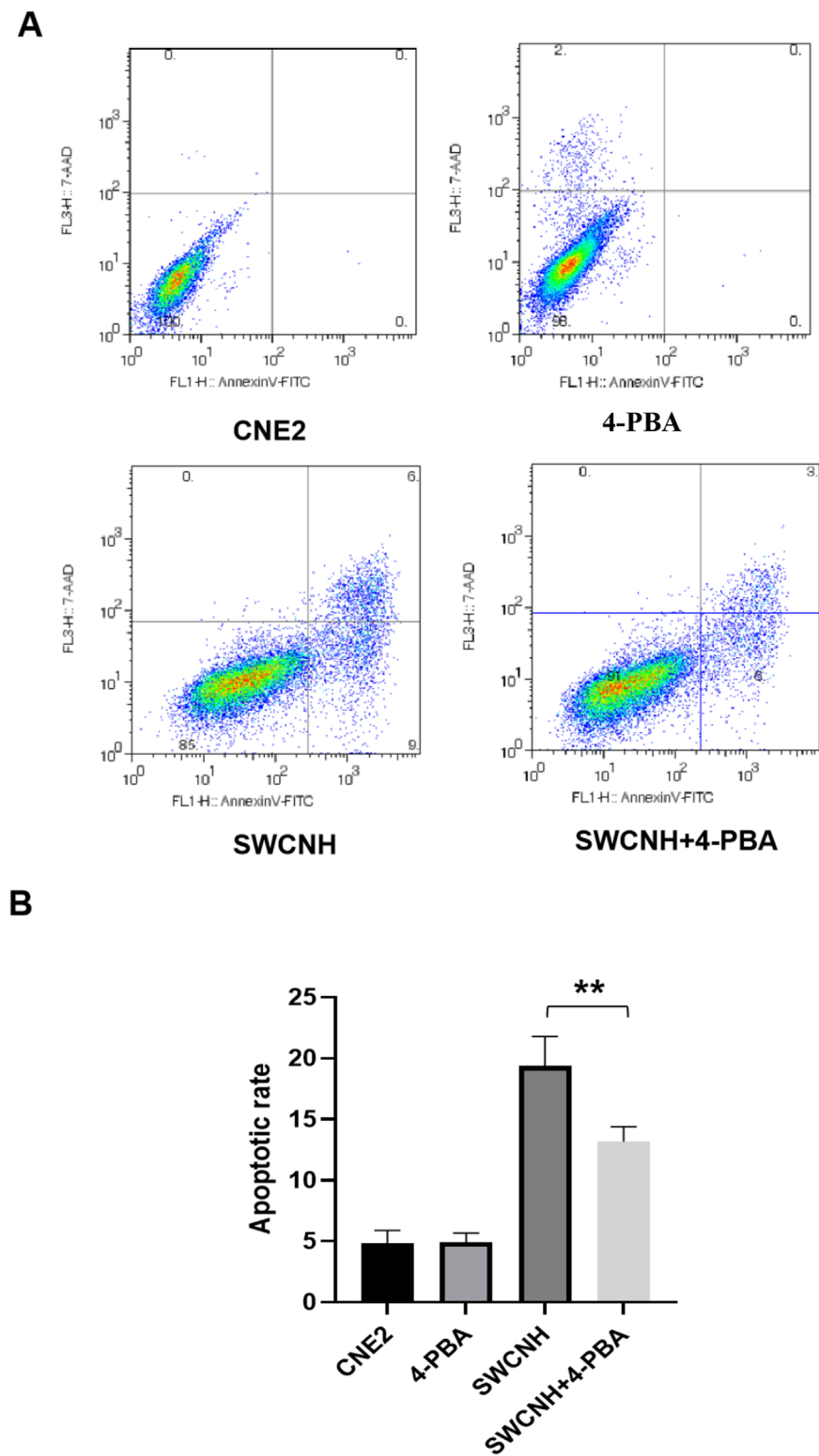


FIGURE 4 SWCNH induced apoptosis of CNE2 cells. The apoptotic rate of CNE2 cells in SWCNH group and SWCNH +4-PBA group was higher than that in control group. The apoptotic rate of CNE2 cells in SWCNH group was higher than that in SWCNH+4-PBA group ($p < 0.05$). The apoptotic rate of CNE2 cells in 4-PBA group was not significantly different from that in control group ($p > 0.05$). All experiments were conducted three times. **(A)** Distribution map of apoptotic cells detected by flow cytometry; **(B)** Percentage of apoptotic cells detected by flow cytometry.

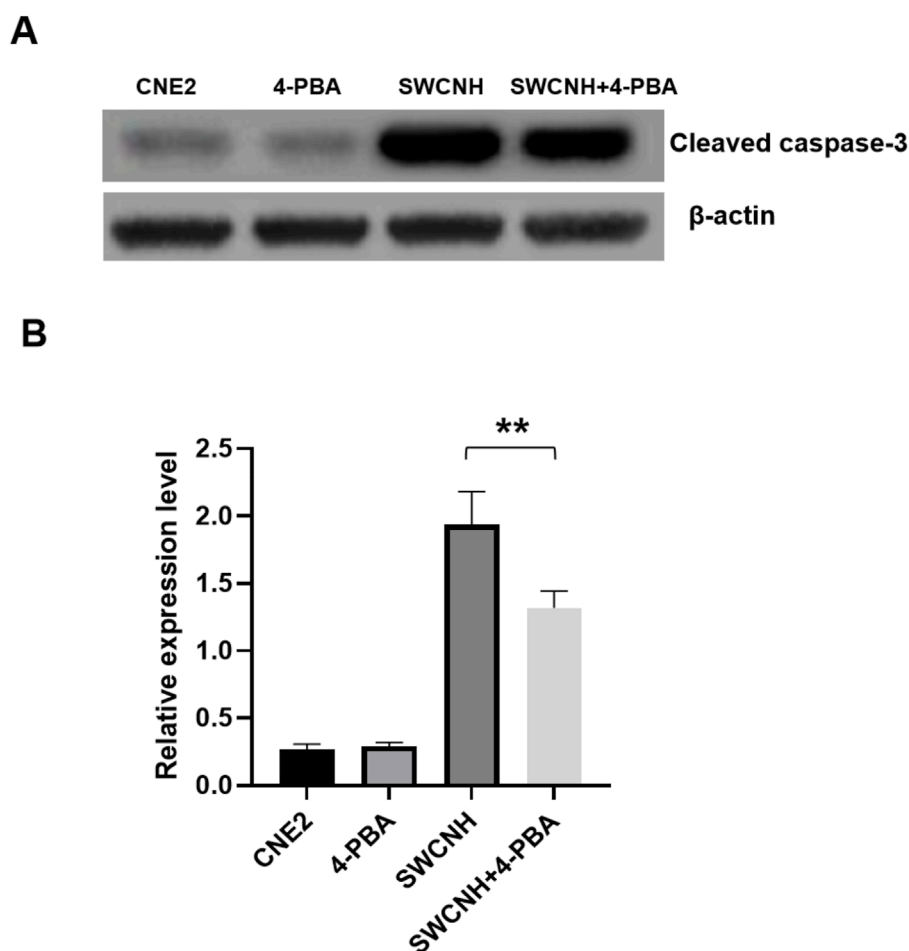


FIGURE 5

Over-expression of caspase-3 induced by SWCNH. The caspase three expression of apoptotic protein in SWCNH group and SWCNH+4-PBA group was higher than that in control group. The caspase three expression level in SWCNH group was higher than that in SWCNH+4-PBA group. The difference was statistically significant ($p < 0.05$). There was no significant difference in caspase three expression between 4-PBA group and control group ($p > 0.05$). All experiments were conducted three times. (A) The expression levels of cleaved caspase-3 detected by western blot; (B) The grayscale analysis of the expression levels of caspase-3 in A.

medium was added to each tube, and the final volume of each tube was 100 μ L, incubated at 37°C for 20 min, washed twice with PBS, and then tested with flow cytometry.

The expression levels of ER pathway proteins

After 48 h of co-culture, proteins were extracted from CNE2 cells. ERS marker proteins eIF2 α , ATF4, CHOP and OS-related proteins NQO-1, GCLC, HO-1, and Nrf2 expression levels were detected Western blot method. Moreover, 4-phenylbutyrate (4-PBA) was selected as a inhibitor of ERS.

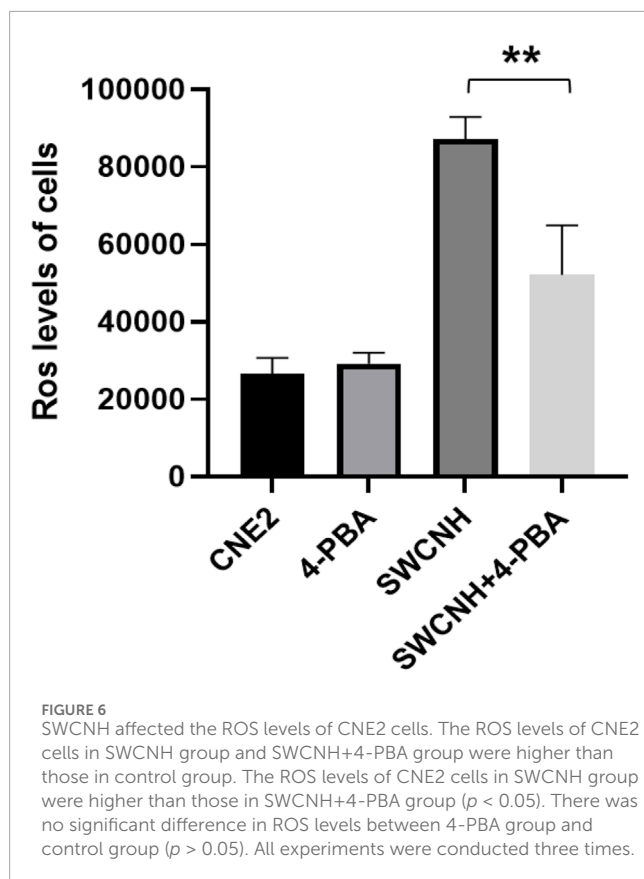
Statistical methods

All data (mean $\pm$ standard deviation) were analyzed with SPSS statistical analysis software (version 20.0). $p < 0.05$ presented statistically significant.

Results

SWCNH characterization

At first, the distribution of particle size or density, the surface adsorptive isotherm, and elemental composition were detected. Then, 95.3% C was found in this material, and each metals was less than 0.1% content, such as Cu (0.0396%), Cr (0.004%), and Fe (0.0863%), etc. Furthermore, 631.55 m^2/g was the surface area of SWCNH with B.E.T method according to the adsorptive isotherm plot (Supplementary Figure S1) (Murata et al., 2000). Besides, at P/P_0 0.994, the diameter was less than 308.7 nm, at the same time, the total pore volume in single point was 1.57 cm^3/g . Moreover, the density of this particle was 1.0077 g/cm^3 , RSD was 0.91%. It implied many closed pores existed in the material. The distribution of SWCNH was showed in Figure 1, which demonstrated the particle size was ranged from 342 to 712 nm in this aqueous suspension. The diameter of the individual SWCNH particle was 80–100 nm, and which was a dalia-like spherical nanohorns aggregate. Thus, in



the aqueous suspension, SWCNH particles were the unit spherical aggregations. Secondly, the images of SEM confirmed that the diameters of this individual spherical SWCNH particles in PS surface were 60–100 nm (Figure 2). Therefore, our results indicated the stronger interactions of π - π stacking on surface of PS was existed between SWCNH and the benzene ring.

SWCNH inhibited mitotic entry of CNE2 cells

The rate of mitotic entry of CNE2 cells in SWCNH group and SWCNH +4-PBA group were lower compared to the control group. Moreover, the rate of mitotic entry of CNE2 cells in SWCNH group was also lower compared to SWCNH+4-PBA group ($p < 0.05$). But, the rate of mitotic entry of CNE2 cells in 4-PBA group was similar to the control group ($p > 0.05$) (Figure 3). 4-phenylbutyrate (4-PBA) was selected as a inhibitor of ERS.

SWCNH induced apoptosis of CNE2 cells

CNE2 apoptotic rate in SWCNH group and SWCNH +4-PBA group were higher compared to the control group. Moreover, CNE2 apoptotic rate in SWCNH group was also higher compared to SWCNH+4-PBA group ($p < 0.05$). But, CNE2 apoptotic rate in 4-PBA group was similar to the control group ($p > 0.05$) (Figure 4). 4-phenylbutyrate (4-PBA) was selected as a inhibitor of ERS.

Over-expression of caspase-3 induced by SWCNH

The caspase three expression of apoptotic protein in SWCNH group and SWCNH+4-PBA group was higher than that in control group. The caspase three expression level in SWCNH group was higher than that in SWCNH+4-PBA group. The difference was statistically significant ($p < 0.05$). There was no significant difference in caspase three expression between 4-PBA group and control group ($p > 0.05$) (Figure 5).

SWCNH affected the ROS levels of CNE2 cells

The ROS levels of CNE2 cells in SWCNH group and SWCNH+4-PBA group were higher compared to the control group. ROS levels in CNE2 cells of SWCNH group were higher than those in SWCNH+4-PBA group ($p < 0.05$). But, ROS levels in 4-PBA group was similar to the control group ($p > 0.05$) (Figure 6).

The effects of SWCNH on ER pathway proteins expression

ER marker proteins expression of ATF4 and CHOP in CNE2 cells of SWCNH group and SWCNH+4-PBA group was higher compared to the control group. The ATF4 and CHOP expression levels in SWCNH group were higher compared to the SWCNH+4-PBA group. Moreover, there was statistically significant difference ($p < 0.05$). But, the expression of ATF4 and CHOP proteins in control group and 4-PBA group could not be found. Besides, it was similar to the eIF2 α expression in CNE2 cells among SWCNH, 4-PBA, and SWCNH+4-PBA group ($p > 0.05$) (Figure 7).

The expression levels of oxidative stress-related proteins caused by SWCNH

The NQO1, GCLC, HO-1, and Nrf2 expression levels in CNE2 cells of SWCNH group were higher compared to the control group. The NQO1, GCLC, HO-1, and Nrf2 expression levels in SWCNH group were higher compared to the SWCNH+4-PBA group, with statistical significance ($p < 0.05$). The expression levels of Nrf2, HO-1, GCLC and NQO1 in 4-PBA group were similar to the control group ($p > 0.05$) (Figure 8).

Discussion

ERS could be caused by the low doses of maternal CB-NP (carbon black nanoparticle) in PVMs (brain perivascular macrophages). Moreover, it could reactive astrocytes around blood vessels of mouse brain. Furthermore, misfolded proteins accumulated and accompanied by ERS, and associated with perivascular neurodegeneration and abnormalities (Onoda et al., 2020).

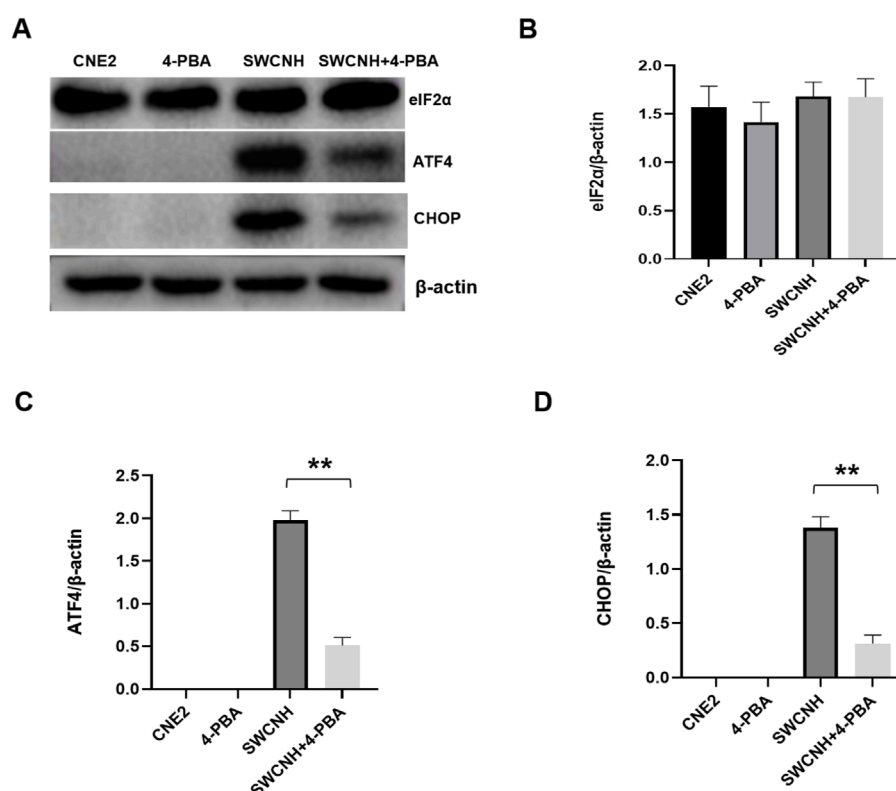


FIGURE 7

The effects of SWCNH on ER pathway proteins expression. The expression of ER marker proteins ATF4 and CHOP in CNE2 cells of SWCNH group and SWCNH+4-PBA group was higher than that of control group. The expression levels of ATF4 and CHOP in SWCNH group were higher than that in SWCNH+4-PBA group. The difference was statistically significant ($p < 0.05$). There was no expression of ATF4 and CHOP proteins in control group and 4-PBA group. There was no significant difference in eIF2α expression in CNE2 cells between SWCNH group, 4-PBA group and SWCNH+4-PBA group ($p > 0.05$). All experiments were conducted three times. (A) The expression levels of ER marker proteins detected by western blot; (B) The grayscale analysis of the expression levels of eIF2α in A; (C) The grayscale analysis of ATF4 expression levels in A; (D) The grayscale analysis of CHOP expression levels in A.

It was found that for rodents, SWCNH had low toxicity (Porter et al., 2007), and there was seldom report about its cytotoxicity (Miyawaki et al., 2008). Ajima et al. confirmed that SWNHox (oxidized SWCNH) could not inhibit proliferation of lung cancer cells (Ajima et al., 2005). Moreover, SWCNH modified with gum arabic did not suppress the viability of Hela cells, too (Fan et al., 2007). In addition, oxSWCNH coated with DPEG could restrained growth of macrophage RAW 264.7 (Tahara et al., 2012). The above research results indicated different behaviors about cell functions, too. Which was associated with the type or characteristic of materials, including curvature, diameter, length, particle size, pore structure, and surface area, even cell types. Furthermore, the modified and unmodified nanomaterials of carbon based on different compounds and groups may induce different bio-effect on cells (Sohaebuddin et al., 2010).

As an important subcellular structure and functional unit of human body, endoplasmic reticulum is the common place for many kinds of intracellular and extracellular signal transduction (Oakes and Papa, 2015; Aitor et al., 2018). Under pathological conditions, endoplasmic reticulum produces a large number of unfolded or misfolded proteins and disorders of Ca^{2+} balance, which results in ERS. At the same time, cells initiate unfolded protein response (UPR) to restore the homeostasis of the cell's internal environment.

When the time and intensity of ERS reach a certain level, UPR did not play a protective role (Seon et al., 2017; Rozpedek et al., 2016), and began to produce cytotoxic effects.

When ERS occurs, eIF2α is the initiation factor of translation will be phosphorylated, then promotes ATF4 (transcription factor), and activates CHOP (transcription factor) expression (Iurlaro and Cristina, 2016). Normally, CHOP mainly exists in the cytoplasm and is expressed at a low level. When cells are under stress, the expression of CHOP increases greatly and accumulates in the nucleus, which promotes the production of ROS. A large number of ROS also induces protein misfolding and Ca^{2+} imbalance in endoplasmic reticulum, which further stimulates the production of ROS and ERS (Cao and Kaufman, 2014). ROS includes superoxide anions, hydroxyl radicals and hydrogen peroxide, its production is related to ERS and UPR (Cao and Kaufman, 2014; Zheng et al., 2018). ROS plays a key role in many cell processes and can be produced in cytosol and various organelles including endoplasmic reticulum and mitochondria. The changes in the redox balance of endoplasmic reticulum are sufficient to induce ERS, which in turn induces the production of ROS in endoplasmic reticulum and mitochondria. Moreover, the persistent role of ROS causes oxidative stress damage, leading to NPC (Cai et al., 2021).

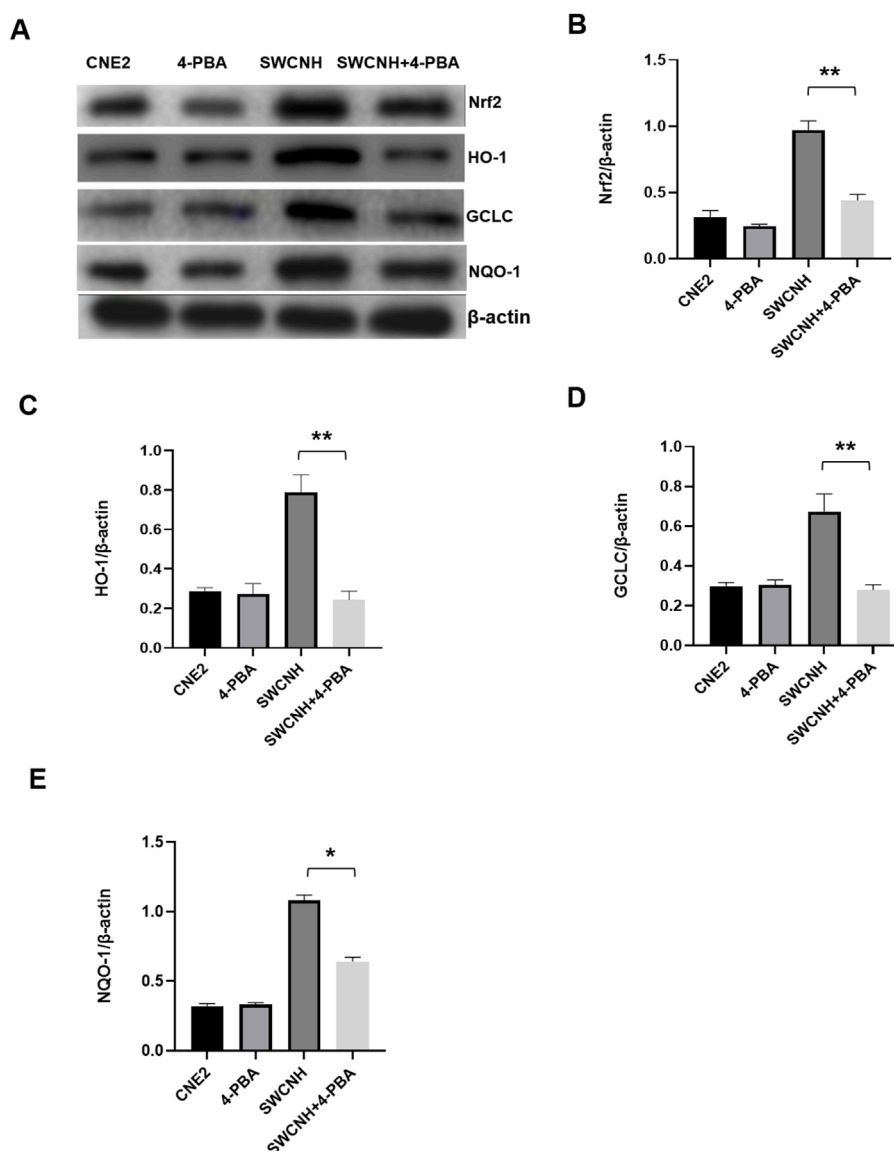


FIGURE 8

The expression levels of oxidative stress-related proteins caused by SWCNH. The expression levels of Nrf2, HO-1, GCLC and NQO1 in CNE2 cells of SWCNH group were higher than those of control group. The expression levels of Nrf2, HO-1, GCLC and NQO1 in SWCNH group were higher than those in SWCNH+4-PBA group, with statistical significance ($p < 0.05$). The expression levels of Nrf2, HO-1, GCLC and NQO1 in 4-PBA group were not significantly different from those in control group ($p > 0.05$). All experiments were conducted three times. (A) The expression levels of oxidative stress-related proteins detected by western blot; (B) The grayscale analysis of the expression levels of Nrf2 in A; (C) The grayscale analysis of HO-1 expression levels in A; (D) The grayscale analysis of GCLC expression levels in A; (E) The grayscale analysis of NQO-1 expression in A.

Therefore, we explored the mechanism of ERS in apoptosis progression in CNE2 cell line. ERS in NPC cell line CNE2 was caused with SWCNH, and 4-phenylbutyrate (4-PBA) was selected as a inhibitor of ERS. CNE2 cells were co-cultured with SWCNH, 4-PBA and SWCNH+4-PBA, respectively. The expression levels of ER pathway protein eIF2 α , ATF4 and CHOP, or OS-related proteins NQO1, GCLC, HO-1, and Nrf2 was detected. At first, our study confirmed that ER pathway proteins ATF4 and CHOP in SWCNH group and SWCNH+4-PBA group were higher compared to the control group. Moreover, these indicators were higher than that in SWCNH+4-PBA group, but these indicators in 4-PBA group was similar to the control group. ER (Endoplasmic reticulum) is

the important cell organelle involved in the protein folding or modification in post-translational level, and assembly of membrane and secretory proteins in eukaryotic cells, and when these processes are affected, it can lead to Unfolded Protein Reaction (UPR) or misfolded proteins in ER. Reticulum accumulation causes ER stress (ERS), which is related to the pathophysiological processes of various human tumors, including tumor migration, invasion and metastasis (Cubillos-Ruiz et al., 2017). Solid tumors have three UPR pathways, PERK-eIF2 α , IRE-1-XBP-1 and ATF6, and solid tumors activate UPR due to hypoxia. UPR makes tumor cells adapt to the stress state and survive by up-regulating the molecular chaperone GRP78/Bip in a more malignant direction. GRP78/Bip in NPC

enables tumor cells to survive through various mechanisms, and is closely related to tumor drug resistance and proliferation, invasion and metastasis (Liu et al., 2022). It is interesting that ERS induced by ROS maybe suppressed by deficiency of PERK, then which lead to apoptosis of tumor cells (Kim et al., 2008). Moreover, the activated c-Jun induced by ROS and ERS regulated cancer cells reciprocally (Su et al., 2012). Some findings indicated that ERS-mediated apoptosis might be one of potential mechanisms for cytotoxicity of cytotoxic substances (An et al., 2023; Xu et al., 2012; Liu et al., 2016).

SWCNH has the huge application prospects. Nethetheless, it is tremendous lack of studies on the mechanism and nanotoxicity of SWCNH. Moreover, SWCNH exhibits the clearly improved biocompatibility over carbon nanotubes (CNT) (Yuan et al., 2011). GPNMB (glycoprotein nonmetastatic melanoma protein B), the key transmembrane protein is discovered to initiate the nanotoxicity (He et al., 2018). At present, there is no literature reporting the biological effects of SWCNH on ROS and apoptosis. However, some researches has indicated the different role of SWCNTs (Single-walled carbon nanotubes). SWCNTs had apparent toxic effects to the protoplasts, including increased ROS generation, changed morphology, inducing apoptosis and necrosis of protoplast cells (Yuan et al., 2011). Furthermoe, Cheng et al. found the generation of ROS was dependent on activation of the mitochondria-apoptosis (Cheng et al., 2011). The apoptosis involved in a great deal of biochemical and morphological hallmarks, such as the activation of caspase-3, internucleosomal DNA fragmentation, chromatin condensation (Yang et al., 2014; Li et al., 2013). Besides, SWCNT could induce the breakage of DNA, the formation of micronuclei (MN), and suppress the growth of cell via the generation of ROS (Kim and Yu, 2014). Low doses of pristine and oxidized-SWCNTs affected mammalian embryonic development. SWCNT could cause genotoxicity, cytotoxicity, and ROS generation (Pietroiusti et al., 2011). SWNHs might be used as a safe anticancer agent, where it is able to trigger mitochondrial dysfunction-induced apoptosis by upregulating SIRT3 expression in HepG2 cells (Li et al., 2018). Although SWCNH are in early preclinical research yet, these nanotube-derived nanostructures demonstrate an interesting versatility pointing them out as promising forthcoming drug delivery systems to target and treat cancer cells (Moreno-Lanceta et al., 2020). The cytotoxicity and hemocompatibility studies prove that the studied materials are acceptable for use in biomedical applications, especially a sample SWCNH-ox-1.5 with the best application potential (Zieba et al., 2021).

In this study, the apoptotic status of CNE2 cells and its ROS levels of reactive oxygen species were determined, and the apoptotic protein expression of caspase 3, the expression levels of ER pathway protein eIF2 α , ATF4 and CHOP, or OS-related proteins NQO1, GCLC, HO-1, and Nrf2 was detected. Finally, our study confirmed that CNE2 apoptotic rate, ROS levels, caspase three expression or ER pathway proteins ATF4 and CHOP, the OS-related proteins levels of NQO1, GCLC, HO-1, and Nrf2 in SWCNH group and SWCNH+4-PBA group were higher compared to the control group. Moreover, these indicators were higher than that in SWCNH+4-PBA group, but these indicators in 4-PBA group was similar to the control group.

Some of the known mechanisms describing the cytotoxicity of nanomaterials are as follows: 1) physical direct interaction of extremely sharp edges of nanomaterials with cell wall membrane

(Akhavan and Ghaderi, 2010); 2) ROS generation (Taposhree et al., 2015) even in dark (Lakshmi Prasanna and Vijayaraghavan, 2015); 3) trapping the cells within the aggregated nanomaterials (Akhavan et al., 2011) for bacteria and (Ehsan et al., 2014) for spermatozoa; 4) oxidative stress (Liu et al., 2011); 5) interruption in the glycolysis process of the cells (Akhavan and Ghaderi, 2012); 6) DNA damaging (Ashutosh et al., 2011); 7) metal ion release (Wang et al., 2014); and recently 8) contribution in generation/explosion of nanobubbles (Jannesari et al., 2023). Therefore, the dominant mechanism occurred in this work should be ROS generation induced by SWCNH. Under pathological conditions, endoplasmic reticulum stress (ERS) occurs in endoplasmic reticulum, which produces a large number of unfolded or misfolded proteins and Ca²⁺ balance disorders. These kinds of processes can be controlled by designing effective anticancer proteins by using artificial intelligent-based methods.

Conclusion

ERS is the key apoptosis mechanism of CNE2 cells induced by SWCNH. After injury of ERS, SWCNH causes oxidative stress injury, which may eventually lead to apoptosis of CNE2 cells.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

XC: Conceptualization, Investigation, Software, Writing—original draft, Writing—review and editing. SG: Formal Analysis, Project administration, Validation, Writing—original draft, Writing—review and editing. SC: Data curation, Methodology, Supervision, Writing—original draft, Writing—review and editing. SH: Funding acquisition, Resources, Visualization, Writing—original draft, Writing—review and editing. TL: Conceptualization, Data curation, Formal Analysis, Writing—original draft, Writing—review and editing. YG: Methodology, Project administration, Validation, Writing—original draft, Writing—review and editing. CF: Software, Supervision, Validation, Visualization, Writing—original draft, Writing—review and editing. JZ: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Writing—original draft, Writing—review and editing.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmats.2024.1455648/full#supplementary-material>

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Modulating oxidative stress: a reliable strategy for coping with community-acquired pneumonia in older adults

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Community-acquired pneumonia (CAP) remains one of the leading respiratory diseases worldwide. With the aging of the global population, the morbidity, criticality and mortality rates of CAP in older adults remain high every year. Modulating the signaling pathways that cause the inflammatory response and improve the immune function of patients has become the focus of reducing inflammatory damage in the lungs, especially CAP in older adults. As an important factor that causes the inflammatory response of CAP and affects the immune status of the body, oxidative stress plays an important role in the occurrence, development and treatment of CAP. Furthermore, in older adults with CAP, oxidative stress is closely associated with immune senescence, sarcopenia, frailty, aging, multimorbidity, and polypharmacy. Therefore, multiple perspectives combined with the disease characteristics of older adults with CAP were reviewed to clarify the research progress and application value of modulating oxidative stress in older adults with CAP. Clearly, there is no doubt that targeted modulation of oxidative stress benefits CAP in older adults. However, many challenges and unknowns concerning how to modulate oxidative stress for further practical clinical applications exist, and more targeted research is needed. Moreover, the limitations and challenges of modulating oxidative stress are analyzed with the aim of providing references and ideas for future clinical treatment or further research in older adults with CAP.

KEYWORDS

community-acquired pneumonia, older adults, oxidative stress, inflammation, aging

1 Introduction

Community-acquired pneumonia (CAP) refers to the infectious inflammation of the lung parenchyma (including the alveolar wall, i.e., pulmonary interstitium in general) acquired outside of hospitals, including pneumonia caused by pathogens with proven latency (1). CAP is one of the most common acute respiratory infections worldwide and can be caused by bacteria, viruses, fungi, or other pathogens alone or in combination. The common causative agents of CAP may vary from country to country, but the older adults and children have always been the primary groups of victims (2, 3). In particular, the global pandemic of *coronavirus disease 2019* (COVID-19) has put unprecedented pressure on healthcare worldwide and incurred non-negligible medical costs (4). Oxidative stress, as

one of the pathogenic mechanisms of CAP, has been widely demonstrated to be closely linked to inflammation and the immune response (5–7). In recent years, most studies related to the treatment of CAP have focused on reducing inflammation and oxidative damage in the lungs. However, immunosenescence, sarcopenia, frailty, multimorbidity, polypharmacy, and other aging-related disease risk factors are also present in older CAP patients. The impact of these factors on the development, treatment and prognosis of CAP in the older individuals and their relationship with oxidative stress are not sufficiently clear. In addition, the potential value and advantages of modulating oxidative stress compared with the traditional preventive and therapeutic modalities of CAP have rarely been mentioned. Therefore, in conjunction with the above unique disease manifestations present in the older population, this review on more links between oxidative stress and CAP in older adults aims to further clarify the possible benefits of modulating oxidative stress in older adults with CAP patients, with the goal of providing a reference for the treatment of CAP in older adults and related research in the future.

2 Occurrence of CAP and activation of the immune response

The lung is a complex microbial ecosystem. The microbes are in complex relationships with each other and with the host, in a state of mutual adaptation and dynamic interaction. Currently, it is recognized that most pathogens invade through the respiratory tract, and infection occurs when host defenses are compromised and/or when the host is exposed to highly virulent microorganisms or large amounts of inoculum. After entering the nasopharynx, pathogens escape recognition and clearance from host immune cells by mimicking the host molecular structure or altering their own antigens, escaping the mucus and adhering to the upper respiratory epithelium (8, 9).

The manner in which bacteria, viruses and fungi induce an immune response after invasion is somewhat different (Figure 1). When bacteria invade the alveoli and then multiply, they are sensed and recognized by *polymorphonuclear leukocytes* (neutrophils), which in turn elicits the body's innate immune response. Bacteria interact with alveolar cells, such as alveolar epithelial cells and

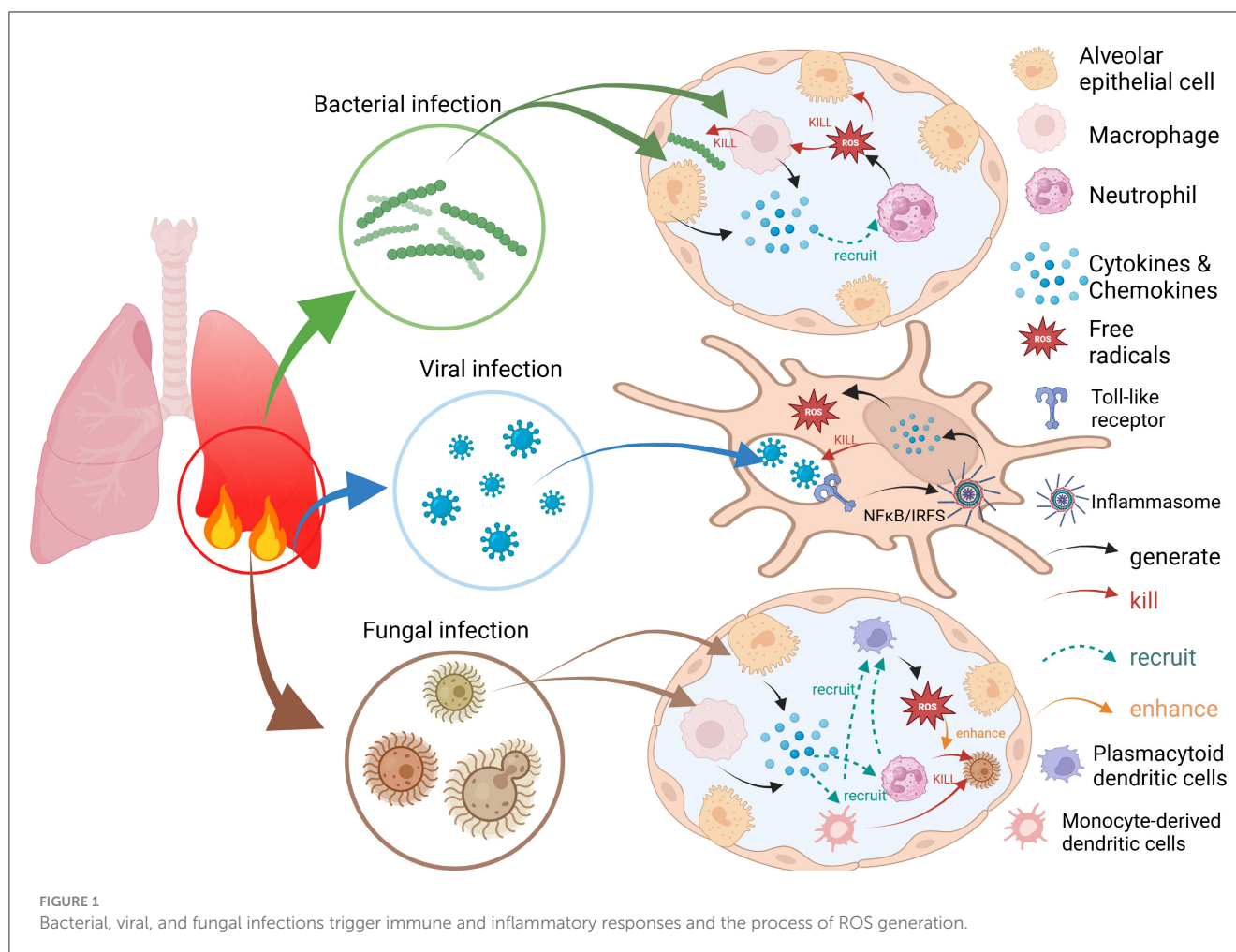
macrophages, which secrete cytokines and neutrophil chemokines and subsequently recruit additional immune cells from the pulmonary circulation to the site of infection. These immune cells produce proteases, *reactive oxygen species* (ROS) and *reactive nitrogen species* (RNS) and act on infected cells to induce necrotic cell death (10). For viral antigens, the adaptive immune response is activated mainly by the encounter of viral particles with antigen-presenting cells or B-cell receptors and further induces a variety of immune cell interactions and the production of cytokines, chemokines, etc., to inhibit viral replication and transmission and protect the host from viral attack (11, 12). Certainly, viruses also evade immune cell recognition and antiviral responses by increasing their affinity for target cells, inhibiting the recognition of relevant receptors and disrupting immune signaling pathways. It can even provoke the immune system to attack its own tissues in the form of inducing autoimmune and autoinflammatory processes that allow it to survive in the host (13). For fungi, within the respiratory tree, inhaled fungal cells are opsonized with soluble pattern recognition receptors, surfactants, complement, and antibodies. Mainly cells such as neutrophils, monocytes, monocyte-derived dendritic cells, *plasmacytoid dendritic cells* (pDCs), and mediators such as cytokines, neutrophil chemotaxis, and interferon are involved in the whole immune process. Among these, pDCs do not bind conidia but enhance the oxidative burst to boost conidial killing in neutrophils (14).

Certainly, there are many other disease-causing pathogens in CAP, but the same is true for the various immune responses of the organism to pathogens, which are usually beneficial to the host (15–17). However, some studies have noted that overzealous activation of the innate immune system can lead to immunopathology, which may result in poor outcomes and even contribute to secondary bacterial infections in the lower respiratory tract, which can lead to pneumonia (18–20). The occurrence of such an excessive immune response may be due to the uncontrolled production of inflammatory cytokines, which trigger the release of large amounts of inflammatory mediators and excessive production of ROS, which exacerbates or prolongs the stimulation and directly or indirectly leads to cell and tissue death and widespread lung injury, thus increasing the burden of CAP (21). Thus, there is still a delicate balance that needs to be regulated between appropriate immunoprotection and excessive pathologic immunity.

The new idea holds that the various microorganisms in the lungs have a complex relationship with the host and with each other that is analogous to an 'adaptive island', where they maintain a dynamic equilibrium and constraints (22). On the basis of this theory, regardless of whether a new pathogen invades, disrupting the balance of existing microorganisms can lead to lung infections. The immune and inflammatory responses of an organism are measured to maintain this balance. Although the whole process is provoked by the pathogen, preinfectious susceptibility to pneumonia, the host's own characteristics, and host–pathogen interactions also play a catalytic role in the onset of CAP and the activation of the immune response (23, 24).

Compared with healthy young adults, it is commonly agreed that older adults have characteristic elevated serum levels of proinflammatory markers (25). During aging, the levels of inflammatory factors and serum proinflammatory markers increase significantly, even in older adults without chronic diseases

Abbreviations: CAP, community-acquired pneumonia; COVID-19, coronavirus disease 2019; ROS, reactive oxygen species; RNS, reactive nitrogen species; GSH, glutathione; CoQ10, coenzyme Q10; VitC, vitamin C; VitE, vitamin E; GPX, glutathione peroxidase; CAT, catalase; SOD, superoxide dismutase; Nrf2, NF-E2-related factor 2; Keap1, kelch-like ECH-associated protein 1; AREs, antioxidant response elements; AMPK, adenosine 5'-monophosphate-activated protein kinase; ATP, adenosine triphosphate; FoxO3a, Forkhead box O3a; HIF-1 α , hypoxia-inducible factor 1 α ; PGC-1 α , peroxisome proliferator-activated receptor γ coactivator-1 α ; ER, endoplasmic reticulum; STING, stimulator of interferon genes; TBK1, TANK-binding kinase 1; NOX2, NADPH oxidase; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; AP-1, activating protein 1; TNF- α , tumor necrosis factor- α ; NG, non-growing; *S. aureus*, *Staphylococcus aureus*; FQ, fluoroquinolones; ARDS, acute respiratory distress syndrome; NAC, N-Acetyl-L-cysteine; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; Th1, T helper 1.



(26). This persistent inflammation, also known as low-grade inflammation, drives the onset of age-related diseases and the exacerbation of chronic conditions such that older adults are more likely to have significant lung inflammation and lung tissue damage (27, 28). In short, multiple components, such as pathogenic microorganisms, inflammatory responses, and immune resistance, are combined to determine whether CAP occurs and how it progresses or subsides. And the impacts on older adult patients are particularly significant and complex.

3 Overview of oxidative stress

Oxidation and antioxidant are two systems in the body that are interdependent but also mutually restrictive, and oxidative stress is the result of a disruption of the pro-oxidant–antioxidant balance and the consequent accumulation of ROS (29). ROS are highly reactive active molecules produced by the interaction of oxygen with certain elements; they hold at least one unpaired electron in their outer shell and are therefore extremely unstable (30). ROS can either originate from outside the body or be produced by certain oxidizing enzymes in the body. However, whether endogenous or exogenous, major cellular macromolecules such as nucleic acids, lipids and proteins can be oxidatively

modified under the effect of ROS, which can lead to structural and functional alterations in cells (31). Therefore, ROS can be involved in both physiological apoptosis and pathological cell death.

Fortunately, the innate cellular antioxidant system also blocks overly intense inflammatory responses and prevents excessive ROS accumulation, thus limiting damage and inhibiting disease (32). Antioxidant defenses can be divided into non-enzymatic and enzymatic antioxidants. Non-enzymatic antioxidants include those naturally produced by cells, such as *glutathione* (GSH), *coenzyme Q10* (CoQ10), and melatonin, and non-naturally produced antioxidants, such as *vitamin C* (VitC), *vitamin E* (VitE) and flavonoids (33). They are characterized by the ability to act directly and quickly inactivate free radicals and oxidants (34). GSH is an essential and important non-enzymatic antioxidant in mammalian cells. It is not only used directly as an antioxidant to protect cells from free radicals and pro-oxidants but also as a cofactor for antioxidant and detoxification enzymes such as *glutathione peroxidase* (GPX), *glutathione S-transferase* and *glyoxalase* (35). The main antioxidant enzymes include *catalase* (CAT), *superoxide dismutase* (SOD), GPX, etc. O_2 is converted to H_2O_2 by SOD, which is then broken down by CAT into O_2 and H_2O , thereby preventing the generation of hydroxyl radicals. GPX converts peroxides and hydroxyl radicals to non-toxic forms by oxidizing

GSH to glutathione disulfide, which is then reduced to GSH by glutathione reductase (36, 37).

The antioxidant response is modulated mainly by the Nrf2/Keap1 system (Figure 2). Nrf2 (*NF-E2-related factor 2*) is a transcription factor, whereas Keap1 (*kelch-like ECH-associated protein 1*) is an Nrf2 repressor. Under physiological conditions, Keap1 acts as a negative regulator of Nrf2, locking Nrf2 in the cytoplasm and preventing it from entering the nucleus. On the other hand, Keap1 mediates the ubiquitination-mediated degradation of Nrf2 to maintain intracellular Nrf2 homeostasis. When stimulated by ROS, the cysteine residues on Keap1 are modified by ROS, and Nrf2 dissociates from Keap1. The activated Nrf2 enters the nucleus and binds to the Maf protein to form a heterodimer. Binding to *antioxidant response elements* (AREs) induces the transcription of genes regulated by AREs, which initiates the expression of genes encoding cytoprotective enzymes, such as antioxidative stress proteins, especially antioxidant enzymes (38). However, upon exposure to oxidative stress, Keap1 loses the ability to ubiquitinate Nrf2, leading to disruption of the redox balance, which may induce damage to cellular mitochondria, proteins, DNA, etc. (39).

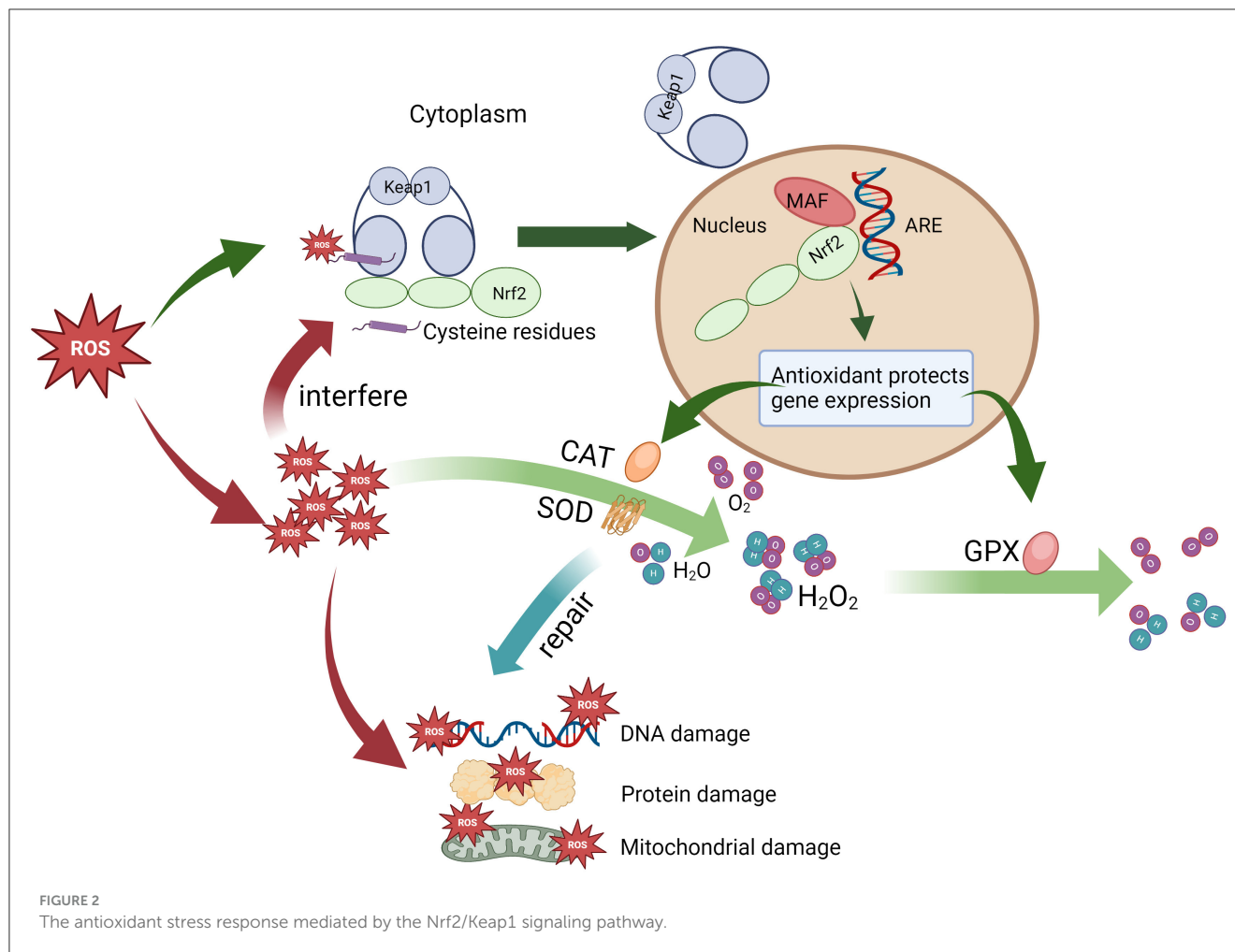
The antioxidant response is also modulated by signaling pathways mediated by *adenosine 5'-monophosphate-activated protein kinase* (AMPK). AMPK is a serine/threonine kinase expressed in all eukaryotic tissues and organs, playing a key role in *adenosine triphosphate* (ATP) synthesis (40). As a central cellular energy sensor, AMPK becomes activated during metabolic perturbations including oxidative stress and inflammatory conditions (40–42). Previous study has shown that AMPK plays cytoprotective roles in glucose and lipid metabolism and protein synthesis (43). AMPK activation also preserves mitochondrial function by stabilizing redox homeostasis and membrane potential, thereby suppressing apoptosis (43). Key AMPK-dependent transcription factors, including *Forkhead box O3a* (FoxO3a), Nrf2, and Sirt1, coordinate the expression of antioxidant defense genes (44–46). Under hypoxia, mitochondria induce hypoxia-inducible factor 1 α (HIF-1 α) expression, a process accompanied by ROS production (47). To modulate cellular energy homeostasis, AMPK enhances FoxO3a transcription (48). Induced FoxO3a mitigates increased ROS levels in hypoxic cells by decreasing HIF-1 α accumulation, exerting anti-oxidative stress effects, and altering the cellular hypoxic response (49). AMPK activation directly causes Nrf2 to migrate and aggregate into the nucleus and indirectly increases nuclear Nrf2 levels, thereby promoting antioxidant enzyme gene expression to combat oxidative damage (32). Furthermore, AMPK increases Sirt1 activity in cells with oxidative stress-induced senescence (50, 51). The sirtuin protein family, particularly Sirt1, acts as a key epigenetic regulator of aging, exerting protective effects against sarcopenia and frailty in older adults and promoting longevity (52). Sirt1-mediated deacetylation of *peroxisome proliferator-activated receptor γ coactivator-1 α* (PGC-1 α) enhances mitochondrial stabilization in skeletal muscle, as shown in *in vitro* and *in vivo* models (53). Meanwhile, Sirt1 exerts anti-oxidative stress effects by enhancing the expression of antioxidants such as MnSOD, CAT, and GPX through deacetylation and activation of pathways like PGC-1 α and FoxO3a (52). Conversely, Sirt1 inhibition increases

ROS production (54). In conclusion, cellular defense against ROS overload involves coordinated mechanisms, with antioxidant enzyme systems playing a pivotal role in ROS scavenging. Furthermore, antioxidant enzymes have repair mechanisms for free radical-induced damage. It can repair damaged DNA and proteins, fight against oxidized lipids, stop chain propagation of peroxyl lipid radicals, and repair damaged cell membranes and molecules (55).

4 Relationship between oxidative stress and CAP

Oxidative stress is an important component of the innate immune system and is part of the defense mechanism against pathogens (56). On the one hand, oxidative stress is considered to be one of the pathogenic mechanisms of CAP and is closely related to inflammation (57). Numerous studies have shown that oxidative stress is greater in patients suffering from CAP than in healthy volunteers (6, 58, 59). Indeed, after the onset of pneumonia, immune cells respond by releasing ROS and inflammatory factors at the site of infection. For example, macrophages are major contributors to the immune response. Upon activation, macrophage TXNIP protein induces ROS production, causing *endoplasmic reticulum* (ER) stress. This, in turn, triggers cellular signaling processes, including redox homeostasis, inflammation, and immune responses (60). During ER stress, *stimulator of interferon genes* (STING), a transmembrane protein predominantly located in the ER, responds to stress signals and promotes inflammasome activation, such as NLRP3, via the STING/TANK-binding kinase 1 (TBK1) pathway, leading to tissue inflammation (61). Neutrophils, another key cell type involved in killing bacteria and other microorganisms, activate *NADPH oxidase* (NOX2) on their cell membranes upon stimulation. This generates large amounts of superoxide and ROS, which react with pathogens in phagosomes and trigger an inflammatory response (62). In contrast, lung cells respond to oxidative responses by activating *nuclear factor kappa-light-chain-enhancer of activated B cells* (NF- κ B) and *activating protein-1* (AP-1), which inhibits oxidative stress while also stimulating the release of cytokines such as *tumor necrosis factor- α* (TNF- α) (57, 63). In this way, while maintaining the balance of the redox system, lung cells are able to generate a certain inflammatory response to trigger an optimal immune response. A review states that ROS generated by immune and inflammatory responses in tissues and organs act as potent killers of various pathogens, and controlled accumulation of ROS serves as an effective weapon against pathogen invasion (32). For example, in neutrophils, pathogens are captured and internalized via phagocytosis, forming membrane-enclosed phagosomes (64). Within these phagosomes, pathogens are exposed to bactericidal peptides, destructive enzymes, and ROS, leading to significant degradation (62). However, once uncontrolled oxidative stress occurs and cytokines are overproduced, not only can the inflammatory state of the lungs be exacerbated, but more ROS will continue to be produced, worsening the situation (Figure 3).

Notably, if the homeostasis of the redox system is persistently disrupted, it also weakens the innate and acquired immune response, exacerbates bacterial and viral infections, causes



cellular and tissue damage, and drives the progression of CAP. Specific modes of damage include affecting the CD4⁺/CD8⁺ T-cell ratio, impairing natural killer cell function and neutrophil migration/phagocytosis, and reducing the stability of the macrophage cytoskeleton to affect phagocytosis (32). At the molecular level, excess ROS can react rapidly and non-specifically with chemical groups in DNA, lipids and proteins, leading to cellular metabolic disorders, loss of function and abnormal apoptosis (65). If a vicious cycle of uncontrolled oxidative stress and an uncontrolled inflammatory response further develops, more trauma to cells and tissues can occur, leading to a poor CAP outcome. For example, when a respiratory viral infection is combined with invasive fungal disease, cytokines attract more immune cells, which in turn produce more cytokines, creating an inflammatory cycle that damages lung tissues and causes the formation of fibrin and scar tissue, which allows fluids to seep into and fill the lung cavities, leading to respiratory failure (66). However, causing oxidative stress is not always detrimental. An early review revealed that oxidative stress is associated with persistent bacterial infections and antibiotic resistance. Pathogenic bacteria may undergo physiological changes by adapting to oxidative stress, either by promoting bacterial survival during antibiotic exposure or by influencing the oxidative stress response mechanism, thereby reducing the effective intracellular

concentration of the antibiotic and allowing the bacteria to survive (67).

Beyond genetically resistant bacteria, persisters, which are genetically susceptible cells that survive antibiotic doses that kill the rest of the clonal population (68). These persisters are *non-growing* (NG) bacteria that coexist with their growing counterparts but are transiently insensitive to antibiotics (69). They are thought to resume growth after antibiotic treatment ceases, leading to chronic infections, recurrences, and subsequent treatment failure (70, 71). Altered adaptation to oxidative stress is a major driver of antibiotic resistance in these persisters (72, 73). A recent research has shown that *in vitro* treatment of stationary-phase *Staphylococcus aureus* (*S. aureus*) with *fluoroquinolones* (FQ) enhances FQ lethality by stimulating ROS production in cells (74). Thus, enhancing oxidative stress might be valuable in cases of persistent bacterial infections and/or antibiotic resistance. However, the role of ROS in controlling bacterial infections and mediating bacterial responses to antibiotics is complex. Studies indicate that in the case of the major Gram-positive pathogen, *S. aureus*, ROS are inhibitory to antibiotic mediated killing and their generation by the host increases *S. aureus* antibiotic tolerance during infection (75, 76). Another study found that after engulfment by macrophages, sustained production of RNS by host cells locks persisters in a NG state via tricarboxylic acid-cycle intoxication, thereby poisoning

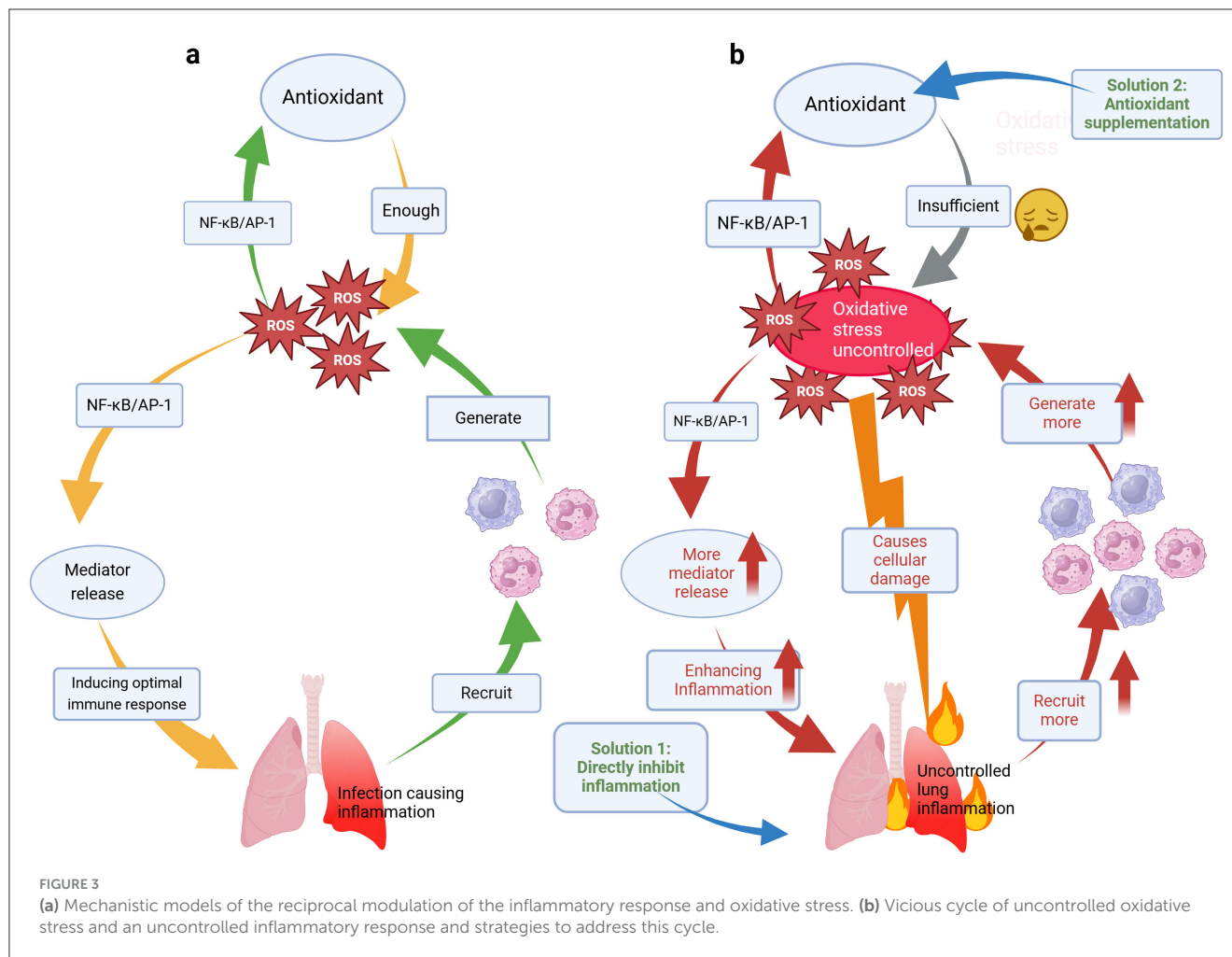


FIGURE 3

(a) Mechanistic models of the reciprocal modulation of the inflammatory response and oxidative stress. (b) Vicious cycle of uncontrolled oxidative stress and an uncontrolled inflammatory response and strategies to address this cycle.

bacterial respiration. Chemical inhibition of RNS production can stimulate persisters growth resumption, thereby resensitizing them to the action of bactericidal antibiotics and improve the effectiveness of treatment (73). Therefore, given that anti-infection therapy remains the core treatment for CAP, it is crucial to modulate oxidative stress to address the diverse bacterial responses to antibiotics.

Viral infections are associated with reduced GSH levels, which cause a cellular redox imbalance and activate a vicious cycle of reduced host defenses, leading to a severe imbalance in the intracellular microenvironment, and adjunctive antioxidant treatment may improve the outcomes of antiviral therapies (77). In fungal infections, an emerging view is that ROS production, regulation, and response are central axes of the host–pathogen interaction (78). Both the host and fungi produce ROS, use conserved mechanisms to detoxify ROS, and leverage ROS in their local environment to mediate defense mechanisms. In conclusion, oxidative stress is one of the important mechanisms in the development and progression of CAP, and the inflammatory response caused by lung lesions can exacerbate oxidative stress. On the basis of this situation, theoretically, exogenous supplementation with antioxidants is necessary along with the modulation of the immune and inflammatory responses. Moreover, an initial trial

demonstrated that altered oxidative stress status is associated with the severity of CAP (5). Consequently, antioxidant therapy has been proposed as a supplementary treatment option.

5 Potential benefits and application value of modulating oxidative stress in older CAP patients

Global aging is already a reality today, putting enormous pressure on every country's healthcare system and representing an enormous challenge to global public health. The number of intensive care unit hospitalizations due to CAP have been rising steadily in recent years, especially in the older population, where CAP is a common cause of hospitalization, readmission, and death (79). A survey indicated that nearly 1 million older people in the U.S. are hospitalized for CAP each year, and nearly one-third of them die within 1 year; the burden of CAP in the older population is enormous (80). Despite geographic differences, *Streptococcus pneumoniae* remains a major pathogen for all ages worldwide (81, 82). And older adults are also more vulnerable to the growing epidemic caused by viruses (83). Compared with

younger patients, older patients have an increased risk of infection with other pneumonia pathogens, and infections with influenza virus and antibiotic-resistant pathogens should also be considered. However, the incidence of so-called atypical pathogens is relatively low, and *Mycoplasma* infections are particularly rare (84). Early and appropriate anti-infective therapy remains a core part of the CAP treatment regimen. However, inappropriate antibiotic use is common in older adults (>65 years old), and older adults are particularly vulnerable to severe antibiotic-related adverse effects (85). Moreover, bactericidal antibiotics (quinolones, β -lactams, and aminoglycosides) have been found to cause ROS overproduction and mitochondrial dysfunction in mammalian cells, leading to oxidative damage to membrane lipids, proteins, and DNA (86).

Additionally, the aging process is accompanied by chronic inflammation and immune senescence (87). It has already been made clear that CAP is closely related to the immune response and inflammation, and the presence of an inadequate immune response together with an inflammatory flare-up can lead to a worse outcome (88). Therefore, older patients are at greater risk. On the basis of these findings, clinical intervention involving immunomodulation and suppression of the inflammatory response in older adults with CAP, such as the use of immunoglobulins and cortisol, would theoretically be beneficial but requires careful evaluation before use and has limited applicability (89, 90). Attention should also be paid to the fact that inappropriate treatment of patients hospitalized with CAP prolongs hospitalization and increases costs and mortality rates (82). In addition, with respect to older age groups, the fact that the nutritional status of older age groups, multiple age-related diseases, and chronic underlying conditions all have an impact on the onset and progression of CAP in older adults cannot be ignored (84). The positive effects of modulating oxidative stress in sepsis, *acute respiratory distress syndrome* (ARDS) and acute lung injury have been clinically demonstrated. It improves oxygenation rates and GSH levels and strengthens the immune response, and it reduces mechanical ventilation time, the length of stay in the intensive care unit, multiple organ dysfunctions, the length of stay in the hospital and mortality rates in acute lung injury/ARDS patients (84).

In conclusion, oxidative stress is involved in several aspects of CAP, including immunity and inflammation. Given the special risk factors and manifestations of CAP in older individuals and the existing studies, modulating oxidative stress may have the following advantages and application value (Table 1).

5.1 Preventive value of CAP in older adults

Regardless of what disease you are facing, the first tool to consider should be prevention. Vaccines, as the only effective preventive medical treatment, can reduce the risk and burden of contracting diseases such as influenza and pneumococcal pneumonia (120). An analysis of national pneumococcal vaccination data in Korea revealed that previous pneumococcal vaccination in the older population effectively improved hospitalization rates and 30-day mortality among patients hospitalized for pneumonia (121). However, the outcomes of pneumococcal vaccine use in older adults remain controversial.

A retrospective study in Japan concluded that the effectiveness of pneumococcal vaccines was significant in people aged 65–85 years but not in the older population (122). However, vaccine coverage is not satisfactory, and increasing vaccination rates remains a difficult challenge (123). Moreover, vaccines do not cover all pathogens and take a certain amount of time to develop. When faced with an outbreak of a worldwide disease such as COVID-19, the use of a vaccine can be caught off guard (124).

Oxidative stress is one of the important mechanisms in the development of CAP, and maintaining the balance of the oxidative state in the body may be able to produce a preventive effect through a different mode of action than vaccines do. Moreover, in the older population, there is a low-grade chronic inflammatory state formed by senescent cells stimulating the secretion of proinflammatory cytokines. Individuals with low-grade chronic inflammation present a dysregulated innate immune system, resulting in an increased risk of infection (125). Meanwhile, the subsequent inflammatory cascade further increases extracellular ROS concentrations and oxidative stress (126). From this perspective, modulating oxidative stress can also play a role in preventing the onset and controlling the progression of CAP in older individuals by controlling chronic inflammation and reducing the accumulation of ROS.

For example, vitamins C and E are the most common antioxidants. VitE is a fat-soluble antioxidant whose main role is to prevent lipid peroxidation, which minimizes the formation of secondary radicals. Alpha-tocopherol is the most potent antioxidant in the VitE composition and can react quickly with free radicals to generate relatively stable tocopheroxyl radicals, thus improving oxidative stress (33). An earlier prospective cohort study also confirmed that VitE supplements prevented rehospitalization in patients (>65 years old) who were first hospitalized for CAP (98). In another follow-up study, VitE (50 mg/d) was administered for 5–8 years to older male smokers (50–69 years old), which revealed that VitE administration reduced the incidence of pneumonia in older men in different subgroups (99). However, some studies have noted that the effect of VitE on the incidence of pneumonia does not seem to be completely uniform (127–129). An early clinical trial found that VitE (50 mg/day) did not affect pneumonia risk in participants with physically demanding jobs but reduced the risk by 50% in those engaging in moderate or heavy exercise during leisure time (129). Another study found that VitE reduced the risk of pneumonia by 69% in participants who smoked the least and exercised in their leisure time but increased the risk of pneumonia by 68% in participants who smoked the most and did not exercise (130). However, the study focused only on Finnish male smokers aged 50–69 years, and long-term high-frequency smoking is more directly and detrimentally associated with the development of pneumonia.

VitC can directly quench free radicals in the aqueous layer and also cooperate with VitE to regenerate α -tocopherol from α -tocopherol radicals in membranes and lipoproteins and increase GSH levels in the cell; thus, it plays an important role in protein thiol group protection against oxidation (33). One study reported that VitC can effectively kill bacteria, mycobacteria, HIV, and HCV because it can generate free radicals and H_2O_2 (131). As such, VitC plays a number of important roles in reducing oxidative stress caused by infection, balancing the immune system, and killing

TABLE 1 Summary of pilot studies on vitamins C and E, NAC, CoQ10, and melatonin discussed in this article.

Constituent	Study design	Description	Main results and finding	Experimental limitations	References
Vitamin C	Clinical trial	Whether 2.0 grams/day of VitC prophylaxis versus placebo over 8 weeks reduces the incidence or severity of the common cold and other respiratory illnesses in 674 marine recruits.	Whole blood ascorbic acid levels were significantly higher in the VitC group 6 weeks after study initiation. No differences in cold incidence or duration were observed between groups, but cold symptoms were less severe in the VitC group. This study and the literature do not support using VitC prophylactically to prevent the common cold.	Study population and age limitations.	Pitt et al. (91)
	Clinical trial	Fifty-seven older adult patients admitted for acute respiratory infections (bronchitis and bronchopneumonia) received either 200 mg/day of VitC or placebo.	This modest oral dose significantly increased plasma and white cell VitC concentrations, even during acute respiratory infection. Patients receiving VitC supplementation showed significantly better clinical outcomes based on a symptom-based scoring system, especially those who were most severely ill at trial onset.	Enrolled patients with bronchitis may affect experimental results.	Hunt et al. (92)
	Cellular experiment (human and mice)	The study included 45 patients: 15 with normal status, 15 with non-severe CAP, and 15 with severe CAP. Lung tissues, blood specimens, and cultured murine alveolar macrophage cell lines were obtained from the patients and treated with lipopolysaccharide (LPS) and VitC. Various indicators of inflammation and oxidative stress were assessed.	An <i>in vitro</i> cellular assay confirmed that VitC reduced ROS and DNA damage in peripheral blood mononuclear cells and decreased TNF- α and IL-6 levels in whole blood cells from severe CAP patients. TNF- α , p38, and phosphorylated p38 levels were increased in LPS-stimulated macrophages. VitC reduced ROS levels, DNA damage, and the expression of TNF- α , p38, and phosphorylated p38 in LPS-stimulated macrophages <i>in vitro</i> .	Not mentioned.	Hunt et al. (93)
	Clinical trial	Ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was used to measure plasma VitC concentrations in 25 COVID-19 patients treated with VitC and 6 untreated, as well as in 60 healthy volunteers (51 for VitC measurement, 6 for blank plasma, and 3 for stability analyses).	COVID-19 patients had mean plasma VitC concentrations nearly five times lower than healthy volunteers. High-dose VitC treatment normalized and even exceeded healthy levels. Thus, 100 mg/kg/day VitC supplementation is essential for COVID-19 patients.	The trial was not a multicenter randomized controlled trial and recruited only non-smoking females to eliminate smoking effects on VitC concentrations.	Xing et al. (94)
	Animal study (mice)	Rats in the hepatic ischemia-reperfusion model received different doses of ascorbic acid (AA) before ischemia (30, 100, 300, and 1,000 mg/kg).	Hepatoprotective effects were observed only at low AA doses (30 or 100 mg/kg), whereas high doses (1,000 mg/kg) exacerbated ischemia/reperfusion-induced hepatic injury via lipid peroxidation. AA primarily acts as an antioxidant at low doses but exhibits pro-oxidant effects at high doses; thus, its use in pharmacological doses should be approached with caution.	Not mentioned.	Seo et al. (95)
	Clinical trial	The study included 60 patients (30 in each group) aged ≥ 18 years with moderate to severe COVID-19, hospitalized in the intensive care unit. The intervention group received intravenous VitC (1g q8h) for 4 days, while the placebo group received saline.	Although the intervention group had more severe cases, mortality was lower. High-dose intravenous VitC may reduce inflammation, improve oxygen support, and lower mortality in COVID-19 patients without adverse effects, suggesting its potential as a promising therapy for moderate to severe cases.	These results did not reach statistical significance due to the small sample size and moderate dose of intravenous VitC.	Kumar et al. (96)
	Clinical trial	Sixty patients with severe COVID-19 were randomized into control and intervention groups. The control group received lopinavir/ritonavir and hydroxychloroquine, while the intervention group received high-dose intravenous VitC (6 g/day) in addition to the same regimen.	On day 3, the intervention group showed greater improvements in mean body temperature and peripheral capillary oxygen saturation compared to the control group ($p < 0.05$), but had a significantly longer median hospital stay ($p = 0.028$). No significant differences were observed between groups in SpO ₂ levels at discharge, ICU length of stay, or mortality. No adverse effects were reported.	Limitations include small sample size and subjective evaluation indicators.	JamaliMoghadamSiahkali et al. (97)

(Continued)

TABLE 1 (Continued)

Constituent	Study design	Description	Main results and finding	Experimental limitations	References
Vitamin E	Clinical trial	Patients aged ≥ 65 years hospitalized for CAP were followed for up to 30 days from initial hospitalization for mortality. Those discharged alive within 30 days were followed for up to 90 days for re-hospitalization.	The study included 717 patients. VitE supplementation reduced the risk of re-hospitalization for any cause following discharge after pneumonia ($P = 0.028$). Neither influenza nor pneumococcal vaccines showed any protective effects on mortality or re-hospitalization.	Limitations included: (1) rare mortality and re-hospitalization events; (2) significant bias in assessing modifiable factors; (3) small sample size and patients lost to follow-up; (4) unconsidered impact of complications, overlapping infections, and adverse events.	Neupane et al. (98)
	Clinical trial	The study included 7,469 male smokers aged 50–69 years who had smoked since at least age 21. Participants received 50 mg/day of VitE for 5–8 years. The primary outcome was the incidence of hospital-treated CAP assessed at follow-up.	Among 2,216 participants who smoked 5–19 cigarettes/day and exercised, VitE supplementation reduced pneumonia incidence by 69% and prevented pneumonia in 12.9% of those taking VitE before age 74. Among 5,253 participants who smoked ≥ 20 cigarettes/day or did not exercise, VitE reduced pneumonia incidence by 14%. Overall, pneumonia incidence was 72% lower in patients using VitE, including those who smoked ≥ 20 cigarettes/day or did not exercise.	Limitations: The study only included older male smokers and did not involve non-smokers.	Hemilä (99)
	Clinical trial	The effect of VitE (50 mg/day) on the onset of pneumonia was examined in 133 male smokers aged 50–69 years, with the first occurrence of hospital-treated pneumonia assessed over a 3-year follow-up period.	VitE (50 mg/day) did not affect pneumonia risk in participants with physically demanding jobs but reduced the risk by 50% in those engaging in moderate or heavy exercise during leisure time	No definitive practical conclusions can be drawn from this subgroup finding, and further studies are warranted among individuals engaging in moderate or heavy exercise.	Milisav et al. (100)
	Animal study (mice)	To establish an aging model, 6-week-old female mice were continuously administered d-galactose (d-gal). The VitE group received intragastric α -Tocopherol (50 mg/100 mL/kg) diluted in highly refined, low-acidity olive oil, along with daily intraperitoneal d-gal for 4 weeks. Levels of advanced glycation end products (AGEs) in the blood, T cell phenotypes in the thymus and spleen, and ROS levels in spleen-derived leukocytes and T cells were measured as indices of aging and immunosenescence.	After 6 weeks of d-gal administration, intracellular ROS levels in the mouse spleen and serum AGEs were significantly higher. Thymic function was reduced, and peripheral T cell subsets redistributed. VitE treatment significantly reduced ROS levels ($P < 0.05$) and decreased serum AGEs, while increasing the naïve to effector-memory T cell ratio in aging mice.	Limitations of this study include not considering the effects of gender, age, and treatment duration on D-gal-induced oxidative stress. Additionally, the research did not provide further evidence of an aging phenotype beyond serum AGE levels.	Lee et al. (101)
	Cellular experiment (human skeletal muscle myoblasts)	Human skeletal muscle myoblasts in the replicative senescent state were treated with tocotrienol-rich fraction (TRF), an ubtype of VitE.	Replicative senescence increased ROS generation and lipid peroxidation in myoblasts. TRF treatment significantly reduced ROS production and lipid peroxidation. It also modulated the gene expression of SOD, CAT, and GPX, increasing superoxide dismutase and catalase activity while reducing glutathione peroxidase activity. TRF improved antioxidant defenses and mitigated oxidative stress associated with replicative senescence in myoblasts.	Not mentioned.	Khor et al. (102)

(Continued)

TABLE 1 (Continued)

Constituent	Study design	Description	Main results and finding	Experimental limitations	References
Vitamin E	Clinical trial	Among 817 eligible older adults categorized into frail and non-frail groups, plasma VitE levels were measured. Confounders assessed included lower extremity muscle strength, cognitive function, diseases, and factors related to VitE metabolism.	In a logistic model adjusted for multiple confounders, participants in the highest VitE tertile were less likely to be frail compared to those in the lowest tertile. Oxidative stress may contribute to frailty development. VitE: Low circulating levels of a key component of the human antioxidant system are associated with frailty. VitE may be associated with frailty through an unknown mechanism independent of its antioxidant properties.	Important factors were not considered, and confounder measures lacked precision, potentially leaving residual confounding. Other antioxidant pathways were not assessed. The cross-sectional study design precludes ruling out reverse causality.	Ble et al. (103)
Vitamin C and Vitamin E	Animal study (mice)	In an oleic acid (OA)-induced ARDS rat model of acute lung injury, control groups received no treatment, while experimental groups ($n = 6$ each) received VitE (100 mg/kg) or VitC (100 mg/kg).	OA increased lung MDA levels and serum TNF- α and IL-1 β levels, while VitE and VitC significantly reduced cytokine levels. OA also decreased lung SOD, CAT, and GSH levels, which were significantly restored by vitamins. Histopathological evidence showed reduced neutrophil infiltration in VitE and VitC groups compared to the OA group. Additionally, these groups exhibited less endothelial and tissue damage, as indicated by decreased bronchial and alveolar septum thickening, vascular hyperemia, and polynuclear cell infiltration.	Not mentioned.	Erol et al. (104)
	Animal study (mice)	The dorsiflexors of the left limbs in young and aged male rats were subjected to repetitive loading exercise. Seven animals from each age group were randomly assigned to either a diet supplemented with VitE (DL-alpha tocopheryl acetate; 30,000 mg/kg) and VitC (L-ascorbic acid; 2% by weight) or normal non-supplemented (NS) rat chow containing 126 mg/kg VitE and no VitC. All animals had free access to rat chow and water. Biomarkers of oxidative stress were measured in the tibialis anterior muscles.	Positive work increased only in aged animals supplemented with VitE and C. Markers of oxidative stress increased in the tibialis anterior muscles of aged and young adult animals subjected to repetitive loading, but VitE and C supplements attenuated this increase. CuZnSOD and CAT activities increased with supplementation in both young adult and aged animals. These data suggest that antioxidant supplementation improves indices of oxidative stress associated with repetitive loading exercise and aging and enhances the positive work output of muscles in aged rodents.	VitE and C levels were not measured in the skeletal muscles of experimental animals.	Ryan et al. (105)
	Clinical trial	Forty male patients with type 2 diabetes aged 40–60 years, receiving antidiabetic therapy with 500 mg of metformin tablets twice daily (BID). The patients were randomly divided into four groups: Control group: 500 mg of metformin + placebo BID; VitC group: 500 mg of metformin + 500 mg of VitC BID; VitE group: 500 mg of metformin + 400 mg of VitE BID; Vitamins C + E group: 500 mg of metformin + 500 mg of VitC + 400 mg of VitE BID. All groups received the treatments for 90 consecutive days.	VitC and/or E improved fasting blood sugar (FBS), HbA1c, lipid profile, insulin, homeostasis model assessment of insulin resistance (HOMA-IR), reduced glutathione (GSH), and Quantitative Insulin Sensitivity Check Index (QUICKI) compared with the diabetic group receiving placebo. This study provides additional evidence that antioxidant vitamin supplementation in type 2 diabetes mellitus (T2DM) can improve clinical outcomes, attenuate or prevent diabetic pathogenesis and complications, and address the imbalance between declining endogenous antioxidants and increasing ROS production, which is exacerbated by poor glycemic control and contributes to oxidant-mediated damage in diabetes.	The experimental sample size was small and included only males.	El-Aal et al. (106)
NAC	Cellular experiment	Intervention with the antiviral drugs raltegravir (Rem), nelfinavir (Nel), and NAC in SARS-CoV-2-infected Vero E6 cells.	SARS-CoV-2 infection impairs cellular glutathione metabolism. NAC and the antiviral drug Nel can prevent this defect <i>in vitro</i> .	A limitation of this study is the use of Vero E6 cells instead of a cell model that more closely represents the lung cell targets of SARS-CoV-2.	Bartolini et al. (107)

(Continued)

TABLE 1 (Continued)

Constituent	Study design	Description	Main results and finding	Experimental limitations	References
NAC	Clinical research	The 217 patients hospitalized with COVID-19 in the intervention group received 1,500 mg of NAC intravenously daily, while the 245 patients hospitalized with COVID-19 in the control group did not receive NAC. Outcomes were assessed after 10 consecutive days of treatment.	Compared with the control group, the NAC group showed significant improvements in median room air SpO ₂ , median SpO ₂ /FiO ₂ ratio, length of hospitalization, and incidence of cough, dyspnea, and loss of appetite ($P < 0.05$). NAC is a safe, tolerable, available, and affordable drug that appears to be a promising adjuvant therapeutic agent for COVID-19.	This study was unicentric, and the efficacy of NAC in combination with remdesivir and steroids was not evaluated.	Afaghi et al. (108)
	Animal study (mice) and cellular experiment (bovine embryo tracheal cells)	Bovine embryonic tracheal cells (EBTr) and a mouse lung injury model, both induced with lipopolysaccharide (LPS), were treated differentially with NAC.	NAC pretreatment attenuated LPS-induced inflammation in both EBTr cells and mouse models. Moreover, LPS suppressed the expression of oxidative-related factors in EBTr cells while promoting the gene expression and secretion of inflammatory cytokines. inflammatory cytokines and decreased their mRNA levels, maintaining stable levels of antioxidative gene expression. Conversely, NAC pretreatment alleviated the secretion of inflammatory cytokines, decreased their mRNA levels, and maintained stable levels of antioxidative gene expression. <i>In vivo</i> , NAC mitigated LPS-induced inflammatory responses and lung injury in ALI mice. Compared with the LPS group, the NAC group exhibited significant decreases in relative protein concentration, total cell count, and percentage of neutrophils in BALF, as well as in the secretion levels of IL-6, IL-8, TNF- α , and IL-1 β , MPO activity, lung injury score, and expression levels of inflammatory-related genes. NAC also ameliorated LPS-induced changes in mRNA levels of antioxidant genes. In conclusion, the findings suggest that NAC modulates both inflammatory and oxidative responses, thereby alleviating LPS-induced inflammation in EBTr cells and lung injury in mice.	Some results observed in EBTr cells were not reproduced in the LPS-ALI mouse model.	Chen et al. (109)
	Clinical trial	COVID-19 patients receiving high-dose oral NAC (600 mg every 8 h) were compared with those not receiving NAC.	The NAC group ($n = 2,071$) had a higher overall baseline risk but showed better performance in reducing mortality. This result remained significant in multivariate analyses adjusted for baseline characteristics and concomitant corticosteroid use. However, no significant differences were observed in mean length of hospitalization, admission to the intensive care unit, or use of invasive mechanical ventilation between the NAC and non-NAC groups. High-dose oral NAC treatment in admitted COVID-19 patients was associated with significantly lower mortality, despite these patients being older, more frequently male, and having more comorbidities. NAC has a favorable safety profile.	The study was observational and lacked complete patient information; thus, a controlled clinical trial is required.	Izquierdo et al. (110)
	Clinical research	In COVID-19 patients admitted to the ICU, 72 patients who received NAC for 3 days were compared with 68 patients who did not receive NAC.	NAC-treated patients exhibited higher PaO ₂ /FiO ₂ ratios after 3 days in the ICU compared with the control group ($p < 0.05$). The administration of NAC improved the clinical and analytical responses in critically ill COVID-19 patients compared with the control group. NAC prevented the decrease in glutathione concentrations.	Limitations included the absence of data on patients with mild symptoms, the single-center design of the trial, and the lack of sensitivity in the GSH measurement method.	Gamarra-Morales et al. (111)

(Continued)

TABLE 1 (Continued)

Constituent	Study design	Description	Main results and finding	Experimental limitations	References
Coenzyme Q10	Cellular experiment (human lung, mice lung)	This study examines the effects of <i>P. aeruginosa</i> on lipid peroxidation in human and mouse lungs, as well as cell death induced by <i>P. aeruginosa</i> in human airway epithelial cells.	Lipid peroxidation was detected in human cystic fibrosis (CF) lungs and correlated with bacterial infection. Incubation of CF bronchial epithelial cells with <i>P. aeruginosa</i> induced an increase in ROS, leading to lipid peroxidation and cell death. Antioxidants, such as CoQ10, inhibited <i>P. aeruginosa</i> -induced cell death. Antioxidants are proposed as a treatment for pneumonia caused by <i>P. aeruginosa</i> infection.	Not mentioned.	Ousingsaw et al. (112)
	Clinical trial	Hospitalized older adults with CAP (diagnosed using defined clinical and radiological criteria) were randomized to receive oral CoQ10 (200 mg/d) or placebo for 14 days, in addition to antibiotics.	A total of 141 patients were included (70 in the CoQ10 group and 71 in the placebo group). Patients receiving CoQ10 recovered faster ($P = 0.0206$) and had a shorter hospital stay ($P = 0.0144$) compared with the placebo group than in the placebo group ($P = 0.0440$). The rate of treatment failure was lower in the CoQ10 group than in the placebo group ($P = 0.0440$). CoQ10 administration has no serious side effects and improves outcomes in hospitalized older adults with CAP; therefore, we recommend it as an adjunctive treatment in this population.	Limitations include the lack of assessment for CoQ10 deficiency, failure to identify bacterial pathogens, and the unexplored effect of specific bacterial pathogens on CoQ10 efficacy. Additionally, the use of clinical cure as the primary outcome is subjective and may introduce bias.	Farazi et al. (113)
Melatonin	Cellular experiment	A mouse model of Klebsiella pneumoniae infection was established. Control mice were left untreated, while experimental mice were treated with 2 mg/kg melatonin via oral gavage for 6 consecutive days. Corresponding tissues and cells were collected after treatment.	Melatonin treatment significantly upregulated telomerase activity in macrophages, which was associated with reduced ROS levels and enhanced cellular energy production, indicating improved mitochondrial function. Moreover, melatonin treatment suppressed NLRP3 inflammasome activation, resulting in reduced IL-1 β secretion. These findings suggest a potential therapeutic role for melatonin in pneumonia treatment.	The experiments were conducted <i>in vitro</i> using macrophage cell lines, which may not fully reflect the complexity of the <i>in vivo</i> environment. Insights into potential mechanisms were provided, but further investigation is needed.	Jiang et al. (114)
	Clinical research	Seventy-four hospitalized patients with mild to moderate COVID-19 were divided into test and control groups (37 patients each). Both groups received standard treatment, while the test group additionally received melatonin (3 mg, three times daily) for 14 days.	A total of 24 patients in the intervention group and 20 in the control group completed the treatment. Compared with the control group, the intervention group showed significant improvements in clinical symptoms (cough, dyspnea, malaise), CRP levels, lung involvement, mean time to discharge, and return to baseline health ($p < 0.05$). There were no deaths or adverse events in either group.	The main limitations of this trial were the small sample size and short-term follow-up, which may introduce bias.	Farnoosh et al. (115)
	Clinical research	Patients admitted to the ICU with severe COVID-19 were divided into two groups: 109 in the melatonin group and 117 in the control group. Both groups received conventional therapy, while the melatonin group additionally received oral melatonin (5 mg, twice daily) for 7 days.	Compared with the control group, patients in the melatonin group showed significant improvements in mortality, rate of mechanical ventilation, median days to discharge, and time to ≥ 2 -point improvement in clinical status ($P < 0.05$). Dizziness was the most common side effect in the melatonin group. Melatonin significantly improved clinical status and was well-tolerated in patients with severe COVID-19 pneumonia.	Further trials with larger sample sizes, diverse populations, and high-quality designs are needed to confirm these preliminary findings.	Ameri et al. (116)
	Clinical research	A total of 40 hospitalized patients with COVID-19 completed the study, with 20 patients in each of the intervention and control groups. Both groups received standard treatment, while the intervention group additionally received oral melatonin (9 mg/day) for 14 days. Blood samples were collected from patients at the beginning and end of the treatment period.	Adjuvant therapy with melatonin controlled and reduced inflammatory cytokines in COVID-19 patients. Melatonin also controlled and modulated the dysregulated genes involved in Th1- and Th2-mediated humoral and cellular immunity. This study demonstrated for the first time that melatonin can serve as an anti-inflammatory adjuvant by regulating the expression of Th1 and Th2 regulatory genes to reduce and control inflammatory cytokines in COVID-19 patients.	Limitations of the study included restricting participants to patients admitted at the start of the outbreak, excluding patients with severe symptoms, not assessing the effect of melatonin dosage, and not examining CD4+, CD8+ T cells, or NK cells.	Hosseini et al. (117)

(Continued)

TABLE 1 (Continued)

Constituent	Study design	Description	Main results and finding	Experimental limitations	References
Melatonin	Clinical research	Seventeen COVID-19 patients receiving melatonin treatment (5 mg, q12h, for 5 days) were compared with 20 healthy subjects.	The activities of antioxidant enzymes were decreased after melatonin treatment ($P < 0.05$). Melatonin treatment enhanced antioxidant enzyme activity, contributing to increased total antioxidant capacity and restoration of redox homeostasis. Thus, melatonin restores redox homeostasis altered in COVID-19 patients and can serve as an adjuvant therapy for SARS-CoV-2 infection.	Limitations of the study include the small sample size of COVID-19 patients, insufficient duration of antioxidant therapy due to budget constraints, and the absence of postmortem samples treated with melatonin using the immune colloidal gold technique to demonstrate prevention of mitochondrial sequestration.	Soto et al. (118)
Melatonin	Animal study (mice)	Middle-aged mice were divided into three groups (six mice per group). The control group was untreated, while the experimental groups received melatonin (MEL) at 5 mg/kg/day and 10 mg/kg/day by gavage for 2 months, respectively.	Melatonin (MEL) alleviated histological damage and increased the cross-sectional area of muscle fibers in gastrocnemius (GA) tissues of middle-aged mice. Furthermore, MEL treatment increased the percentage and size of normal mitochondria and mtDNA copy number while reducing MDA and ROS levels in GA tissues of middle-aged mice.	Not mentioned	Fang et al. (119)

Note that the findings presented in this table are not exhaustive, and some experiments do not mention study limitations.

microorganisms by generating free radicals. However, the role of VitC in preventing pneumonia is not very certain and is still only a reliable speculation that lacks confirmation from more reliable and relevant experimental studies. In an earlier study, 674 Marine Corps recruits received 2 g/day of VitC for 8 weeks. The study found that VitC did not significantly alter the incidence or duration of colds, but it did reduce the severity of cold symptoms in the treatment group (91). A recent meta-analysis emphasized that the current evidence is insufficient to affirm the efficacy of VitC supplements in preventing pneumonia because of the small number of trials and the very low quality of the available results (132). However, a recent review confirmed the preventive role of VitC in viral pneumonia through a different lens. Owing to pneumonia, viral infections can reduce the level of ascorbic acid because they create oxygen (O) and nitrogen (N) species (133). Therefore, VitC can prevent pneumonia. In addition, an analysis of nationally representative data from more than 34,000 participants revealed that low serum levels of antioxidant vitamins were associated with increased respiratory morbidity and/or mortality in U.S. adults (134). These results underscore the importance of antioxidant vitamins in respiratory health.

There are many other important antioxidants, including the previously mentioned GSH, CoQ10, which is present in all membranes of the cell, and melatonin. The powerful antioxidant effects of GSH are not repeated. Increasing GSH levels for the prevention and inhibition of COVID-19 are also widely recognized (135). *N-Acetyl-L-cysteine* (NAC) is a precursor of reduced GSH. NAC has better oral and topical bioavailability than GSH, and has an excellent safety profile and is also a mucolytic agent (136). NAC is able to act as a potent antioxidant scavenger of free radicals directly but also reduces downregulated inflammation and lowers the level of oxidative stress through stimulation of Nrf2 and inhibition of the NFκB pathway (137). NAC, a pleiotropic drug, has been proposed for the treatment and/or prevention of a variety of diseases involving GSH depletion and altered redox status. For example, *severe acute respiratory syndrome coronavirus 2* (SARS-CoV-2) infection impairs the metabolism of cellular GSH, and NAC can prevent such defects *in vitro* (107).

CoQ10 has potent antioxidant effects on the mitochondrial membrane, as well as other membranes in the cell and in the plasma and cytoplasm. Its most important and relevant role is the ability to switch between the redox forms (ubiquinone, semiquinone, and ubiquinol) and maintain a balance between the redox forms outside the mitochondria through enzymatic action (138). A molecular review revealed that CoQ10, which reduces important inflammatory cytokines and prevents organ damage due to massive oxidative stress, has been tested for the prevention of a wide range of diseases, especially those with an inflammatory pathogenesis. CoQ10 supplementation could prevent COVID-19-induced morbidities and potentially has a protective role against the deleterious consequences of the disease (139).

Melatonin is secreted mainly by the pineal gland of the brain, and its secretion is extremely high in infants and adolescents and much lower in older individuals. Melatonin scavenges free radicals directly and also stimulates a variety of antioxidant enzymes, including SOD, GPX, glutathione reductase, and CAT, inhibits pro-oxidant enzymes and increases intracellular GSH levels. There is also evidence that melatonin stabilizes cell membranes, which may

help fight oxidative damage (33). A review summarizes the potent bacteriostatic effects of melatonin, such as resistance to pathogenic bacterial infections *in vivo* via various pathways, including NF- κ B and ROS; antimicrobial activity against classical gram-negative and gram-positive bacteria, even members of other bacterial groups such as *Mycobacterium tuberculosis*; and antiviral effects (140). Although there is no experimental confirmation of the effectiveness of the antioxidant effects of melatonin in the prevention of CAP. However, melatonin has demonstrated satisfactory results in terms of being a possible prophylactic measure for COVID-19 infection (141). This may be related to its ability to reduce mitochondrial oxidative stress in posterior lung cells after COVID-19 infection while conferring a general antioxidant effect (142). A retrospective cross-sectional study analyzed data from a closed population of 110 adult patients treated with melatonin for a variety of sleep disorders until the onset of the COVID-19 pandemic (143). The patients in general were mostly older adults treated with a mean dose of melatonin >40 mg/day for various sleep disorders, mainly for complaints of insomnia, for more than 12 months. COVID-19 infection was recorded in 15 patients (13.5%) requiring hospitalization, 5 of whom were infected, only one with severe pneumonia and no deaths due to COVID-19. These results are consistent with a possible preventive effect of melatonin in the context of the COVID-19 pandemic.

The previously mentioned representative antioxidants, while playing an antioxidant role, are also nutrients needed by humans and can indirectly improve the state of malnutrition in the older population, further affecting immune function and playing a preventive role (65). Certainly, the modulation of oxidative stress works in an indirect, slow, and gentle manner as opposed to the direct stimulation of the immune response by vaccines. In addition, some vaccines produce effects by exerting antioxidative stress effects (144). Theoretically, vaccines are synergistic with antioxidative stress, and the significance and value of modulating oxidative stress in preventing CAP in older individuals may be better realized on the basis of the low-grade chronic inflammatory state and the chronic accumulation of ROS in older individuals. However, because we have only just experienced COVID-19, there will be a relatively greater number and depth of articles on prophylactic research related to viral CAPs such as pneumonia with COVID-19. This enthusiasm should be maintained for research on other pathogens, including bacteria and fungi. The more we understand about biology, the better we are equipped to meet sudden public health challenges such as the COVID-19 pandemic (145).

5.2 Reducing lung inflammation

Inflammation and oxidation are apparently interrelated processes, as excessive or uncontrolled free radical production induces an inflammatory response, while free radicals are inflammatory effectors. In fact, both oxidation and inflammation occur when the immune system responds to pathogen invasion (146). An analysis of endogenous oxidative damage markers and their associations with pulmonary involvement severity in patients with SARS-CoV-2 pneumonia indicated that SARS-CoV-2

pneumonia is significantly associated with increased endogenous oxidative damage. Oxidative damage seems to be associated with the severity of pulmonary involvement (147). Many compounds with antioxidant effects, such as those mentioned above, that protect lung tissue from CAP by attenuating oxidative stress-induced damage and inhibiting inflammatory responses have been identified.

According to the results of an animal study in rats, the oral administration of antioxidant vitamins such as VitE and VitC may help inhibit the development of ARDS (104). An early randomized, double-blind trial of 57 older adult patients admitted to the hospital for acute respiratory infections (bronchitis and bronchopneumonia) using a clinical scoring system based on major symptoms of the respiratory condition revealed that VitC-supplemented patients fared significantly better than placebo-supplemented patients did (92). An *in vitro* assay confirmed that VitC reduced ROS and DNA damage in peripheral blood mononuclear cells and decreased TNF- α and IL-6 levels in whole blood cells from patients with severe CAP (93). A research has found that COVID-19 patients have an average plasma VitC concentration nearly five times lower than that of healthy volunteers. Therefore, it is recommended that COVID-19 patients receive VitC supplementation at a dose of 100 mg/kg per day (94). NAC is a safe, tolerable, available and affordable drug which seems like a promising adjuvant therapeutic agent for COVID-19 (108). In animal experiments, NAC was found to attenuate lipopolysaccharide-induced inflammation in bovine embryonic tracheal cells and lung injury in mice (109). In the clinical trial, the group of COVID-19 patients treated with NAC had a greater risk at baseline, such as being older (average age over 70) and suffering from more comorbidities such as hypertension, diabetes, and COPD. However, patients who received NAC had a significantly lower mortality rate than did COVID-19 patients who did not receive NAC, which was attributed to the antioxidative stress effect of NAC (110). Treatment with NAC in critically ill patients with COVID-19 (mean age >60 years in both experimental and control patients) improved patients' PaO₂/FiO₂ indices, and it was hypothesized that NAC prevented the reduction in GSH and thus affected clinical outcomes (111). In mouse experiments, CoQ10 significantly inhibited *Pseudomonas aeruginosa*-induced cell death; thus, antioxidants such as CoQ10 have been proposed for the treatment of pneumonia caused by *Pseudomonas aeruginosa* infection (112). CoQ10 treatment in older adults with CAP accelerated symptom resolution, shortened the duration of antibiotic therapy, and also performed well in the subgroup of patients with severe pneumonia (113). In cellular experiments, melatonin can upregulate telomerase activity in macrophages by decreasing ROS levels, increasing cellular energy production, and inhibiting NLRP3 inflammatory vesicle activation in macrophages, suggesting that melatonin has therapeutic potential for pneumonia (114). In clinical trials, adjunctive treatment with melatonin has demonstrated efficacy in patients with mild, moderate, and severe COVID-19 (115, 116). Melatonin can be used as a medicinal adjuvant with an anti-inflammatory mechanism to reduce and control inflammatory cytokines by regulating the expression of *T helper 1* (Th1) and Th2 regulatory genes in patients with COVID-19 (117). The results of a recent clinical trial revealed that melatonin restores redox homeostasis, which is altered in

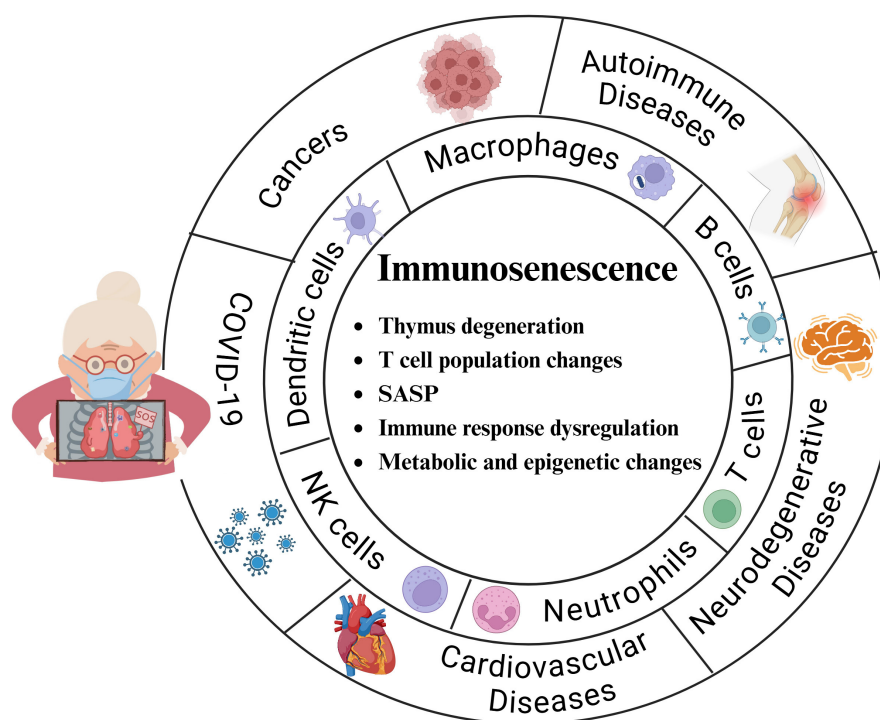


FIGURE 4

Main features of immunosenescence and the major cells and diseases affected. **Thymic degeneration:** The thymus gland is essential for T-cell development, but it gradually shrinks and degenerates with aging, leading to a decrease in the production of new T cells. **T-cell population changes:** An increase in memory T cells and a decrease in initial T cells weaken the ability to respond to new antigens. **Senescence-associated secretory phenotype (SASP):** This phenotype promotes cellular senescence, including immune cells, and promotes the secretion of proinflammatory cytokines, chemokines, and proteases that lead to chronic inflammation (inflammatory aging). **Immune response dysregulation:** Decreased immune surveillance, poor response to vaccines, increased susceptibility to infections, and increased incidence of autoimmune diseases and cancers. **Metabolic and epigenetic changes:** Alterations in metabolic and epigenetic pathways significantly affect immune system aging and T-cell senescence.

COVID-19 patients and can be used as an adjuvant therapy for SARS-CoV-2 infection (118).

Indeed, modulating oxidative stress plays a role primarily in controlling the inflammatory response component of CAP. A range of lung damage caused by inflammation and oxidative stress can be reduced, which can improve clinical symptoms in older patients. Previous studies have theoretically and experimentally demonstrated the positive therapeutic effects of modulating oxidation in viral CAPs, such as COVID-19, but there is a relative lack of more relevant clinical studies to further clarify the clinical efficacy in CAP infection caused by other pathogens.

5.3 Immunosenescence-related benefits

With respect to older adults, the topic of aging, especially immune aging, cannot be avoided. The immune system is widely believed to undergo quantitative and qualitative changes during the process of aging (148). This age-related decline in immune function, called immune senescence, leads to alterations in the cytokine microenvironment as well as impaired innate and adaptive immunity and is closely related to oxidative stress (149). According to existing studies, immune senescence is characterized mainly

by major features, such as thymic degeneration, changes in T-cell populations, senescent secretory phenotypes, dysregulation of immune responses, and metabolic and epigenetic changes, which affect cardiovascular diseases, neurodegenerative diseases, COVID-19, autoimmune diseases, and cancer[(150–154), Figure 4].

In general, all immune cells are affected by aging (155). Compromised immune cells lose their ability to modulate their own redox and inflammatory homeostasis, which is characterized by mononuclear immune cells infiltrating different tissues and producing excessive amounts of ROS and proinflammatory mediators (156, 157). High levels of ROS in cells are considered the driving force behind the deleterious effects of the aging process (153). This may lead to impaired phagocytosis of macrophages, decreased production of lymphocyte factors and antibodies, dysregulation of inflammatory processes, and an impaired immune response (87). If these affected immune cells are not well modulated, they further increase oxidative and inflammatory stress, creating a perpetual cycle that increases the rate of aging (158). These immune senescence changes further reduce the ability of the lungs to respond to infection and repair damage, increasing vulnerability to respiratory disease and slowing recovery from illness (159).

In addition, one of the hallmarks of immune senescence is “inflammation,” which refers to an age-related systemic state of

sterile, chronic and low-grade inflammation characterized by the upregulation of blood markers of inflammation and is considered to be a consequence of increasing chronological age, as well as a hallmark of biological senescence, multimorbidity and mortality risk (87, 153). And oxidative stress plays an important role in the development and maintenance of low-grade inflammation in age-related diseases. The ROS generated by oxidative stress activate the NF- κ B signaling pathway, which drives the expression of many proinflammatory cytokine genes as transcription factors, leading to chronic sterile inflammation and significantly affecting the aging process (160). In fact, unstimulated neutrophils and lymphocytes isolated from older adults patients accumulate greater amounts of ROS, present decreased SOD activity, and are less resistant to cell death compared to those cells obtained from young individuals (161). In contrast, the feedback loop between ROS and NF- κ B exacerbates oxidative damage and inflammation, leading to immune cell senescence and a decrease in tissue function. Together, oxidative stress and immune senescence form a vicious cycle in which impaired immune function increases susceptibility to oxidative damage, and oxidative stress further weakens the immune response (162).

In conclusion, the relationships among immune senescence, chronic inflammation and oxidative stress are intertwined and complex. While the links have not been fully elucidated, reducing systemic oxidative stress likely reduces the risk of stress-induced cellular senescence and associated inflammation (87). Notably, immune senescence is particularly detrimental to the respiratory system of older adults, who are easy targets for exogenous infections (162). Furthermore, due to immune senescence, older patients have an inadequate inflammatory response to infection, which may lead to an underestimation of the severity of pneumonia (163).

Some existing studies have also confirmed the effectiveness of counteracting immune senescence from the perspective of modulating oxidative stress. For example, flavonoids, which are natural antioxidants present in various foods, can act as antioxidants through signaling pathways such as the NF- κ B pathway (164). Quercetin, a type of flavonoid, has been widely reported for its antioxidant activity (165). In an *in vitro* cellular assay, aged peripheral blood mononuclear cells treated with quercetin presented a strong proliferative response comparable to that of young cells and increased GSH antioxidant defense levels, significantly reducing the expression of immune senescence markers (166). Another botanical bioflavonoid composed of *Scutellaria baicalensis* and *Acacia catechu* improved innate and adaptive immunity through antioxidant effects in a rat model of immune senescence and has great potential for the treatment of respiratory disorders due to immune stress and aging (162). In immunosenescent mice, although not showing any statistical difference, the administration of VitE increased the ratio of naïve to effector-memory T cells, suggesting that VitE may be effective in restoring oxidative stress-induced immunosenescence (101). There are also studies confirming that melatonin supplementation can prevent or delay the deterioration of immune system function that accompanies aging and may restore it to a “youthful” state (149).

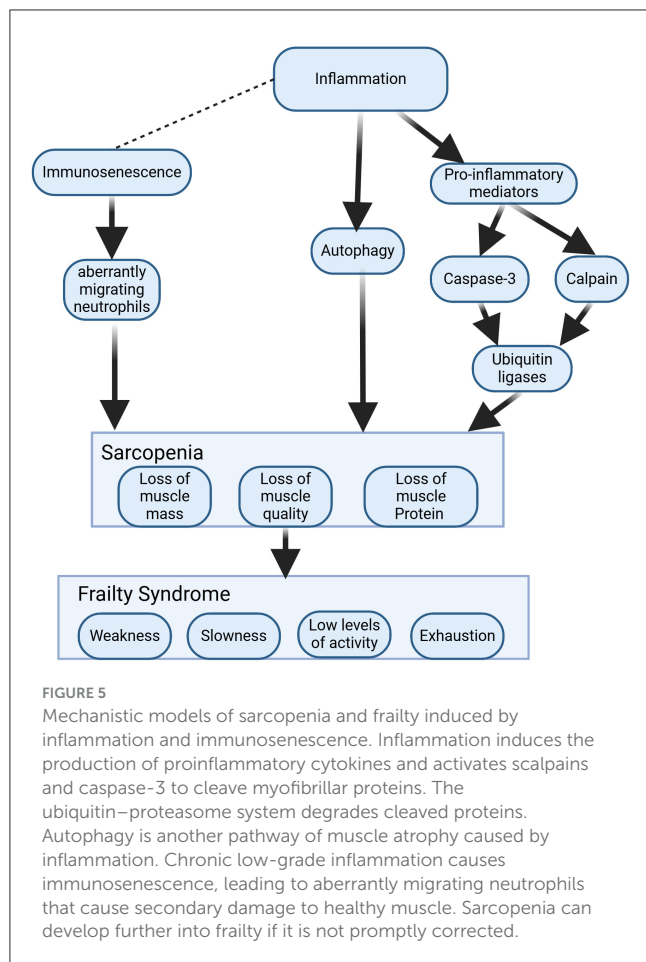
Indeed, chronic oxidative stress is only one of the many factors that contributes to immune senescence in the older population. Basic experiments have demonstrated that modulating stress can

improve or even reverse immune senescence. However, this is destined to be a long process, and long-term clinical studies are needed to fully substantiate the value of antioxidant therapy.

5.4 Improvements in sarcopenia and frailty

In the case of older adults with CAP, to seek effective treatment strategies, it is necessary to consider not only pathogen infection but also functional decline. Cough is the most common and primary manifestation of CAP and is important for clearing secretions from the airways. The strength of the cough is regulated by the respiratory muscles, and a weakened cough reflex can lead to the development of pneumonia, especially aspiration pneumonia, in older individuals (167). Therefore, respiratory muscle weakness has also been shown to be a risk factor for the development of pneumonia and for the recurrence of pneumonia in older individuals (168). In addition, the role of the swallowing reflex is also important. It is another protective airway reflex, such as coughing, and its strength is related to the swallowing muscles (169). Both impaired reflexes are recognized as major causes of aspiration pneumonia in older individuals (170). A high-quality review confirmed that aging weakens respiratory and swallowing muscles and causes sarcopenia in multiple ways; thus, older adults with CAP may be more challenging (171). Sarcopenia is an aging-related change in which skeletal muscle mass decreases, skeletal muscle strength decreases, and physical function decreases with age (172). The results of a meta-analysis indicate that sarcopenia is highly prevalent in individuals with respiratory disease (173). The onset of sarcopenia is closely linked to inflammation and immune senescence and eventually leads to frailty (Figure 5).

Inflammation leads to muscle proteolysis by affecting elevated levels of TNF- α , IL-6, IL-1, and CRP and inhibits protein synthesis through activation of the NF- κ B and ubiquitin–proteasome pathways, which affects the balance between anabolic and catabolic processes in muscle, resulting in sarcopenia (174). A study has postulated that muscle injury in an older person combined with immune aging would cause secondary damage to healthy muscle from aberrantly migrating neutrophils, resulting in myocyte damage and apoptosis and loss of muscle fibers, which could result in functional loss and physical weakness associated with frailty (175). Frailty is an extremely relevant geriatric condition in an aging society, and its occurrence increases with age (176). It can be defined simply as the clinical state in which an individual increases vulnerability to the development of negative health-related events when exposed to endogenous or exogenous stressors (177). It can be viewed as a progressive age-related decline in physiological systems, leading to a reduction in intrinsic capacity reserves, making people highly vulnerable to stress and increasing the risk of a range of adverse health outcomes (178). Sarcopenia is a high risk factor for frailty, leading to increased mortality (172). Frailty syndromes might result in the following: poorer response to somatic and/or psychiatric stressors, increased risk of hospitalization, adverse disease outcomes, hospitalization, and premature death (179). Hence, both sarcopenia and frailty are extremely challenging for



older adults with CAP. Previous studies have revealed relationships among oxidative stress, sarcopenia and frailty (52, 180). In addition to the previously mentioned immune senescence- and inflammation-related mechanisms, sarcopenia is also associated with oxidative stress in skeletal muscle. An animal study found that mice deficient in Cu/ZnSOD exhibited sarcopenia (181). Oxidative stress promotes muscle atrophy, so skeletal muscle atrophy can be treated by targeting antioxidative stress (182). For example, an early animal study noted that VitE and C supplementation reduced oxidative stress and improved antioxidant enzymes and positive muscle work in chronically loaded muscles of aged rats (105). There are also studies summarizing the preventive and therapeutic potential of a particular type of VitE, tocotrienol, whose superior antioxidant capacity improves antioxidant defense mechanisms in muscle-forming cells, enhances musculoskeletal health and addresses age-related musculoskeletal problems (102, 183). According to a recent study, melatonin targets mitochondria, scavenges free radicals and reduces oxidative damage, with a potential and promising therapeutic role in limiting muscle degeneration (mainly mitochondrial function) and sarcopenia (184). This conclusion has also been confirmed in a recent animal study. Following melatonin treatment, histological damage was alleviated, and the cross-sectional area of muscle fibers in the gastrocnemius tissues of middle-aged mice increased. Furthermore,

the percentage and size of normal mitochondria increased, while the levels of MDA and ROS decreased in these tissues (119).

Previous studies have suggested that oxidative stress may be associated with frailty by affecting RNA oxidation, absolute telomere length shortening, increased markers of oxidative stress, and mitochondrial dysfunction and it was also found that the deleterious effects of both oxidative stress and frailty increase linearly with aging (185, 186). Therefore, it may be feasible to intervene in frailty by modulating oxidative stress. An early clinical trial involving 827 older adults revealed an association between low circulating levels of VitE, one of the most important components of the human antioxidant system, and the presence of frailty. Participants with the highest VitE levels were less likely to be frail than those with the lowest VitE levels were (103). The results from a 12-year Framingham Heart Follow-Up Study suggest that higher dietary quercetin intake is particularly strongly associated with lower odds of frailty onset in adults (187).

Surely, the onset and development of sarcopenia and frailty are not determined by only oxidative stress, and oxidative stress is only one of the important mechanisms. However, relevant studies have been able to affirm, to some extent, the positive significance of modulating oxidative stress in sarcopenia and frailty, which could be instructive for the future of older CAP patients with related diseases.

5.5 Dealing with aging, multimorbidity and polypharmacy in the older population

Aging is characterized by a progressive loss of tissue and organ function caused by genetic and environmental factors, nutrition and lifestyle. Oxidative stress is one of the most important mechanisms of increased cellular senescence and frailty, leading to a wide range of diseases that are common in the older population, including chronic lung disease, cardiovascular disease, chronic kidney disease, skeletal muscle dysfunction and cancer (188, 189). In such cases, a public health problem arises—multimorbidity—in which individuals develop two or more chronic pathologies. It is estimated that more than half of the population over the age of 60 is affected (190). The requirement of medications as first-line treatment for these chronic conditions increases the potential cost of treatment, leaving older adults in a polypharmacy dilemma and causing many negative effects. It is also directly correlated with the development of frailty, which may further compromise the antioxidant defense system (191). Risk factors such as malnutrition, multimorbidity and polypharmacy also increase the risk of severe illness and death in older CAP patients (192). In cases where oxidative stress is the pathophysiological basis, some natural antioxidant products work through different mechanisms than conventional drugs do to enhance therapeutic efficacy, avoiding the need to add new drugs to basal therapies, thus achieving therapeutic goals at lower doses of standard therapies (191). One study revealed that the use of vitamins C and/or E in men taking daily metformin improved insulin resistance and fasting blood glucose levels, perhaps without the need to add another hypoglycemic agent (106). A meta-analysis revealed that VitC supplementation in patients with chronic obstructive pulmonary disease (COPD)

increased serum antioxidant levels (VitC and GSH) and improved lung function [FEV1% and FEV1/FVC; (193)]. Another review discussed the beneficial health effects of VitE in the prevention or treatment of hyperlipidemia, diabetes, osteoporosis, and cancer (194). Quercetin can be a potential treatment agent for a variety of inflammatory conditions, and it can also lower blood pressure, lower cholesterol levels, improve endothelial function, and even exhibit anticancer effects by inhibiting cancer cell proliferation and inducing apoptosis (195). Some reviews affirmed the importance of the antioxidant effects of melatonin in ameliorating chronic kidney disease and diabetic chronic kidney disease and proposed that endogenous antioxidants have fewer side effects than do exogenous antioxidants (196, 197). CoQ10 supplementation has a positive effect on mitochondrial deficiency syndrome and some of the symptoms of aging, whereas cardiovascular disease and inflammation are mitigated by the antioxidant effects of CoQ10 (198).

Additionally, some studies have indicated that certain medications for underlying conditions, commonly used in older adults, can lead to nutrient deficiencies. For example, individuals with hyperlipidemia treated with statins exhibit significant reductions in CoQ10, α -tocopherol, and other important antioxidants. These reductions are thought to drive statin-induced myopathy (199). Coadministration of CoQ10 may ameliorate these effects, but additional studies are needed to confirm this, particularly in older adults experiencing or at risk of polypharmacy (200). Long-term, high-dose aspirin use has been associated with decreased VitC levels, which can lead to gastric mucosal thinning, gastritis, peptic ulcer disease, nausea, anorexia, and malnutrition (199, 201). However, the doses used for primary and secondary cardiovascular disease prophylaxis are much lower. Moreover, there is currently no evidence suggesting a similar reduction in VitC levels or a need for VitC supplementation in chronic low-dose aspirin users (200).

In fact, patients with CAP cannot avoid the risk of multimorbidity and polypharmacy, and some chronic diseases are even detected during the treatment of CAP. Owing to various changes in the modern environment, diet and life structure, this situation can hardly be solved at the source and plagues the treatment of many diseases, not only CAP. Multiple medications may interact with each other and, to some extent, may be detrimental to the treatment of CAP or even affect the treatment plan for CAP. However, oxidative stress has been linked to many diseases in older individuals, and it might be a good attempt to intervene in chronic diseases from the perspective of modulating oxidative stress. In particular, many natural antioxidant compounds have relatively safe compositions and a wide therapeutic range, and we are confident in their further development and use in the future.

6 Impact of COVID-19

Since the worldwide outbreak of SARS-CoV-2, oxidative stress has attracted a great deal of attention, and there has been a proliferation of scientific studies on the theory and practice of its relationship with pneumonia (202, 203). As more is known about the disease and a vaccine has been developed, the threat of

COVID-19 is diminishing, but some patients experience persistent symptoms or sequelae even after recovering from COVID-19 (204, 205). The estimated overall global prevalence of COVID-19 sequelae is approximately 43%, with major clinical manifestations, including pulmonary, cardiovascular, renal, neuropsychiatric, and other related symptoms (206). Moreover, COVID-19 appears to have long-term health effects, which may put strain on the health care system. This long-term COVID-19 affects a large proportion of people in all age groups, and older adults are characterized by age-specific manifestations and are more likely to have persistent symptoms or sequelae, increasing susceptibility to the disease and affecting quality of life (207). A Korean study revealed that COVID-19 negatively affects physical activity, quality of life, and memory in older adults (> 55 years old), leading to mental diseases such as insomnia and depression (208). Therefore, after the onset of COVID-19, older CAP patients deserve more attention, and providing more comprehensive therapeutic measures and modulating the oxidative stress state may be a good choice.

For example, melatonin reduces the long-term inflammatory and oxidative effects of viruses, stimulates the suppressed immune system, and has the potential to ameliorate inflammation and inhibit the cytokine storm induced by SARS-CoV-2 (209). Treatment with antioxidant supplements such as NAC, VitC, VitE, and melatonin in COVID-19 patients reduces biomarkers of oxidative stress and inflammation, such as total systemic antioxidant capacity, CRP, IL-6, and lipid peroxidation, and improves the survival score (210). However, a recent study of markers of oxidative stress in older adults with SARS-CoV-2 infection revealed that oxidative stress was associated with fatal events as well as near-term prognosis in older adults with SARS-CoV-2 infection but was less important than other predictors (e.g., cardiovascular factors) of long-term prognosis at 2 years (211). Notably, the unique active ingredients in various natural traditional Chinese medicine (TCM) herbs also play important roles in the prevention and treatment of COVID-19 through their anti-inflammatory, antioxidant and antiapoptotic effects (212). In fact, the respiratory tract and lungs bear the brunt of this worldwide epidemic. Even if most older adults are clinically cured, the respiratory damage caused by COVID-19 should not be ignored. In conjunction with existing research, modulating oxidative stress homeostasis can indeed benefit older adults to some extent and increase their resistance to future threats of challenges such as COVID-19.

7 Limitations and challenges of modulating oxidative stress in older CAP patients

Oxidative stress has been demonstrated to be associated with CAP in older individuals through various theories and practices. Free radicals act as physiological signaling messengers to oxidatively modify biologically essential molecules to maintain normal cellular physiology and pathology. The current concern is that excessive or inappropriate antioxidant interventions in aging CAP instead disrupt normal cell signaling pathways and

inhibit the beneficial effects of ROS and lipid peroxidation products on disease, such as by mediating the physiological apoptosis of immune cells (213). This, in turn, deviates from the original intent of antioxidant therapy and negatively affects inflammation, the immune response, etc., driving the progression of CAP and increasing the risk of CAP. In particular, older people also have chronic low-grade inflammation, so antioxidants should be carefully applied. Moreover, not all conditions are suitable for antioxidative stress treatment. Researches have indicated that some pathogens respond to host oxidative stress by producing GSH to survive in the environment and the host. This mechanism involves most gram-negative bacteria and a few gram-positive bacteria and is more prevalent and essential in eukaryotic cells (214). Experiments have also demonstrated that GSH biosynthesis-deficient *Pseudomonas aeruginosa* mutants are less virulent in an acute pneumonia infection model (215). Therefore, analyzing from the other side, increasing the oxidative stress response of the cells can serve to deplete part of the GSH defense produced by the pathogen, thus reducing its virulence. It has also been suggested that the development of chronic bacterial infections and antibiotic resistance is associated with altered adaptation of pathogens to oxidative stress, suggesting that chronic infections and antibiotic resistance can be counteracted by increasing oxidative stress in cells (67). However, controlling the degree of enhancement to inhibit the pathogen while avoiding excessive oxidative stress in the organism is still a current challenge.

Studies show that antioxidants can have dichotomous roles in ROS production. They are easily oxidized and can act as oxidants to induce damage at high concentrations (100). An early animal study demonstrated that VitC primarily acts as an antioxidant at low doses, whereas pro-oxidant effects predominate at high doses. Thus, its use at pharmacological doses should be approached with caution (95). Such differences are also observed in clinical trials. For example, the effects of different high doses of VitC are not uniform in patients with severe COVID-19 (96). Administering 3 g/day of VitC to moderate and severe COVID-19 ICU patients reduced inflammation and mortality without adverse effects. However, a dose of 6 g/day has no significant impact on ICU length of stay or mortality (97). Earlier studies have shown that in healthy volunteers, daily doses of VitC exceeding 500 mg tend to decrease its bioavailability. Safe daily doses are generally less than 1000 mg, and doses above 400 mg daily offer no additional benefit (216). However, the optimal dose of VitC that is both safe and effective for antioxidant activity in disease states remains unclear. In addition, studies have shown that NAC can protect melanocytes from oxidative stress and damage and delay the onset of ultraviolet-induced melanoma in mice. However, high doses may increase Nrf2 nuclear translocation, potentially promoting metastasis (217, 218).

In summary, we advocate the healthy modulation of oxidative stress in older CAP patients to achieve the desired therapeutic assistance rather than simply inhibiting and scavenging free radicals. However, although common antioxidants such as vitamins, NAC, and Co Q10 are commonly used as drugs in the clinic, the emphasis is still on their primary pharmacological effects, and their antioxidant effects remain at the research stage. The wide variety of antioxidants that have been identified are constantly being depleted, as they work with free radicals, and

different antioxidants are often accompanied by other biological effects. Therefore, the choice of antioxidants to be used to treat older adults with CAP, the dosage and timing of their use, and the optimal mode of administration are still not very clear, and more targeted studies are needed.

Given existing studies, we propose focusing research on adjunctive treatments for CAP with combined antibiotics to alleviate clinical symptoms while considering the unique characteristics of older adult patients. Most studies have focused on COVID-19 patients, with fewer addressing CAP caused by other pathogens, limiting generalizability. To avoid drug interactions, the study population should initially consist of older CAP patients without comorbidities, then expand to include those with one or more comorbidities, malnutrition, or immunodeficiencies. Drug selection should prioritize single-agent therapies with high safety profiles, clear mechanisms of action, high bioavailability, and suitability for CAP treatment, such as NAC. Determining drug dosage, timing, and mode of administration should begin with *in vitro* experiments, reference clinically established safe dosages, and integrate drug toxicology. Oxidative stress involves multiple signaling molecules and reactive enzymes; thus, detection indices should be both experimentally valuable and universally applicable. Incorporating the effects of pathogenic microorganisms, inflammation, and immune responses into the study design would further enrich the experimental content.

8 Conclusion

Global aging has resulted in an enormous increase in the population of older people and an enormous challenge to global public health. The impact of CAP, one of the most common respiratory system diseases, on the older population is obvious. The onset and development of CAP are closely related to several pathophysiological aspects, including pathogenic microorganisms, inflammation, immunity, and oxidative stress. In older CAP patients, there are also multiple age-related risk factors, such as immune senescence, sarcopenia, and polypharmacy, that influence the clinical presentation, development, and prognosis. Anti-infective therapy is still the primary treatment for CAP, but inappropriate use of antibiotics is common in older individuals, and older individuals are particularly susceptible to antibiotic-associated adverse effects. Modulating the signaling pathways that cause the inflammatory response and improve the immune function of patients has become the focus of reducing inflammatory damage in the lungs, especially in older adults with CAP. Oxidative stress, as an important mechanism of CAP, has been widely demonstrated to be related to inflammation and immunity. Especially after the occurrence of COVID-19, a number of studies related to oxidative stress have been conducted, but they tend to be limited to inflammation and immunity only, ignoring the possible additional links with the older population, who are the most infected with CAP. Therefore, we attempted to further clarify the link between oxidative stress and age-related risk factors such as immune senescence, sarcopenia, frailty, aging, and multimorbidity in the context of older adults CAP patients, as well as the greater potential value and prospects of modulating oxidative stress in treating older adults with CAP.

Clearly, there is no doubt that targeted modulation of oxidative stress benefits older adults CAP patients. However, many challenges and unknowns concerning how to modulate oxidative stress for further practical clinical applications exist, and more targeted research is needed. This review is limited in length, and we mainly discuss toward the characteristics of older adults CAP, emphasizing the value of modulating oxidative stress; thus, we only briefly describe the various immune, inflammatory, and aging mechanisms and only cover the study of common antioxidants, such as GSH, NAC, vitamins, CoQ10, and melatonin. If readers need further information, please refer to the outstanding articles cited in the text for relevant content.

Author contributions

WM: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Writing – original draft, Writing – review & editing. XL: Conceptualization, Data curation, Resources, Software, Writing – original draft, Writing – review & editing. SF: Conceptualization, Formal analysis, Investigation, Resources, Supervision, Visualization, Writing – review & editing. RZ: Data curation, Investigation, Methodology, Supervision, Validation, Writing – review & editing. ML: Conceptualization, Data curation, Investigation, Writing – review & editing. SX: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

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Development and validation of predictive models for meige syndrome patients based on oxidative stress markers

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Background: Meige syndrome (MS) is a complex neurological disorder with unclear etiology. Accurate prediction of MS risk is essential for facilitating early diagnosis. This study aimed to develop and validate a nomogram for predicting the risk of MS based on oxidative stress markers.

Methods: This retrospective, cross-sectional study included 424 patients with MS and 848 age- and sex-matched healthy controls, with data collected from January 2022 to December 2023. Clinical and laboratory data were extracted from electronic medical records. The MS patients and healthy controls were randomly allocated to the training and validation sets at a 7:3 ratio using random stratified sampling. A nomogram was developed using a multivariate logistic regression model based on data from the training set. Model performance was validated through fivefold cross-validation, receiver operating characteristic (ROC) curves, calibration plots, and decision curve analysis (DCA).

Results: Univariate and multivariate logistic regression analyses identified albumin, gamma-glutamyl transferase (GGT), total bilirubin (TBIL), and the urea nitrogen-to-creatinine ratio as independent predictors of MS. A nomogram was constructed based on these four variables. The cross-validation confirmed the model's reliability. The model demonstrated high predictive accuracy, with an area under the curve (AUC) of 0.930 for the training set and 0.914 for the validation set. The calibration curve and DCA results indicate that the model has strong consistency and significant potential for clinical application.

Conclusions: This study developed a nomogram based on four risk predictors, GGT, TBIL, albumin, and the urea nitrogen-to-creatinine ratio, to forecast the risk of MS and enhance the accuracy of MS risk prediction.

KEYWORDS

meige syndrome, oxidative stress marker, nomogram, albumin, gamma-glutamyl transferase, total bilirubin, the urea nitrogen-to-creatinine ratio

Introductions

Meige syndrome (MS), initially reported by the French neurologist Henri Meige in 1910, is an infrequent and debilitating neurological condition characterized by involuntary contractions of the eyelids and facial muscles (1), which ultimately leads to significant functional impairment and psychosocial distress for affected individuals. Generally, the condition is more prevalent among individuals aged 40 to 70, with a higher incidence in women than in men (2). Currently, the etiology and pathogenesis of MS remain unknown. It has been suggested that a disruption in the balance of neurotransmitters in the brain, particularly dopamine and acetylcholine, may be involved (3). This neurochemical imbalance has been associated with multiple factors, such as specific medications, psychological stress, dental interventions, and trauma (4). Diagnosis of MS currently depends largely on patients' clinical symptoms, and there is no definitive diagnostic test available. Consequently, there is a pressing need to identify reliable biomarkers for MS diagnosis.

Impairment of the basal ganglia and related neural pathways plays a key role in the development of MS (5). Research indicates that MS may be linked to an imbalance between acetylcholine and dopamine, while other studies suggest that changes in neuronal plasticity and decreased cortical inhibition could contribute to the condition (3, 6). A variety of neurological disorders have been linked to neuronal cell death due to oxidative stress (7, 8). Many studies have demonstrated that oxidative stress is a key factor in the progression of various neurological disorders, including Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), Huntington's disease (HD), ischemic stroke, and multiple sclerosis (9). The central nervous system (CNS) exhibits heightened sensitivity to oxidative stress, a result of its high metabolic rate, rich presence of substances susceptible to oxidation, and constrained antioxidant capabilities (10). Reactive nitrogen species (RNS) and reactive oxygen species (ROS) reactive can lead to oxidative stress, which inflicts damage on cellular constituents such as lipids, proteins, and DNA, ultimately resulting in cellular impairment and apoptosis (11). Oxidative stress can also modify the activity of ion channels, which are essential for preserving neuronal excitability and signal transmission (12). Oxidative stress-induced alterations in ion channel regulation can exacerbate the progression of neurological diseases (13).

In recent years, biomarkers such as albumin, gamma-glutamyl transferase (GGT), total bilirubin (TBIL), uric acid (UA), and the urea nitrogen-to-creatinine ratio have emerged as significant indicators of oxidative stress in the context of neurological diseases (14–16). Although these markers have been extensively studied in various contexts, their potential as predictors for MS has not been fully explored. The present study innovatively investigates the relationship between these oxidative stress markers and MS, revealing their potential as novel predictive indicators for MS. To the best of our knowledge, this is the first study to explore the association between these oxidative stress markers in blood and MS, with the largest sample size of MS cases reported to date. Given the

lack of definitive diagnostic tests for MS and the critical importance of early diagnostic for improved patient outcomes, our nomogram—a powerful predictive tool—provides a quantitative and reliable method for clinicians to assess MS risk based on these oxidative stress markers. This innovation facilitates early intervention and personalized management strategies.

Materials and methods

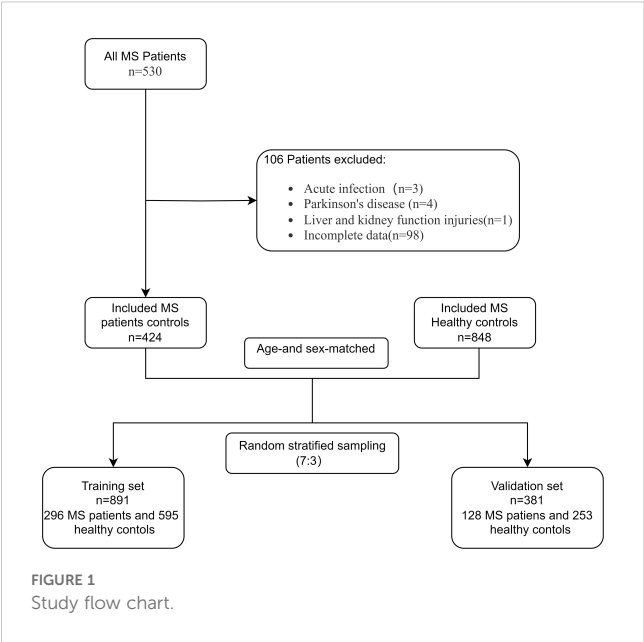
Study population

This retrospective, cross-sectional study included 530 consecutive patients diagnosed with MS who were admitted to the Third People's Hospital in Henan Province, from January 2022 to December 2023. The study received clearance from the Research Ethics Committee at the Third People's Hospital of Henan Province (2024-SZSYKY-009) and adhered to the Declaration of Helsinki, as well as the pertinent institutional protocols. Due to the retrospective and observational design of the study, the requirement for informed consent was waived. To safeguard participant privacy, all data extracted from the medical record system were anonymized, ensuring the protection of patient confidentiality.

Participants were included if they were diagnosed with primary MS by a clinician based on clinical criteria. Exclusions were made for patients with: (1) acute infections, (2) malignant tumors, (3) severe hepatic or renal insufficiency, and (4) incomplete data for key variables such as albumin, GGT, TBIL, indirect bilirubin (IBIL), direct bilirubin (DBIL), total protein, UA, creatinine (CREA), and urea nitrogen (UREA). After applying these exclusions, the study ultimately comprised 424 individuals with MS. Furthermore, data from 848 healthy individuals, matched for age and sex, who had routine medical examinations at the same hospital were gathered for the study. Using a random stratified sampling with a 7:3 ratio, the MS patients and healthy controls were divided into two groups: a training set comprising 296 MS patients and 595 healthy controls, and a validation set consisting of 128 MS patients and 253 healthy controls (Figure 1).

Data extraction

Clinical and laboratory data for all patients were extracted from the electronic medical record system. The clinical data included information on age, sex, and BMI. The laboratory data included GGT (reference range, 7 to 58 U/L), TBIL (reference range, 2 to 20.5 $\mu\text{mol/L}$), DBIL (reference range, 0.4 to 6.9 $\mu\text{mol/L}$), IBIL (reference range, 1.7 to 13.2 $\mu\text{mol/L}$), total protein (reference range, 60 to 85 g/L), albumin (reference range, 35 to 55 g/L), CREA (reference range, 44 to 106 $\mu\text{mol/L}$), UREA (reference range, 1.7 to 8.2 mmol/L), and UA (reference range, 149 to 369 $\mu\text{mol/L}$) (Beckman AU680, USA). The urea nitrogen-to-creatinine ratio was calculated as follows: urea nitrogen-to-creatinine ratio = urea nitrogen/creatinine. Data related to epidemiology, clinical assessments, and laboratory tests were collected from each patient's single visit.



Statistical analyses

The Shapiro-Wilk test used to evaluate the normality of the data. In cases where the data conformed to a normal distribution, we documented the mean and standard deviation (mean ± SD) and employed the Student’s t-test to discern differences between the two groups. When the data did not follow a normal distribution, we

applied the Wilcoxon-Mann-Whitney U test and presented the results as median and interquartile range, denoted as [M (P25, P75)]. For categorical data, comparisons were conducted using Pearson’s chi-square test, and the results were expressed in terms of frequency (percentage). To initially filter variables, a univariate logistic regression analysis was performed, and in combination with the Spearman’s rank correlation coefficient and the variance inflation factor (VIF), predictors for the final model were chosen. Subsequently, the RMS package in R was employed to create nomograms, and the internal validity of the model was confirmed by conducting fivefold cross-validation. The performance of the nomogram was appraised through the analysis of receiver operating characteristic (ROC) curves, calibration plots, and decision curve analysis (DCA). Statistical analyses were performed using SPSS version 25.0, and model construction and validation were conducted using R software (version 4.2.1). The threshold for statistical significance was established at $P < 0.05$.

Results

Patient characteristics

A total of 530 patients with MS were initially screened for eligibility. Of these, 3 patients with infectious diseases, 4 patients with PD, and 1 patient with renal insufficiency impairment were excluded. Additionally, 98 patients with incomplete data were excluded from the analysis. Ultimately, this study included 424 MS patients and 848 healthy controls (HCs) (Table 1). There were no significant differences

TABLE 1 Characteristics of the patients with MS and healthy controls.

Characteristics	Meige syndrome N=424	Healthy control N=848	P value
Sex			1.000
Male	102 (24.1%)	204 (24.1%)	
Female	322 (75.9%)	644 (75.9%)	
Age	59 (53, 65)	59 (53, 65)	1.000
BMI	24.26 (22.3, 26.318)	24.3 (22.5, 26.6)	0.554
GGT (U/L)	16 (13, 22)	20 (16, 28)	< 0.001
TBIL (μmol/L)	13.7 (11.6, 16.7)	16.15 (13.4, 18.3)	< 0.001
DBIL (μmol/L)	3.7 (3, 4.6)	4.5 (3.6, 5.9)	< 0.001
IBIL (μmol/L)	10 (8.475, 12)	11.5 (9.7, 12.6)	< 0.001
TP (g/L)	67.4 (64.4, 70.4)	74.55 (72.1, 77)	< 0.001
ALB (g/L)	40.6 (38.675, 42.5)	44.8 (43.4, 46.6)	< 0.001
UA (μmol/L)	288.85 (245.1, 331.88)	300.3 (256.6, 346.1)	0.005
CREA (μmol/L)	51.8 (44.5, 60.15)	56.85 (50.375, 65.525)	< 0.001
UREA (mmol/L)	5.23 (4.42, 6.015)	4.835 (4.09, 5.61)	< 0.001
UCR ×100	9.865 (8.25, 12.22)	8.26 (7.11, 9.6425)	< 0.001

Medians and interquartile ranges are utilized to represent continuous variables, while frequencies and percentages are employed to depict categorical variables. GGT, gamma-glutamyl transferase; TBIL, total bilirubin; DBIL, direct bilirubin; IBIL, indirect bilirubin; TP, total protein; ALB, albumin; UA, uric acid; CREA, creatinine; UREA, urea nitrogen; UCR, urea nitrogen-to-creatinine ratio.

regarding sex, age, and BMI between the groups ($P > 0.05$). The MS group exhibited higher levels of UREA and the urea nitrogen-to-creatinine ratio compared to HCs ($P < 0.05$), whereas levels of albumin, total protein, GGT, IBIL, DBIL, TBIL, UA, and CREA were significantly lower in the MS group ($P < 0.05$). Subsequently, MS patients and HCs were randomly allocated to the training and validation sets at a 7:3 ratio using random stratified sampling. No significant differences were observed between the two groups of MS patients (Table 2).

Logistic regression analyses in the MS

We conducted a univariate analysis to identify variables with statistically significant differences ($P < 0.05$). These variables were subsequently incorporated into a multivariate logistic regression analysis to ascertain the independent predictors ($P < 0.05$) (Supplementary Figure S1). To assess multicollinearity, we employed VIF assessment ($VIF < 5$) and Spearman correlation analysis ($r > 0.5$) (Supplementary Table S1, Supplementary Figure S2). After excluding variables with multicollinearity, the multivariate logistic regression identified albumin, GGT, TBIL and urea nitrogen-to-creatinine ratio as independent predictive factors. Specifically, lower levels of albumin (OR, 0.453; 95% CI, 0.410–0.500; $P < 0.001$), GGT (OR, 0.941; 95% CI, 0.919–0.964; $P < 0.001$), and TBIL (OR, 0.928; 0.877–0.982; $P = 0.009$), along with higher levels of the urea nitrogen-to-creatinine ratio (OR, 1.461; 95% CI, 1.352–1.579; $P < 0.001$), were recognized as independent predictors of disease risk for MS (Table 3). Patients with MS were

categorized into two groups based on disease duration and whether they had received botulinum toxin injections. The impact of these factors on the four independent variables predicting disease risk for MS was compared. Results indicated that neither disease duration nor botulinum toxin injection status significantly affected the aforementioned indicators (Supplementary Table S3, Supplementary Table S4).

Construction of the MS-predicting nomogram

In the multivariate analysis, four variables with statistically significant differences ($P < 0.05$), including albumin, GGT, TBIL, and the urea nitrogen-to-creatinine ratio, were used to construct a nomogram for predicting the risk of MS using data from the training set. Based on the nomogram, a comprehensive scoring table was developed, with the total score for the model reaching 260 points. At total score thresholds of 197, 203, and 208 points, the respective predicted probabilities for the development of MS were 30%, 50%, and 70%. In our study, the nomogram was developed using the subsequent formula: prediction probability = $1/(1+\exp(0.84464 \times \text{albumin} + 0.051268 \times \text{GGT} + 0.040896 \times \text{TBIL} - 0.3057 \times \text{the urea nitrogen-to-creatinine ratio} - 34.263))$. To illustrate its application, a patient was randomly selected from the dataset for development purposes. The patient had a GGT value of 14 U/L. Additionally, the TBIL value was 11.9 $\mu\text{mol/L}$, the albumin value was 41.1 g/L, and the urea nitrogen-to-creatinine ratio value was 9.92. From the calculations using the formula, the predicted probability of MS was

TABLE 2 Characteristics of patients with MS in the training and validation sets.

Characteristics	Training set Meige syndrome N=296	Validation set Meige syndrome N=128	P value
Sex			0.765
Male	70 (23.6%)	32 (25.0%)	
Female	226 (76.4%)	96 (75.0%)	
Age	59 (53, 66)	58 (53, 65)	0.307
BMI	24.26 (22.308, 26.045)	24.28 (22.285, 26.67)	0.679
GGT (U/L)	16 (12.75, 22)	16.5 (13, 21)	0.812
TBIL ($\mu\text{mol/L}$)	13.6 (11.6, 16.925)	13.85 (11.6, 16.175)	0.854
DBIL ($\mu\text{mol/L}$)	3.7 (3, 4.7)	3.75 (3, 4.5)	0.892
IBIL ($\mu\text{mol/L}$)	10.05 (8.4, 12.1)	10 (8.575, 11.8)	0.891
TP (g/L)	66.9 (64.4, 70)	68 (64.4, 70.9)	0.399
ALB (g/L)	40.539 $\pm$ 2.5195	40.988 $\pm$ 2.8769	0.127
UA ($\mu\text{mol/L}$)	288.9 (244, 329.9)	285.9 (247.67, 347.27)	0.884
CREA ($\mu\text{mol/L}$)	51.85 (44.1, 60.65)	51.55 (44.9, 59.225)	0.820
UREA (mmol/L)	5.22 (4.39, 6.0625)	5.28 (4.57, 5.95)	0.414
UCR $\times 100$	9.81 (8.1, 12.287)	10.055 (8.7075, 12.1)	0.484

GGT, gamma-glutamyl transferase; TBIL, total bilirubin; DBIL, direct bilirubin; IBIL, indirect bilirubin; TP, total protein; ALB, albumin; UA, uric acid; CREA, creatinine; UREA, urea nitrogen; UCR, urea nitrogen-to-creatinine ratio.

TABLE 3 Univariate and multivariate logistic regression analyses for MS.

Characteristics	Univariate analysis		Multivariate analysis	
	OR (95% CI)	P value	OR (95% CI)	P value
Male	1.000 (0.761 – 1.314)	1.000		
Age	1.000 (0.987 – 1.014)	1.000		
BMI	0.980 (0.943 – 1.019)	0.317		
ALB (g/L)	0.470 (0.432 – 0.511)	< 0.001	0.453 (0.410 – 0.500)	< 0.001
TP (g/L)	0.628 (0.596 – 0.662)	< 0.001		
GGT (U/L)	0.915 (0.898 – 0.932)	< 0.001	0.941 (0.919 – 0.964)	< 0.001
IBIL (μmol/L)	0.832 (0.788 – 0.879)	< 0.001		
DBIL (μmol/L)	0.642 (0.583 – 0.707)	< 0.001		
TBIL (μmol/L)	0.858 (0.827 – 0.891)	< 0.001	0.928 (0.877 – 0.982)	0.009
UA (μmol/L)	0.997 (0.995 – 0.999)	0.001	1.003 (1.000 – 1.005)	0.058
CREA (μmol/L)	0.951 (0.940 – 0.962)	< 0.001		
UREA (mmol/L)	1.381 (1.244 – 1.533)	< 0.001		
UCR ×100	1.341 (1.274 – 1.412)	< 0.001	1.461 (1.352 – 1.579)	< 0.001

GGT, gamma-glutamyl transferase; TBIL, total bilirubin; DBIL, direct bilirubin; IBIL, indirect bilirubin; TP, total protein; ALB, albumin; UA, uric acid; CREA, creatinine; UREA, urea nitrogen; UCR, urea nitrogen-to-creatinine ratio.

0.776 (Figure 2). This score offers crucial information for subsequent clinical evaluations and decision-making processes.

Internal prediction model validation

To validate the reliability and robustness of our predictive model, we conducted internal validation using a fivefold cross-validation method. This approach involves dividing the dataset randomly into five equal subsets. During each round of cross-validation, one of the subsets is designated as the validation set, and the other four subsets are combined to form the training set. This cycle is iterated 100 times, making certain that each data point is utilized once for training and once for validation. The performance of our model was evaluated using the area under the curve (AUC). The AUC values obtained from each of the five folds were averaged to provide a single estimate of the model's performance. The mean AUC across all folds was 0.93, indicating a high discriminatory power of our model in predicting MS risk based on oxidative stress marker (Supplementary Figure S3).

Validation of the MS-predicting nomogram

The nomogram demonstrated robust discriminative ability across both datasets. For the training set, the nomogram achieved an AUC of 0.930 (95% CI: 0.913–0.948), with a sensitivity of 83.8%, specificity of 89.2%, positive predictive value (PPV) of 84.6%, negative predictive value (NPV) of 88.0%, and a Brier score of 0.092. For

the validation set, the AUC was 0.914 (95% CI: 0.885–0.942), with a sensitivity of 78.90%, specificity of 87.70%, PPV of 80.7%, NPV of 86.5%, and a Brier score of 0.110 (Figure 3, Supplementary Table S2). The calibration curves were constructed to compare the nomogram's predicted probabilities with actual outcomes in both datasets. Predicted probabilities were categorized into bins, and the mean predicted probability for each bin was plotted against the corresponding observed frequency. The resulting curves closely approximated the 45-degree line, indicating good calibration. Calibration slopes were close to 1 and intercepts were close to 0, further confirming the nomogram's reliability for outcome prediction (Figures 4A, B). Furthermore, DCA was used to evaluate the practical application of the nomogram for predicting MS. With a range of threshold probabilities from 0 to 0.97, the net benefit was positive, indicating the model's superior clinical value for MS prediction (Figures 4C, D). These results indicate that the nomogram possesses robust predictive and discriminative capabilities.

Discussion

Studies have suggested that oxidative stress plays a significant role in the pathological mechanisms of various neurological disorders (9). Given its high metabolic activity and oxygen demand, the brain is particularly susceptible to oxidative stress. Excessive ROS, including superoxide, hydrogen peroxide, and hydroxyl radicals, can damage vital biomolecules such as proteins, lipids, and DNA (16). This damage initiates a cascade that can result

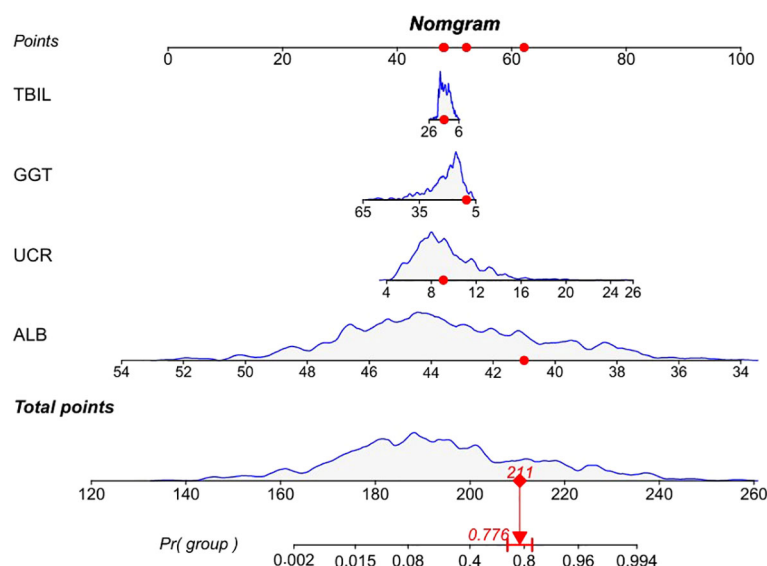


FIGURE 2

A predictive nomogram for MS. A score is allocated to each variable on a point scale, with the aggregate of these scores yielding the total points. These total points are then mapped to the predicted probability of MS. The red lines represent values of a randomly selected patient from the development dataset who is GGT of 14 U/L (52 points), with TBIL of 11.9 $\mu\text{mol/L}$ (49 point), ALB of 41.1 g/L (62 points), and UCR of 9.92 (48 points). These values when plotted, correspond to the points scale and points are then summed to give a total point score. The presented patient has a total point's score of 211. The red line indicates that the MS predicted probability corresponding to the total score of this example is 0.776. GGT, gamma-glutamyl transferase; TBIL, total bilirubin; ALB, albumin; UCR, urea nitrogen-to-creatinine ratio.

in cellular dysfunction and, ultimately, neurodegeneration. However, current evidence does not link peripheral blood oxidative stress markers to MS. Thus, investigating the relationship between these markers and MS is crucial.

In this study, compared with HCs, the serum levels of antioxidants, such as GGT, TBIL, albumin, and UA were

significantly reduced in patients with MS, while the pro-oxidant the urea nitrogen-to-creatinine ratio was markedly increased. This finding lends credence to the hypothesis that oxidative damage could be a significant factor in the development of MS. Multivariate logistic regression analysis identified GGT, TBIL, albumin, and urea nitrogen-to-creatinine ratio as independent risk predictors for MS. These biomarkers are readily accessible and have potential for broad clinical application. We integrated these variables into a nomogram to develop a predictive model. The model demonstrated strong discriminative ability, with ROC curve areas of 0.930 (95% CI: 0.913–0.948) for the training set and 0.914 (95% CI: 0.885–0.942) for the validation set. Calibration curves and DCA indicated that the model has good consistency and clinical utility.

Serum albumin, the most abundant protein in circulation, has antioxidant properties that significantly mitigate reoxygenation injury (17–19). albumin's antioxidant properties are due to its molecular structure. The reduced Cys34 residue, the largest pool of thiols in circulation, enables albumin to scavenge nitric oxide (NO), ROS, other reactive nitrogen species, and prostaglandins via this cysteine residue (20–22). Albumin exhibits neuroprotective effects, partly due to its modulation of intracellular signaling in neuronal and glial cells and its antioxidant capabilities (23). In recent years, an increasing amount of evidence has indicated that albumin plays a crucial role in neurological diseases. Some studies have linked lower serum albumin levels to a higher risk of cognitive impairment and dementia (24). Furthermore, research has shown that serum albumin levels are notably reduced in patients with PD and are considered independent risk factors for the condition (25). Similarly, we observed that MS patients had significantly lower albumin levels in their peripheral blood compared to HCs. This

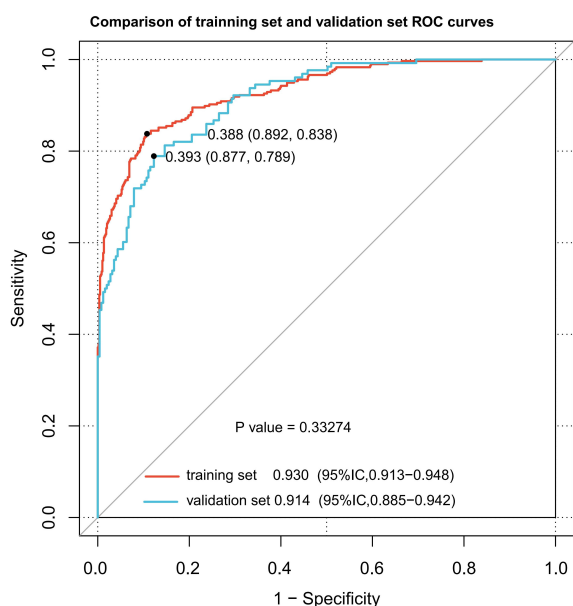


FIGURE 3

ROC curves for the training and the validation sets. The dot on the curve indicates the point corresponding to the optimal cutoff value.

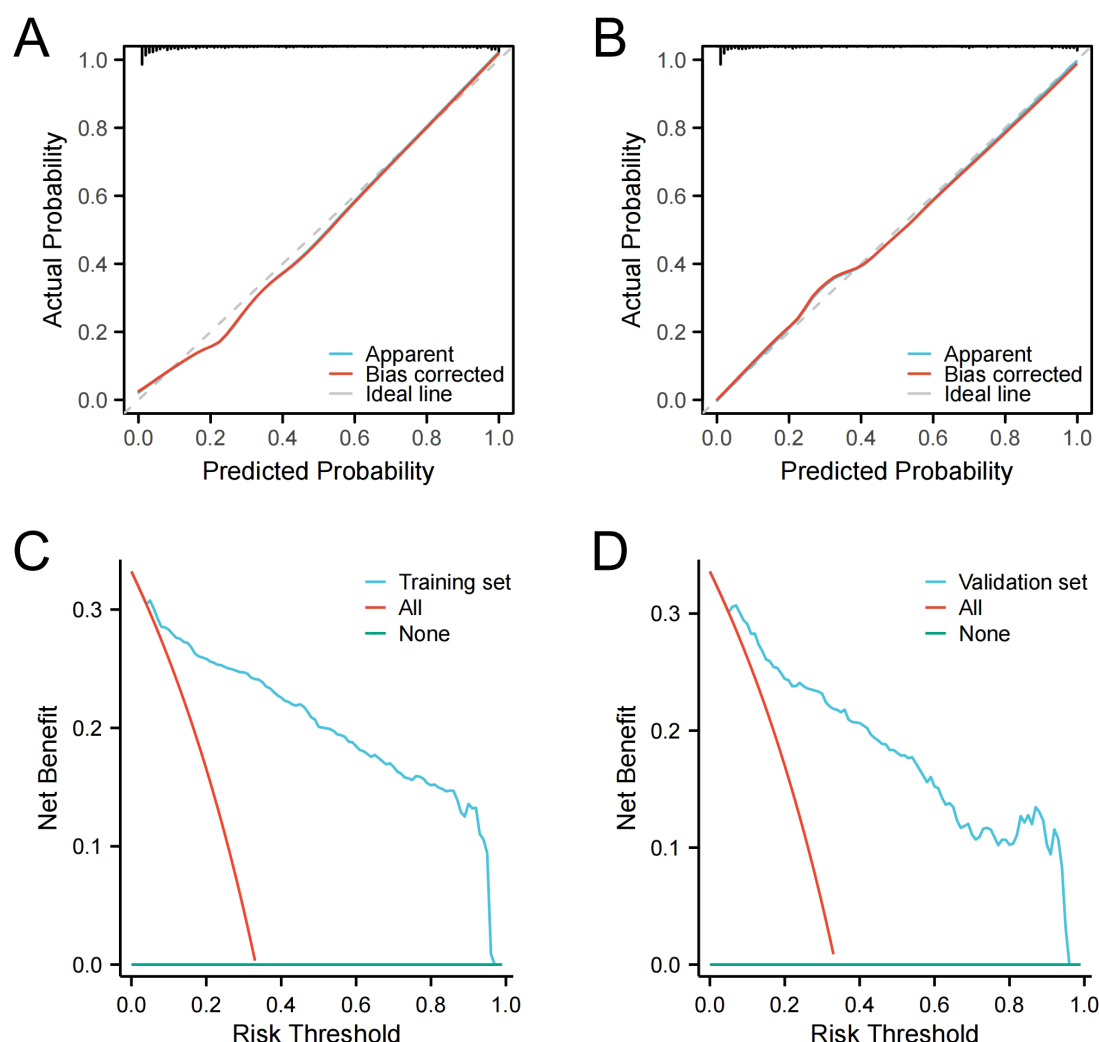


FIGURE 4

Calibration curve for the training set (A). Calibration curve for the validation set (B). DCA curve for the training set (C). DCA curve for the validation set (D).

reduction in albumin implies a weakened antioxidant capacity, which may exacerbate oxidative stress, induce neural tissue damage, and impede neural recovery, thereby potentially promoting MS development. The potential link between albumin levels and MS risk underscores the importance of further investigating albumin as a biomarker for MS diagnosis and risk assessment.

Bilirubin, a potent endogenous antioxidant, is found in biological fluids throughout the body and ranks among the strongest natural antioxidants (25). TBIL is the breakdown product of aging red blood cells (26), exhibits potent antioxidant capabilities, enabling it to neutralize harmful oxidative stress byproducts, shield the body's organs from injury (27), and has direct antioxidant properties in the brain (28). Under normal conditions, the human body possesses an antioxidant defense mechanism that maintains a dynamic equilibrium between oxidizing agents and antioxidants, thereby preventing harm to the body. However, a disruption in this balance can result in heightened oxidative processes, which can lead to tissue

damage (29). Our research indicated that compared to the HCs, the TBIL levels in MS are reduced, aligning with prior studies (15). This reduction may be associated with increased oxidative stress and tissue damage in MS. Additionally, elevated TBIL levels have been reported in patients with Parkinson's disease (PD), potentially due to dopaminergic substitution therapy (30). Two plausible explanations for the altered TBIL levels in neurological disorders are: First, bilirubin exhibits cytoprotective effects against injury caused by oxidative stress. Second, bilirubin has anti-inflammatory properties. The latter has been particularly evident in brain tissue, as demonstrated in models of experimental autoimmune encephalomyelitis, where bilirubin exhibits potent anti-inflammatory activity (31). Therefore, the deficiency of TBIL in MS patients could exacerbate oxidative stress and inflammation, contributing to disease progression. Further investigation into the relationship between TBIL and MS may provide valuable insights into the pathogenesis of MS and identify TBIL as a potential biomarker or therapeutic target for the disease.

GGT primarily facilitates the “glutathione cycle,” which operates at the plasma membrane and is crucial for maintaining cysteine homeostasis, thereby preserving intracellular glutathione levels and the cell’s redox balance (32). Recent studies indicate that GGT contributes significantly to antioxidant defense and the metabolism of xenobiotics (33). As a key antioxidant, GGT serves as an indicator of oxidative stress, with numerous studies linking its activity to the development of a range of diseases, including cardiovascular disease, cancer, lung inflammation, and neurological disorders (14). In our study, MS patients exhibited lower GGT levels compared to HCs, implying that reduced GGT might be a risk factor for MS. However, this finding differs from the results observed in other neurological disorders. Previous studies have shown that higher GGT levels have been correlated with dementia risk (34). The observed discrepancy may be attributed to the dual nature of GGT, which can promote pro-oxidant activity through the degradation of glutathione in the extracellular space under specific conditions (14). In MS, the reduced GGT levels could indicate a diminished antioxidant capacity, potentially exacerbating oxidative stress and contributing to disease progression.

UREA and CREA are end products of nitrogen-containing compounds in the human body and serve as accessible biomarkers for renal function in clinical settings (35). The urea nitrogen-to-creatinine ratio, a laboratory biomarker, has traditionally been used to assess dehydration (36). Recently, it has also been identified as an independent prognostic factor for adverse outcomes in patients with various conditions, such as stroke (16) and heart failure (37). Other research has determined that a reduced level of the urea nitrogen-to-creatinine ratio could potentially elevate the risk of ischemic stroke (38). However, there is inconsistency in the effects of urea nitrogen-to-creatinine ratio on various diseases, and the role of the urea nitrogen-to-creatinine ratio in MS patients remained uncertain. In MS, the role of the urea nitrogen-to-creatinine ratio was previously unexplored. Our study revealed significantly higher serum the urea nitrogen-to-creatinine ratio levels in MS patients compared to HCs, suggesting its potential as a biomarker for MS susceptibility. Previous studies suggested that urea nitrogen-to-creatinine ratio levels correlate with oxidative stress (39), abnormal inflammation (40), and endothelial dysfunction (41) in patients, which could be potential mechanisms underlying the association between elevated the urea nitrogen-to-creatinine ratio and MS risk. Given the established links between the urea nitrogen-to-creatinine ratio and various adverse outcomes, as well as its association with oxidative stress and inflammation, our findings highlight the potential importance of the urea nitrogen-to-creatinine ratio as a biomarker for MS risk.

This study was the first to build an MS prediction model based on oxidative stress markers. The independent predictors included in our model are easily accessible, thereby enhancing the ease of using the model. However, given that this study is a retrospective, single-center analysis, it also has certain limitations. The single-center origin of the data limits the generalizability of the study results, and

the lack of external validation further underscores the need for future studies to employ multicenter data for further verification. Moreover, due to its retrospective nature and the fact that the normal controls were individuals undergoing health check-ups, we were unable to accurately obtain data regarding several potential confounding factors, such as medication history prior to admission and dietary habits.

Conclusions

Our study successfully developed a nomogram incorporating GGT, TBIL, albumin, and the urea nitrogen-to-creatinine ratio to predict the risk of MS. This model offers a clinically valuable tool for risk assessment, demonstrating robust performance with high AUCs in both training and validation cohorts. The calibration curves and the DCA results demonstrate that the model exhibits strong concordance and possesses significant clinical application value, ultimately highlighting its potential utility in MS predict.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by the Third People’s Hospital of Henan Province (2024-SZSYKY-009). The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from primarily isolated as part of your previous study for which ethical approval was obtained. Written informed consent for participation was not required from the participants or the participants’ legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

YZ: Conceptualization, Writing – original draft. XF: Conceptualization, Formal analysis, Writing – original draft, Supervision. RF: Conceptualization, Formal analysis, Writing – original draft. ZW: Data curation, Methodologies, Writing – review & editing. XZ: Data curation, Investigations, Writing – review & editing. PF: Data curation, Investigations, Writing – review & editing. HD: Validation, Resources, Writing – review & editing. WL: Validation, Resources, Supervisions, Writing – review & editing. All authors: read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2025.1536109/full#supplementary-material>

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Oxidative stress products and managements in atopic dermatitis

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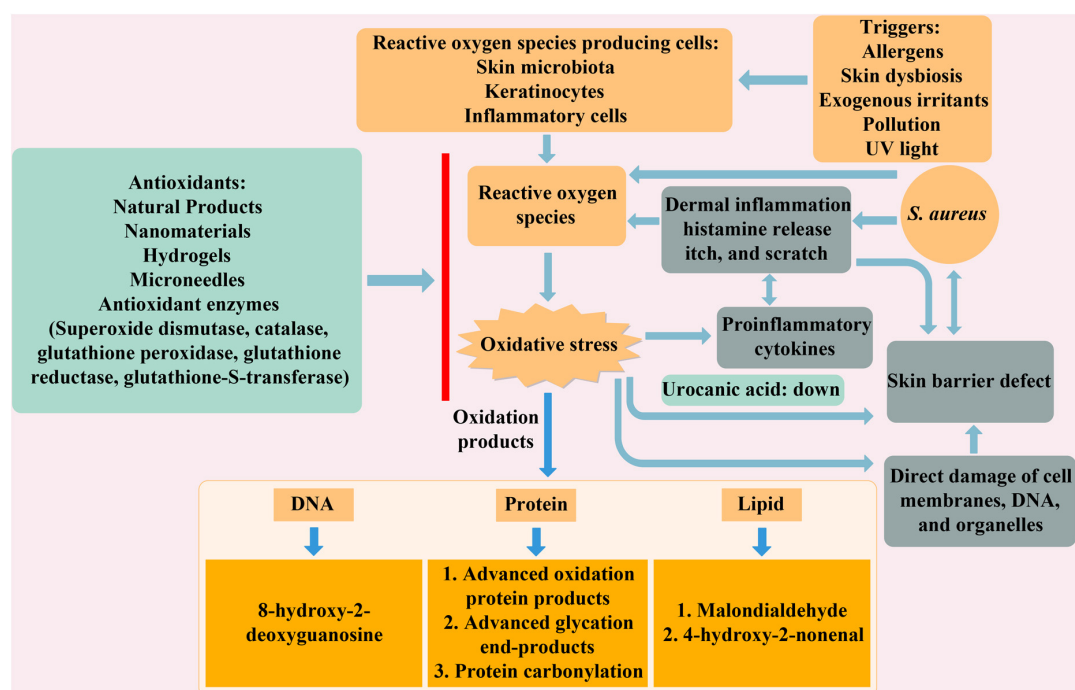
Atopic dermatitis (AD) is a chronic inflammatory skin disorder that affects a significant portion of the global population, severely impacting the quality of life and causing physical and psychological distress of patients. Oxidative stress, resulting from an imbalance between oxidation and antioxidation activities, plays a pivotal role in the pathogenesis of AD. Monitoring oxidative stress products can offer valuable insights into the development of AD and highlight essential clinical and therapeutic effects. Additionally, evidence suggests that antioxidant strategies can alleviate or avert oxidative damage induced by free radicals and offer significant promise in the treatment of AD. In addition to directly utilizing natural products and nanomaterials for antioxidant interventions, these can also be incorporated into hydrogels, which help repair the skin barrier and support the sustained release of therapeutic agents. Furthermore, microneedles provide a minimally invasive method for delivering antioxidants to the deeper layers of the skin, enhancing treatment efficacy. This review aims to summarize the role of the oxidative stress in the pathogenesis of AD, focusing in the main oxidative products (DNA, protein, and lipid oxidation products), as well as antioxidant therapeutic approaches involving natural products, nanomaterials, hydrogels, and microneedles. Understanding these biomarkers and antioxidant therapy approaches provides important insights into the management of AD.

KEYWORDS

atopic dermatitis, skin barrier, oxidative stress, oxidation products, oxidative stress marker, antioxidant therapy

1 Introduction

Atopic dermatitis (AD), also recognized as atopic eczema (AE), presents as a chronic, recurrent, and inflammatory skin disorder (1). Affecting approximately 20% of children and up to 10% of adults worldwide (2), AD often manifests at birth or within the first years of life (3), with the majority of cases presenting before the age of two. In the past four decades, the global prevalence of AD has sharply risen, especially in developed countries (4). Predominant symptoms include skin dryness and intense pruritus, often accompanied by erythema, rash, which significantly impact mental health and quality of life (5). Otherwise, AD is associated with various comorbidities, such as food allergies (6), asthma (7), and allergic rhinitis (8).



GRAPHICAL ABSTRACT

Generation of oxidative stress products and antioxidant therapy in atopic dermatitis (AD). External stimuli trigger the production of reactive oxygen species (ROS), leading to oxidative stress and the oxidation of DNA, proteins, and lipids. This process subsequently induces skin inflammation, the release of proinflammatory cytokines, and disruption of the skin barrier. Antioxidant therapies involving natural products, nanomaterials, hydrogels, microneedles, and antioxidant enzymes help alleviate these effects and improve the condition of AD.

The pathogenesis of AD is complex and not completely understood. Its multifactorial origins include genetic predisposition (9), environmental factors (10), *Staphylococcus aureus* colonization (11), and neurogenic inflammation (12), all contributing to the AD development. Deficiencies in epidermal barrier function and immune imbalance are intricately linked, with mutations in structural epidermal barrier proteins and immune regulatory factors playing pivotal roles in the pathogenesis of AD (13). Reduced expression of filaggrin is often associated with *S. aureus* colonization. *S. aureus* aggravates AD skin lesions (14) and induces pruritus and skin damage through the V8-PAR1 axis (15). One study has reported that filaggrin mutations are linked to infantile eczema and AD. Individuals carrying filaggrin gene mutations are not only at a higher risk of developing skin dryness on the trunk and extensor surfaces of the limbs in infants aged 3–6 months, but they also face an increased risk of developing eczema and AD (16). During the onset of AD, particularly in the acute phase, changes in the skin barrier activate a Th2-mediated immune response. Chronic skin inflammation in AD patients is caused by persistent Th2 inflammation and skin barrier disruption, accompanied by significant reactive oxygen species (ROS) production (17).

Reactive oxygen species are generated in response to different triggers such as allergens, cutaneous dysbiosis, exogenous irritants, pollutants and UV light (18). In particular, ROS are primarily derived from activated immune cells, including neutrophils and macrophages, as well as keratinocytes under oxidative stress conditions. The accumulation of ROS disrupts the balance between ROS generation and the antioxidant defense mechanisms.

Eventually, the excessive oxidative stress is related to the progression of AD (19, 20). In the pathogenesis of AD, ROS derived from activated keratinocytes act in an autocrine and paracrine manner; being important mediators in the main functions of the keratinocyte: maintenance of the skin barrier function, interaction with the skin microbiome and triggering the immune response, including the recruitment of inflammatory cells from the dermis. However, elevated levels of ROS cause excessive oxidative stress and damage cellular components. Additionally, excessive ROS leads to oxidative damage to DNA and proteins, as well as lipid peroxidation of cell membranes, ultimately resulting in cell death (21). Research has indicated that biomarkers linked to oxidative stress, such as urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG), malondialdehyde (MDA), nitrite (NO_2^-), nitrate (NO_3^-), and biopyrrin, are significantly increased in AD patients (22–24). Furthermore, oxidative stress and inflammation mutually reinforce each other (25), with inflammatory cells producing ROS exacerbating oxidative stress, while ROS and oxidative stress products promote inflammatory responses.

To mitigate or avert oxidative damage induced by free radicals, the body has developed an intricate antioxidant defense mechanism. The skin, in particular, has its own antioxidant defense system that eliminates excess ROS through both enzymatic and non-enzymatic pathways, thereby maintaining redox balance and prevent the damage of ROS to cellular tissues (26). Supplementation with external antioxidants is a crucial strategy to combat oxidative stress, especially in AD patients, enhancing their ability to manage oxidative stress (27). The use of exogenous antioxidants is thus a significant component of antioxidant

therapy for AD. Currently, various antioxidant methods have been formulated for the management of AD, including the use of natural product like pterostilbene (28), nanomaterial such as HC-HT-CSNPs (29), SINH-liposome-hydrogel (30), and lipid microparticles loaded with quercetin on microneedles (31), among other antioxidants.

Although ROS contribute to oxidative stress and inflammation, they are also essential for normal physiological functions. Besides direct microbial killing, ROS are also involved in immune responses and emerging as central signaling molecules in the inflammatory response (32). Excessive reduction of ROS may impair these fundamental physiological functions, potentially weakening host defense mechanisms and disrupting normal skin repair processes. Additionally, it introduces the role of antioxidants in the treatment of AD from four therapeutic approaches: natural products, nanomaterials, hydrogels, and microneedles. This review primarily analyzes oxidative stress products in AD at three levels: DNA, protein, and lipid. Additionally, it introduces the role of antioxidants in the treatment of AD from four perspectives: natural products, nanomaterials, hydrogels, and microneedles. Our study aims to explore oxidative stress markers and antioxidant treatment strategies in AD, thereby providing additional therapeutic opportunities for AD management in the future.

2 Role of oxidative stress in AD

Atopic dermatitis is a chronic inflammatory skin disease that commonly occurs in children (33). It is characterized by immune activation, epidermal hyperplasia, and defects in barrier function, reflecting potential changes in keratinocyte differentiation (34). The relationship between skin barrier changes and AD has been confirmed in the pathogenesis of the disease (35). Keratinocytes are key contributors to skin barrier function, playing a central role in the formation of the lipid bilayer and the production of filaggrin. Filaggrin is subsequently degraded into urocanic acid, an essential component of natural moisturizing factors that helps maintain skin hydration. Due to the reaction of reactive substances produced in keratinocytes to the environment and endogenous pro-oxidants, the skin has become the main target of response to oxidative stress. As the largest organ and a vital barrier separating the body's internal environment from the external milieu, the skin is constantly exposed to a variety of external substances, leading to the generation of oxidative and inflammatory mediators (36). Uncontrolled production of ROS and cytokines results in oxidative stress and inflammation. While ROS production is a natural response to environmental changes, prolonged exposure to elevated ROS levels or oxidative stress facilitates the occurrence and exacerbation of skin diseases (37).

Oxidants encompass free radicals or any species containing unpaired electrons, including ROS. Oxidative stress assumes a critical role in AD and other dermatological conditions, evidenced by increased oxidative stress marker levels and diminished antioxidant levels in affected individuals (38). The pathogenesis of AD involves heightened ROS production, as evidenced by elevated ROS levels in skin biopsy specimens from AD patients, assessed using chemiluminescence techniques (39). The

colonization of *S. aureus* frequently observed on the skin of AD patients, is associated with the generation of ROS through bacterial enzymes binding to the aryl hydrocarbon receptor (AHR) (40). AHR contributes to skin homeostasis by upregulating barrier-related proteins, including filaggrin (FLG), loricrin (LOR), and involucrin (IVL). However, the protective effects of AHR activation must be balanced against the antagonistic IL-13/IL-4–JAK–STAT6/STAT3 signaling pathway, which disrupts barrier integrity and promotes oxidative stress in AD. Moreover, ROS-induced high-mobility-group-protein B1 (HMGB1) secretion from keratinocytes facilitates *S. aureus* colonization and persistence by disrupting skin barrier integrity through the downregulation of epidermal barrier genes (41). Protein and lipid peroxidation products generated by oxidative stress in keratinocytes contribute to skin barrier dysfunction and exacerbate the progression of AD (42). Inflammatory responses and ROS production are closely intertwined (43). Inflammatory responses enhance the production of ROS, which further amplify inflammation in turn.

Beyond keratinocytes, oxidative stress also influences various immune cells. Dendritic cells exposed to ROS undergo activation, leading to the secretion of pro-inflammatory cytokines that amplify the immune response in AD (44). In the acute phase of AD, Th2-mediated immune reactions initiate the release of pro-inflammatory cytokines, further perpetuating the inflammatory cascade (45). Additionally, keratinocyte-derived cytokines can also activate Th2-mediated responses under oxidative stress conditions, contributing to skin inflammation in AD (46). In addition, oxidative stress promotes the polarization of macrophages toward a pro-inflammatory M1 phenotype, contributing to sustained inflammation (47). Oxidative stress disrupts the balance between regulatory T cells and effector T cells, leading to immune dysregulation and sustained inflammation in AD (48). Oxidative stress also plays a critical role in modulating cellular signaling events in multiple cell types involved in AD pathogenesis. ROS influence signaling pathways such as NF- κ B, JAK-STAT, and MAPK, which are involved in keratinocyte function, immune cell activation, and cytokine production (49). The activation of these pathways by oxidative stress exacerbates inflammation and skin barrier dysfunction, further promoting AD progression.

3 Marker of oxidative stress in AD

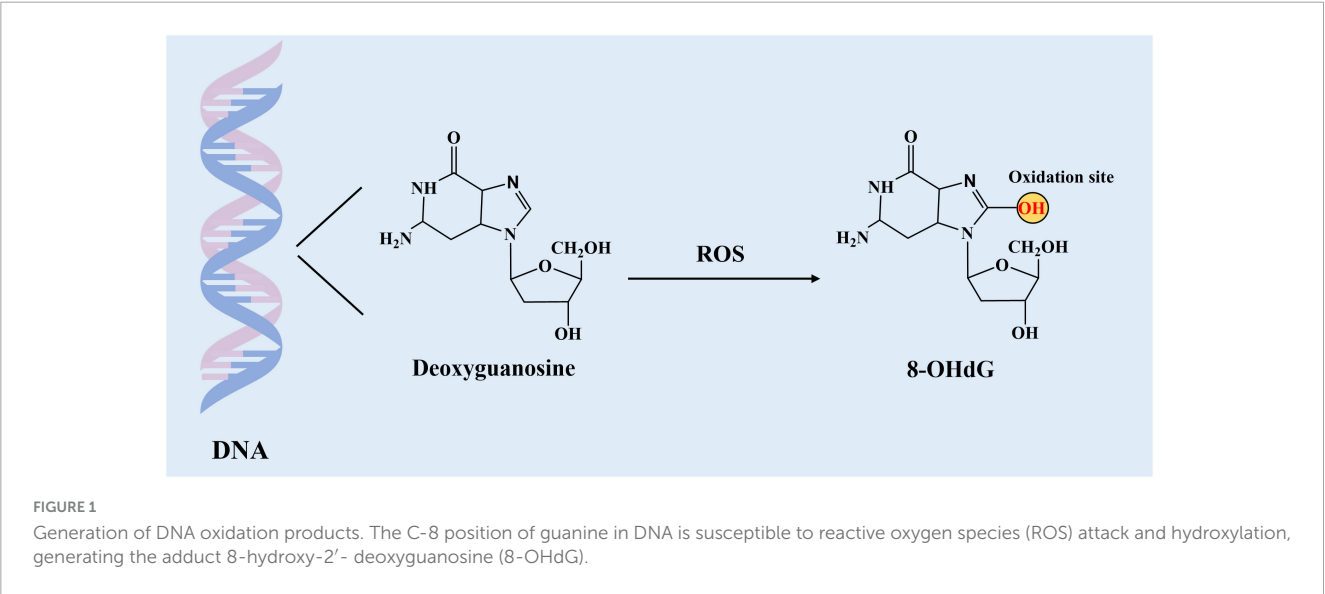
Oxidative stress affects DNA, proteins, and lipids, leading to the formation of various oxidative products in AD. The major oxidative stress markers in AD are summarized in [Table 1](#).

3.1 DNA oxidation products-8-hydroxy-2'-deoxyguanosine

Oxidative stress-induced DNA damage can be assessed using nucleoside derivatives, which act as indicators of oxidative damage. One of the biomarkers is 8-hydroxy-2'-deoxyguanosine (8-OHdG), generated by the oxidation of the deoxyguanosine at the C-8 position ([Figure 1](#)). This biomarker is characterized with sensitive, stable and holistic biomarker of oxidative stress *in vivo* (67, 68). 8-OHdG, a product of damaged DNA is released into the bloodstream

TABLE 1 Oxidative stress markers in atopic dermatitis.

Study population	Type	Oxidative stress markers	References
Children, adult	DNA oxidation	8-hydroxy-2'-deoxyguanosine (8-OHdG)	(22, 50, 51)
Children, adult	Protein oxidation	Advanced oxidation protein products (AOPPs)	(52)
Children, adult		Advanced glycation end- products (AGEs)	(53, 54)
Children, adult		Protein carbonylation (PC)	(55, 56)
Children, adult	Lipid peroxidation	Malondialdehyde (MDA)	(57- 59)
Children, adult		4-hydroxy-2-nonenal (4-HNE)	(52, 60)
Infant, children	Others	Nitric oxide (NO), nitrite and nitrate	(61, 62)
Infant, children		Thiol/disulfide balance	(63, 64)
Children, adult		Biopyrrin	(24, 65, 66)



as a result of the action of the repair enzyme DNA glycosylase, and it is subsequently eliminated in urine (69).

Detecting levels of 8-OHdG in urine can serve as a means to evaluate oxidative damage to DNA in the evaluation of AD (50, 70). Studies comparing 8-OHdG levels in the urine of AD patients and healthy controls have demonstrated positive correlations between 8-OHdG levels and dermatitis scores, indicating disease severity. Additionally, urinary 8-OHdG levels are markedly elevated in children with AD compared to controls, further supporting its utility as a biomarker for AD. A large-scale study involving 200 children diagnosed with AD found that urinary levels of 8-OHdG were considerably increased in the AD group relative to healthy controls ($p < 0.001$) (22). Children with chronic AD exhibited urinary 8-OHdG levels that were 1.6 times higher compared to in healthy controls, with a trend toward decreasing levels as the patients began to heal (51). However, these observations may not be exclusive to acute exacerbations of AD but could instead reflect general changes seen in inflammatory or infectious disorders (65). Moreover, investigations into psoriasis, another chronic refractory skin disease, have also detected 8-OHdG in the urine of affected individuals (71). The results showed that DNA oxidative damage also existed in psoriasis, and 8-OHdG could also be used to monitor

the incidence of psoriasis, with expression levels comparable to those observed in AD.

3.2 Protein oxidation products

3.2.1 Advanced oxidation protein products

Advanced oxidation protein products (AOPPs) constitute a class of complex protein compounds composed of dimethyltyrosine, pentosidine, and carbonyl residues. AOPPs originate from oxidative stress reactions involving plasma proteins and chlorinated oxidants. The principal mechanism driving AOPPs generation involves the activated myeloperoxidase- H_2O_2 -chloride system in neutrophils, with myeloperoxidase serving as the sole enzyme capable of generating chlorinated oxidants (Figure 2) (72, 73). Hypochlorous acid (HOCl) produced by this system is indicative of AOPPs production. In hemodialysis patients, elevated levels of AOPPs have exhibited a positive correlation with plasma myeloperoxidase activity (74) and oxidized fibrinogen was identified as a principal molecule contributing to the positive chemical reaction to AOPPs (75, 76). In 1996, the first detection of this biomarker of oxidative stress was proposed for plasma in patients with chronic uremia (77). Compared to healthy

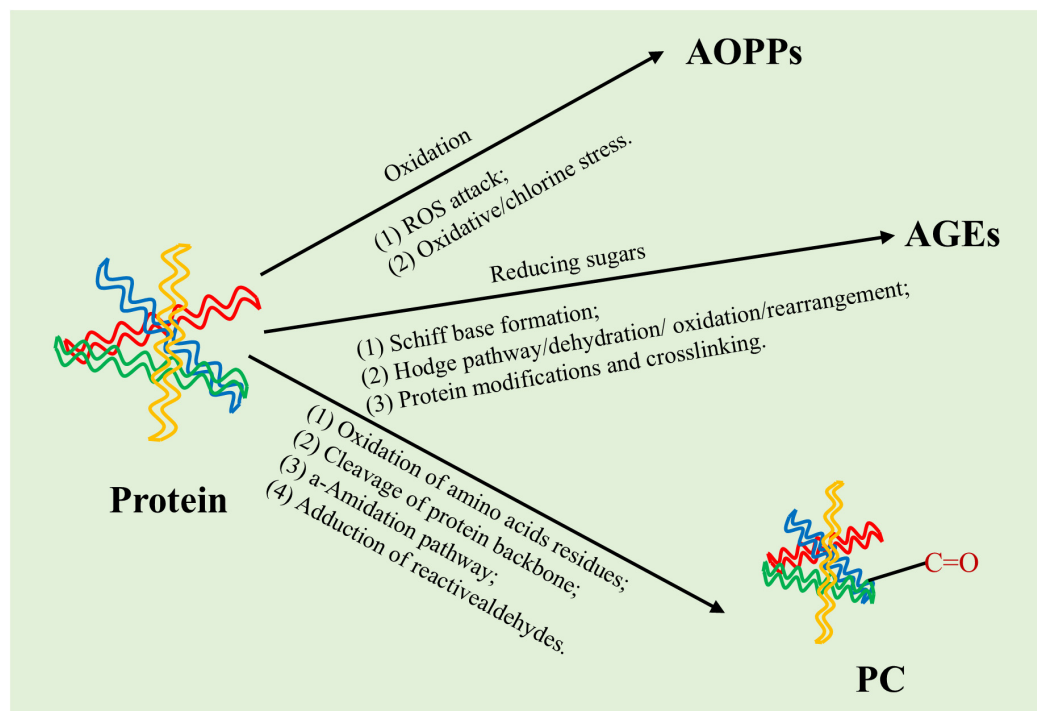


FIGURE 2

Generation of protein oxidation products. The principal mechanism driving advanced oxidation protein products (AOPPs) generation involves the activated myeloperoxidase- H_2O_2 -chloride system in neutrophils. AGFs are primarily generated through Maillard reaction, which is roughly divided into initial stage, intermediate stage and final stage. Protein carbonylation (PC) may occur due to direct oxidation of amino acid residues by reactive oxygen species (ROS) or non-oxidative reaction with carbonyl containing oxidized lipids.

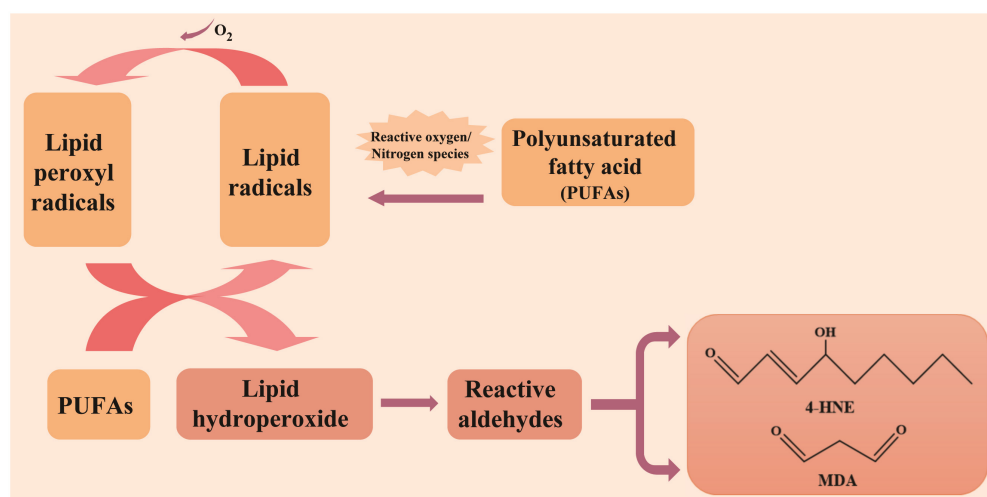


FIGURE 3

Generation of lipid oxidation products. 4-hydroxy-2-nonenal (4-HNE) and Malondialdehyde (MDA) are prevalent lipid oxidation products in atopic dermatitis (AD). Lipid oxidation is closely related to polyunsaturated fatty acids (PUFAs), typically progresses through three stages: initiation, propagation, and termination.

individuals, AOPPs levels in patients with advanced chronic kidney failure who had not yet undergone dialysis were nearly threefold higher.

As markers of oxygen-mediated protein damage, AOPPs have been identified as indicators of oxidative protein damage and proinflammatory mediators (78, 79). Elevated AOPPs levels have

been associated with the advancement of various human diseases and their associated complications, thus serving as markers of oxidative stress across diverse pathologies. Monitoring AOPPs can help predict the development of diseases associated with oxidative stress. In addition to significantly elevated plasma AOPPs levels observed in patients with chronic uremia, similar findings have

been demonstrated in other conditions such as cutaneous burns (80). In both groups of second and third degree thermal burns, AOPPs levels were reduced following treatment as a result of decreased levels of oxidative stress. Notably, ROS may contribute to oxidized protein damage within the stratum corneum, thereby disrupting barrier function and exacerbating AD (55). Currently, studies have shown that compared to healthy individuals, patients with AD and chronic urticaria exhibit elevated levels of AOPPs (52). Moreover, an association between AOPPs levels and age has been documented in patients with AD, underscoring the potential utility of AOPPs as biomarkers for disease severity and progression (52).

3.2.2 Advanced glycation end- products

Advanced glycation end-products (AGEs) and AOPPs share structural similarities and exert comparable biological effects. Their accumulation in biological systems results in analogous clinical outcomes. In the context of protein oxidation, the relationship between AGEs and the generation of AOPPs is noteworthy (75). Both AOPPs and AGEs possess similar structures that induce comparable biological effects, and their accumulation in biological systems leads to similar clinical consequences. AGEs, a diverse group of biologically active compounds, were initially identified by French researchers. They are produced through a non-enzymatic glycation process called the Maillard reaction, which uses the carbonyl groups of reducing sugars and the free amino groups of proteins as substrates (Figure 2) (81). AGEs are categorized into endogenous and exogenous sources (82). Endogenous AGEs are generated during normal physiological processes and aging, while exogenous AGEs primarily originate from dietary sources, with their content varying across different foods.

AGEs are produced in a slow and controlled process, accumulating in the body, including the skin (83). The skin is particularly reactive to changes in AGE levels. Their accumulation increases free radical production, stimulates the release of pro-inflammatory factors, and exacerbates inflammatory reactions. AGEs disrupt the dynamic balance of the skin (84), alter the normal substance composition and structure of different skin layers, impair the skin barrier function, and trigger skin issues. The skin barrier function is essential in the onset and progression of AD. Research has documented that AGEs levels are elevated in the keratinocytes of AD patients compared to healthy individuals, with severe AD patients exhibiting significantly higher levels than those with mild AD. However, no significant difference in serum AGE levels was found between typical AD patients and healthy controls (53). Pentosidine, a specific AGE, is closely associated with oxidative stress, with accelerated production observed in oxidative stress-related diseases (85). Pentosidine can serve as a marker for detecting AD (54). Urine tests in AD patients have shown significantly higher levels of pentosidine compared to the healthy controls. Pentosidine levels were significantly higher in AD patients in the acute phase, but reduced during the recovery process, mirroring trends observed for another AD biomarker, 8-OHdG. The consumption of exogenous AGEs increases the risk of developing AD (86), with pregnant women consuming high-AGEs foods potentially exposing their fetus to a higher AGEs environment, thereby increasing the likelihood of AD development.

3.2.3 Protein carbonylation

In addition to AOPPs and AGEs, protein carbonylation (PC) serves as another marker of protein oxidation in patients with AD. From a medical perspective, protein carbonylation has been emphasized as markers of protein oxidation, oxidative stress, and disease progression (52). The oxidative modification of proteins, characterized by the formation and/or introduction of carbonyl groups into proteins, represents a primary indicator of oxidative damage to proteins (87–89). PC, an irreversible oxidative modification (88), is classified into two types based on the origin of the carbonylated product (Figure 2). Consequently, changes in protein conformation following modification typically result in the loss of protein function (90). Proteins undergoing carbonylation exhibit diverse biological significance within biological systems, leading to varied biological effects (88, 91–93). PC is reflected in various diseases, including brain diseases, inflammatory diseases, autoimmune diseases, and aging (94–96), showing important connections between carbonyls in oxidized proteins, oxidative stress, and disease.

Oxidative stress plays an important role in the pathogenesis of AD, with studies demonstrating elevated levels of PC in dry skin and AD skin lesions (56). Levels of PC in the skin of AD patients are elevated and positively correlated with the severity of the disease (55). Sampling from AD patients utilizing the tape stripping method has revealed increased levels of PC in the stratum corneum, a phenomenon similarly observed in patients with psoriasis (97). The accumulation of PC in the skin contributes to transepidermal water loss and altered dermal matrix accumulation (98). ROS are implicated in inducing damage to cuticular oxidation proteins, disrupting skin barrier function, and exacerbating the development of AD.

3.3 Lipid peroxidation products

3.3.1 Malondialdehyde

Lipids are the most impacted biomolecules in oxidative stress-induced damage, and the identification of these end-products in inflammatory diseases suggest that lipid peroxidation is crucial in such diseases (99). Malondialdehyde (MDA), an important metabolite of arachidonic acid and unsaturated fatty acids possessing multiple unsaturated C-C double bonds in lipids, was the main and most widely studied compound (100) derived from lipid peroxidation following oxidative damage from oxygen radical attack (Figure 3) (101, 102). Upon exposure to oxidative stress, excessive accumulation of ROS disrupts the structure and function of the cell membrane, alters its permeability, and causes lipid peroxidation, inducing the production of MDA.

Malondialdehyde is produced *in vivo* through both enzymatic and non-enzymatic oxidation, making it a widely monitored biomarker for oxidative stress across various diseases and particularly favored for characterizing oxidative stress in AD patients (57). A likely correlation exists between serum antioxidant levels and MDA in individuals with eczema (58), with an inverse correlation between antioxidant levels and MDA levels and decreased serum levels of antioxidant vitamins in patients than in healthy persons. In children with AD, serum levels of MDA were found to be on average 0.055 units higher, while

melatonin (a hormone with antioxidant activity) levels were approximately 3.05 units higher compared to controls. The increase in serum melatonin in AD might represent a compensatory mechanism to reduce skin inflammation by attempting to alleviate excessive oxidant production (59). For the antioxidant properties of quercetin, liposomes incorporating quercetin gel have demonstrated both protective and therapeutic effects on skin eczema (103). Treatment with quercetin-loaded liposomes resulted in significantly reduced skin pathological symptoms compared to untreated counterparts, accompanied by reduced levels of MDA in both liver and skin tissues.

3.3.2 4-hydroxy-2-nonenal

4-hydroxy-2-nonenal (4-HNE) is a lipid peroxide produced by polyunsaturated fatty acids (PUFAs) in response to oxidative stress (Figure 3). It belongs to the same class of lipid peroxidation products as MDA. Both 4-HNE and MDA are extensively studied markers of lipid peroxidation, with MDA recognized as a mutagenic product of lipid peroxidation and 4-HNE recognized as the most toxic product of lipid oxidation (104), worsening the damage resulting from oxidative stress (105, 106). 4-HNE is classified as an α , β -unsaturated aldehyde and possesses three functional groups: carbon-carbon double bonds, carbonyl groups and hydroxyl groups. Due to the existence of the conjugated system of C = C bonds and carbonyl groups, 4-HNE can provide partial positive charge to the carbon at position three and become efficient electrophiles, allowing it to react with essential biological molecules, including proteins and DNA (107–109). 4-HNE can also be generated through both enzymatic and non-enzymatic reactions from the breakdown of ω -6 PUFAs. However, the chemical reaction mechanism of 4-HNE formation remains unclear. Several mechanisms have been proposed over the years. The earliest suggestion was that 4-HNE was formed from PUFAs catalyzed by transition metal ions (102, 110). The formation mechanism of 4-HNE is attributed to the observation of hydroperoxides as an intermediate product.

As a secondary messenger of free radicals and growth regulators, 4-HNE participates in various pathophysiological processes and act as a bioactive marker, especially in oxidative stress related to diseases (111). Skin homeostasis plays an important role in the development of AD. Studies have found that cigarette smoke can trigger the generation of ROS (112). Exposure to cigarette smoke can affect skin homeostasis due to oxidative and inflammatory reactions, as well as inducing lipid peroxidation, leading to increased levels of 4-HNE (113). In a study examining oxidative stress markers in exhaled breath condensates of children with AD, 4-HNE levels were elevated in AD patients but did not differ significantly from those in healthy children (60). Another study found a similar phenomenon by measuring serum 4-HNE levels in AD patients, with 4-HNE concentrations comparable to those in healthy subjects.

3.4 Others

3.4.1 Nitric oxide, nitrite and nitrate

Nitric oxide (NO), a free radical gas containing unpaired electrons, is the smallest biologically active molecule produced

by mammalian cells. It exhibits lipophilicity and possesses a high degree of diffusivity in tissues and cells (114). NO is primarily synthesized in organisms by nitric oxide synthase (NOS), an enzyme divided into three subtypes: inducible iNOS, endothelial eNOS, and neurogenic nNOS (115). Additionally, NO can be produced via non-canonical pathways, either through the reduction of nitrite to NO or by the sequential conversion of nitrate to nitrite and then to NO (116). With diverse biological functions, NO is essential for regulating the body in both healthy and disease states (117). In dermatology, NO is implicated in mechanisms underlying inflammatory or immune-mediated dermatoses, skin infections, skin cancers, and wound healing (118). Skin inflammation is significantly affected by NO (119). Keratinocytes, fibroblasts, and immune cells synthesize NO, and each contributing to the occurrence of inflammatory responses. The importance of NO as a mediator of skin inflammation is underscored by the delicate balance between its production and degradation, which is closely related to the onset and development of inflammatory skin diseases. Overproduction of NO has been linked to conditions such as AD and psoriasis (120–122).

Direct measurement of nitric oxide (NO) presents challenges due to its extremely short half-life in the bloodstream, typically disappearing within seconds, and its involvement in primary biochemical reactions within living organisms (123, 124). NO is highly reactive owing to its unpaired electrons, capable of engaging in oxidative stress by reacting with free radicals, with the primary oxidative metabolites being nitrite and nitrate (125). A study found elevated levels of IgE, nitrite, and nitrate in the plasma of AD patients (61). Furthermore, a study involving 88 cases of AD and 12 cases of non-AD founded that serum nitrate levels not only showed a significant increase in children with AD, but also associated with the severity of the disease (62).

3.4.2 Thiol/disulfide balance

Thiols have emerged as a novel marker of oxidative stress, representing a class of organic compounds containing sulfhydryl groups. These sulfhydryl groups, commonly known as thiols, consist of sulfur and hydrogen atoms bonded to carbon atoms (126). The sulfhydryl groups within thiols provide protection against oxidative stress by scavenging ROS through enzymatic or non-enzymatic mechanisms. Thiols serve as physiological agents for neutralizing free radicals and other ROS (127). Through a series of reactions, thiols undergo modifications and react with oxidants to form disulfide bonds (128). The oxidation of thiols to disulfides is facilitated by through various processes, crucially involving three distinct mechanisms (129). Disulfide compounds formed as a result of these reactions can undergo reversible conversion to a thiol structure, thereby maintaining the dynamic balance of thiol/disulfide equilibrium (130).

Disruption of this balance has associated with the development of certain inflammatory diseases. In infants diagnosed with AD, thiols have been found to be significantly reduced compared to in comparison to healthy controls, while disulfide levels were markedly elevated, indicative of dysregulation in the thiol/disulfide balance favoring peroxidation (63). However, contrasting findings were observed in another study (64), which serum disulfide levels were observed to decrease in AD children in comparison to healthy children, leading to a reduction in the disulfide/natural thiol and disulfide/total thiol ratios.

3.4.3 Biopyrrin

Biopyrrin is a metabolite derived from the oxidation of bilirubin. Bilirubin, acting as a potent ROS scavenger, reacts with ROS, thereby exerting a robust antioxidant effect (131). Upon oxidation, bilirubin is converted into various forms of bilirubin oxidation metabolites (BOMs), comprising at least seven hydrophilic metabolites that are promptly excreted into urine because of their hydrophilic nature (132). These metabolites, including biopyrrin, can be detected by anti-bilirubin monoclonal antibody 24G7 (133, 134). A key advantage of using biopyrrin as an oxidative stress marker is its ability to provide real-time insights into the dynamic changes of oxidative stress through urine detection, thereby reflecting the oxidative stress status associated with various disease states.

Biopyrrin holds promise as a novel biomarker for AD (66). Urinary excretion of biopyrrin was markedly increased in pediatric patients experiencing acute exacerbations of AD, with levels averaging 1.8 times higher than those observed in healthy individuals (65). Bilirubin oxidation was enhanced in the diseased skin of patients with AD and levels of the oxidized metabolite biopyrrin detected in urine correlated with the severity of AD (24). Moreover, urinary biopyrrin levels were positively correlated with serum IgE and TARC/CCL17 expression. Biopyrrin expression was higher in AD lesions compared to normal skin, as detected by the 24G7 antibody. These findings highlight the potential utility of biopyrrin as a valuable biomarker for assessing oxidative stress and the severity of AD.

4 Manage oxidative stress in AD

Immunity and inflammation are pivotal in the pathogenesis of AD. Current therapeutic strategies predominantly focus on anti-inflammatory or immune-modulating agents. While the majority of patients respond favorably to topical corticosteroids (135), calcineurin inhibitors (136), and immunosuppressants (137), these treatments are often associated with significant adverse effects during long-term use (138). Oxidative stress is positively correlated with factors influencing the onset and advancement of AD. Moreover, prolonged exposure to oxidative stress impacts the condition of keratinocytes, leading to alterations in skin barrier function and cell death. Therefore, considering the use of antioxidants to mitigate AD is an essential strategy in the management of AD.

4.1 Natural products

Plants are the primary source of natural antioxidants (139), with antioxidant compounds primarily synthesized as secondary metabolites. Numerous plants and their derivatives exhibit antioxidant properties and frequently possess other significant biological activities. Due to their low toxicity, these natural antioxidants have been extensively utilized in the prevention and management of diseases related to oxidative stress (140). Plant-derived antioxidant compounds can be categorized into several groups: phenolic acids, phenolic diterpenes, flavonoids, volatile oils, carotenoids, and anthocyanins (141, 142). These compounds are

abundant in herbs, spices, seeds, essential oils, fruits, and vegetables (143). Additionally, plants and foods containing vitamins and certain trace minerals contribute to the antioxidant process and constitute essential components of natural antioxidants (144, 145).

4.1.1 Natural extracts

Plants typically contain several highly active antioxidant compounds that can be extracted using various technical methods (146). The antioxidant effects of plant extracts are related to the chemical and physical properties of these compounds and operate through multiple mechanisms (147–149). The therapeutic potential of natural extracts for treating AD has been thoroughly explored through both *in vivo* and *in vitro* studies. A study involving 20 patients with mild to moderate AD demonstrated that a cream containing 100,000 IU of superoxide dismutase (SOD) and 4% plant extracts significantly alleviated AD symptoms and was effective across all phases of the disease (150). The therapeutic efficacy of this cream is attributed to the synergistic actions of SOD and the plant extracts, including antioxidant, anti-inflammatory, and additional beneficial properties. Resveratrol, a naturally occurring polyphenol abundant in grapes and berries, has shown positive therapeutic effects on skin disorders (151), potentially affecting inflammation through its antioxidant activity and free radical scavenging properties (152). Intragastric administration of resveratrol has been shown to ameliorate AD in mice induced by dinitrochlorobenzene (DNCB), by downregulating chemokine and proinflammatory factor levels and upregulating the expression of skin barrier proteins (153). Another study also demonstrated that topical formulations based on the antioxidant properties of resveratrol can reduce ROS, inhibit inflammatory responses, and improve skin barrier function (154). Analysis of the chemical composition of *Lentinula edodes* ethanolic extract revealed that polyphenols are the main antioxidant components, along with flavonoids, β -carotene, and lycopene. This ethanolic extract has shown to decrease serum IgE levels, downregulate the expression of inflammatory cytokines, and alleviate AD symptoms (155). Additionally, some natural extracts can exert antioxidant and anti-inflammatory effects and regulate the Nrf2/HO-1/NQO1 and NF- κ B/MAPK signaling pathways to treat AD (156, 157).

4.1.2 Vitamins

Vitamins are a group of organic compounds crucial for maintaining normal physiological functions in the body and can be categorized into fat-soluble and water-soluble groups (158). Most vitamins are obtained through the daily diet. The primary sources of vitamins A, C, and E are fresh vegetables and fruits, while vitamin D is primarily biosynthesized through the skin under sunlight. These four vitamins inherently possess antioxidant properties, allowing them to function as antioxidants (159, 160).

There is a significant connection between vitamins and skin diseases. Maintaining a reasonable and stable vitamin level is crucial for preserving normal skin health (161). β -carotene (provitamin A) exhibits antioxidant and immunomodulatory effects, enhancing skin barrier function and reducing inflammation levels in hairless mice with oxazolone-induced AD (162). Vitamin C contributes to the formation of skin structure and skin antioxidation (163), ameliorating chronic inflammation and positively impacting AD. In groups supplemented with vitamin E, levels of oxidative

stress markers were decreased, and a reduction in vitamin E concentration contributed to the progressing of AD in dogs (164). Another study supports that vitamin E supplementation can lower IgE levels in AD patients and improve AD symptoms (165). Vitamin D supplementation is beneficial for AD treatment and can be used to treat AD in children and dogs (166, 167). Furthermore, these vitamins can work synergistically to enhance AD treatment. For instance, the dermatitis score was lower in the group receiving both vitamin D and vitamin E compared to groups receiving either vitamin alone (168). However, there are differing views on the efficacy of vitamins in improving AD. Diets rich in antioxidant compounds can reduce the risk of AD. Taking in β -carotene and vitamin E is negatively correlated with AD, whereas vitamin C intake does not show a consistent correlation (169). Additionally, higher concentrations of vitamin C in breast milk are linked to a lower risk of atopy in infants, whereas vitamin E shows no consistent relationship with AD (170).

4.1.3 Minerals

Trace minerals, also known as trace elements, play an important role in maintaining overall nutrition and health (171), playing vital roles in the metabolism and physiological processes of the body. Several trace elements are involved in the redox reaction process. Selenium (Se) is an essential trace mineral that forms a key part of selenoproteins, which primarily exert their nutritional functions through a family of 25 selenoproteins. Se is an enzymatic antioxidant that has no antioxidant effect by itself but participates in selenoproteins as redox-active selenoenzymes to safeguard against oxidative damage (172, 173). Inhibiting iron death by regulating selenoprotein GPx4 plays a significant role in improving skin inflammation (174). Keratinocytes, which are integral to the skin barrier function, are also implicated in skin disorders such as AD. The supplementation of Se and selenoprotein SEPP1 can alleviate the oxidative stress and toxicity of 4-CIBQ-induced keratinocytes (175). Nevertheless, a 12 weeks study revealed that selenium-enriched yeast supplementation did not result in significant improvements in AD severity, indicating no substantial difference before and after supplementation (176).

Zinc, another essential nutrient for skin health, is abundantly present in the epidermis (177). Although zinc itself is not an antioxidant and is redox-inert, it contributes to oxidative defense through several mechanisms (178, 179). Decreased zinc levels have been observed in AD patients, taking zinc supplements orally may help those who are zinc deficient to manage AD (180). Zinc is commonly used as a nutritional supplement for cosmetic purposes and in the management of AD. However, caution is warranted regarding zinc concentration, as excessive intake can lead to zinc toxicity (181). The efficacy of zinc supplementation in treating AD remains controversial, with some studies suggesting no significant benefit, while others indicate potential therapeutic effects (182).

4.2 Nanomaterials

Nanotechnology has seen extensive development across various disciplines and facilitates the synthesis of nanoparticles via bottom-up and top-down strategies (183). Conventional antioxidants frequently encounter challenges, including limited permeability,

poor aqueous solubility, instability, and low bioavailability (184). Consequently, nanomaterials have become a pivotal area of research dedicated to improving the efficacy of antioxidants. The utilization of nanotechnology presents a promising avenue for overcoming the limitations associated with conventional antioxidants, thereby exhibiting significant potential in the realm of antioxidant therapy. Antioxidant nanomaterials can be broadly categorized into two types: those with inherent antioxidant properties and antioxidant delivery nanomaterials. The first type includes nanomaterials that possess antioxidant properties independently, without the need for functionalization with antioxidants. The second type comprises nanomaterials that do not inherently have antioxidant properties but can be used to load and deliver antioxidants, thereby exerting antioxidant effects.

4.2.1 Nanomaterials with intrinsic antioxidant activity

Among antioxidant nanomaterials, several types possess inherent antioxidant properties, most of which are metal nanoparticles. These nanomaterials can mimic the efficacy of antioxidant enzymes like catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx). The antioxidant enzyme activity of nanomaterials is influenced by factors including size, morphology, surface modification, and composition (185). Catalase-like nanoenzymes, a category of nanomaterials with intrinsic CAT activity, operate by decomposing H_2O_2 into H_2O and O_2 (186). Superoxide radicals ($\text{O}_2^{\bullet-}$), a type of ROS generated during metabolic processes, are converted into H_2O_2 and O_2 by SOD, using metal as a cofactor (187). GPx, the final antioxidant enzyme, catalyzes the reduction of H_2O_2 or organic hydrogen peroxide to H_2O or alcohol in the presence of reduced glutathione (188). Cerium oxide nanoparticles (189) exhibit SOD-like activity, and their PEGylation can enhance the survival rate of keratinocytes while significantly reducing intracellular ROS levels. Cobalt oxide nanoparticles, synthesized via a one-pot method, demonstrate three enzymatic catalytic activities. These nanoparticles can protect keratinocytes from hydrogen peroxide-induced ROS and toxicity, alleviating the symptoms of AD (190). Furthermore, hematoxylin and eosin and toluidine blue staining indicated a decrease in epidermal thickness and a reduction in the number of mast cells in the treated group.

4.2.2 Antioxidant delivery nanomaterials

Most antioxidants have low bioavailability due to their inherent properties (191, 192). However, nanotechnology can enhance the antioxidant effect by preparing nanoparticles as carriers for these antioxidants, allowing for targeted and controlled release (193). Currently, a variety of antioxidant delivery systems have been developed to transport natural and synthetic antioxidants, antioxidant gases, genes, and other antioxidant compounds, thereby significantly expanding the scope of antioxidant delivery nanomaterials (194). Nanoparticles prepared with the natural polyphenol antioxidant hydroxytyrosol, hydrocortisone, and chitosan have shown significant improvement in the pathological characteristics of AD in mice. Compared to the AD group, the treatment group exhibited decreased expression levels of IgE, histamine, PGE2, VEGF- α , and AD-related Th1 and Th2 cytokines. Histological examination also demonstrated that HC-HT-CS-NPs exerted a therapeutic effect on AD (195).

Additionally, HC-HT-CS-NPs demonstrated favorable safety in healthy individuals (196).

4.3 Hydrogels

Hydrogel is polymer material with a three-dimensional porous structure formed by physical or chemical crosslinking of polymer chains. Hydrogels exhibit notable hydrophilicity, enabling them to absorb water and biological fluids, and they possess excellent moisturizing and air permeability properties (197). Due to their high water content, structural similarity to natural tissues, and favorable biocompatibility, hydrogels are widely used in biomedical fields, particularly for drug delivery, facilitating controlled release and enhancing efficacy (198–200). Additionally, the physical and chemical properties of hydrogels can be easily modified to impart various functions, including antioxidant properties (201). Antioxidant hydrogels can be categorized into self-antioxidant hydrogels and those combined with antioxidant components (202).

These hydrogels have been applied in skin diseases and have shown promise as carriers for the local treatment of AD. Lignin, a polyphenol-containing substance extracted from lignocellulosic biomass, has the capability to scavenge ROS. Hydrogels prepared by crosslinking lignin with polyethylene glycol exhibit CAT and superoxide SOD enzyme-mimicking properties and possess antioxidant capacity (203). These hydrogels can treat AD by reducing skin oxidative stress. They protect HaCaT cells from oxidative stress damage caused by H_2O_2 . In DNCB-induced AD mice, treatment with these hydrogels reduced dermatitis scores and epidermal thickness, inhibited inflammation, alleviated DNA oxidative damage, and decreased Th2 cytokine levels.

Furthermore, cerium oxide nanoparticles known for their high ROS scavenging ability, have been incorporated into hydrogels. By adjusting pH and crosslinking sodium alginate polymer with Ca^{2+} , a CENP-sodium alginate hydrogel was prepared. This hydrogel effectively mimics CAT and SOD activity, protecting cells from oxidative stress damage. In AD mice, treatment with this hydrogel reduced epidermal thickness, decreased 8-OHdG accumulation, lowered Th2 cytokine and IgE levels, and reduced mast cell infiltration, showing its therapeutic potential for AD management (204).

4.4 Microneedles

The skin is composed of three primary layers: the epidermis, dermis, and subcutaneous tissue (205). The stratum corneum, an integral part of the skin barrier, is formed through the differentiation of keratinocytes and serves as the primary protective layer against external injury and stimulation (206). However, this physiological structure presents a challenge for transdermal drug delivery, limiting the bioavailability of drugs administered through the skin. Microneedles, ranging in length from 25 to 2,000 μm (207), offer a promising transdermal technology by piercing the stratum corneum and crossing the skin barrier to reach all layers of the skin. This technology has been widely used in the treatment of skin diseases, enabling the delivery of antioxidants into the skin or using the inherent antioxidant properties of the microneedles to address oxidative stress-related skin conditions (208–210).

In the treatment of AD, microneedles loaded with epigallocatechin gallate, a potent antioxidant, and L-ascorbic acid as a stabilizing reductant, prepared from poly- γ -glutamic acid, exhibit multiple beneficial functions. These microneedles improve DNCB-induced AD in mice through antioxidant, anti-inflammatory, and immunomodulatory effects (211). Treatment outcomes include reduced epidermal thickness, decreased mast cell infiltration, and lower levels of serum IgE and histamine. Microneedles incorporating natural polyphenols such as curcumin and gallic acid, prepared from PLGA/HA in a double-layer configuration, enable rapid treatment and long-term management of AD (212). The curcumin and gallic acid -loaded microneedles provide immediate antioxidant and anti-inflammatory effects, alleviating AD symptoms in the short term, while the embedded PLGA needle mediates the sustained release of curcumin for long-term improvement. Post-treatment, AD mice exhibited reduced dermatitis scores and improved pathological conditions, with a significant decrease in ROS levels in the lesional skin after 56 days. Additionally, a novel polydopamine nanozyme integrated with near-infrared-responsive microneedles was developed using natural dopamine. Hyaluronic acid was used for the backing layer, and hyaluronic acid methacrylate was employed for the tip, enabling antioxidant treatment of AD (213). PDA MNs + NIR treatment alleviated AD symptoms and inhibited Th2 immune-related reactions. Measurement of the DNA oxidative stress marker 8-OHdG revealed that the PDA MNs + NIR group significantly downregulated its levels.

5 Conclusion

Atopic dermatitis is a chronic inflammatory skin disorder characterized by disruptions in skin barrier integrity and immune dysregulation. The complex pathogenesis of AD involves oxidative stress, which induces several cellular damages in keratinocytes, impairs skin barrier function, and exacerbates the inflammatory response. This oxidative damage results to modifications of DNA, proteins, and lipids, culminating in the formation of various oxidation products. While several antioxidant strategies, such as the use of natural products, nanomaterials, hydrogels, and microneedles have shown promise in mitigating oxidative stress and alleviating AD symptoms, there remain knowledge gaps. Further research is required to fully elucidate the specific oxidative modifications underlying AD pathology, the precise mechanisms of action of antioxidant therapies, and the strategies for optimizing these treatments for clinical application. Addressing these gaps will be essential for developing more effective therapeutic strategies and monitoring tools to improve the management of AD.

Author contributions

YL: Writing – original draft, Writing – review and editing. JH: Conceptualization, Writing – review and editing. ZZ: Conceptualization, Writing – review and editing. YZ: Visualization, Writing – review and editing. YW: Funding acquisition, Supervision, Writing – review and editing. JS: Supervision, Writing – review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Linking oxidative stress biomarkers to disease progression and antioxidant therapy in hypertension and diabetes mellitus

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Oxidative stress (OS) is increasingly recognized as a key factor linking hypertension (HTN) and diabetes mellitus (DM). This review summarizes recent evidence regarding the dual role of OS as both an instigator and an amplifier of cardiometabolic dysfunction. In HTN, reactive oxygen species (ROS) produced by NADPH oxidases (NOXs) and mitochondrial dysfunction contribute to endothelial impairment and vascular remodeling. In DM, hyperglycemia-induced ROS production worsens beta-cell failure and insulin resistance through pathways such as the AGE-RAGE signaling, protein kinase C (PKC) activation, and the polyol pathway. Clinically validated biomarkers of OS, such as F2-isoprostanes (which indicate lipid peroxidation), 8-OHdG (which indicates DNA damage), and the activities of redox enzymes like superoxide dismutase (SOD) and glutathione peroxidase (GPx), show strong correlations with disease progression and end-organ complications. Despite promising preclinical results, the application of antioxidant therapies in clinical settings has faced challenges due to inconsistent outcomes, highlighting the need for targeted approaches. Emerging strategies include: 1. Mitochondria-targeted antioxidants to enhance vascular function in resistant HTN; 2. Nrf2 activators to restore redox balance in early diabetes; and 3. Specific inhibitors of NOX isoforms. We emphasize three transformative areas of research: (i) the interaction between the microbiome and ROS, where modifying gut microbiota can reduce systemic OS; (ii) the use of nanotechnology to deliver antioxidants directly to pancreatic islets or atherosclerotic plaques; and (iii) phenotype-specific diagnosis and therapy guided by redox biomarkers and genetic profiling (for example, KEAP1/NRF2 polymorphisms). Integrating these advances with lifestyle modifications, such as following a Mediterranean diet and exercising regularly, may provide additional benefits. This review outlines a mechanistic framework for targeting OS in the comorbidity of HTN and DM while identifying critical knowledge gaps, particularly regarding the timing of antioxidant signaling and the development of personalized redox

medicine, which may serve as a reference for researchers and clinicians working in this area.

KEYWORDS

oxidative stress, biomarkers, hypertension, diabetes, disease progression, antioxidant therapy, clinical trials, precision redox medicine

1 Introduction

Hypertension (HTN) and diabetes mellitus (DM) are interconnected metabolic disorders driving global cardiovascular morbidity, with oxidative stress (OS) implicated as a key pathological link (Mysko et al., 2025). OS arises from a disruption in redox homeostasis, where an overproduction of reactive oxygen species (ROS) overwhelms endogenous antioxidant defenses, leading to oxidative damage to lipids, proteins, and DNA (Sies et al., 2024). This imbalance accelerates endothelial dysfunction, insulin resistance, and end-organ damage, thereby perpetuating the progression of HTN and DM (Griendling et al., 2021; Yousef et al., 2023; Rais et al., 2024). Under physiological conditions, cells maintain redox homeostasis through tightly regulated systems, including enzymatic antioxidants (e.g., superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPx) and non-enzymatic scavengers (e.g., glutathione, vitamin E) (Lushchak, 2014). ROS, such as superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), also serve as signaling molecules at low levels, modulating metabolic and vascular functions (Sies et al., 2020). However, in HTN and DM, chronic hyperglycemia, angiotensin II activation, and mitochondrial dysfunction exacerbate ROS production (Vejendla et al., 2024). The resulting oxidative overload disrupts redox-sensitive pathways (e.g., NF- κ B, Nrf2), promoting inflammation, fibrosis, and cellular apoptosis (Fountoulakis et al., 2025).

Elevated levels of oxidative biomarkers—such as malondialdehyde (MDA), 8-hydroxy-2'-deoxyguanosine (8-OHdG), and F₂-isoprostanes—reflect systemic OS and correlate with HTN severity and DM complications (Duni et al., 2017). For example, plasma MDA predicts endothelial dysfunction in HTN (Camargo et al., 2025), while urinary 8-OHdG is a surrogate for diabetic nephropathy (Spoto et al., 2025). Conversely, diminished antioxidant capacity (e.g., reduced SOD/GPx activity) is associated with β -cell failure and poor glycemic control (Newsholme et al., 2019). Despite their potential, biomarker variability and lack of standardized assays limit clinical adoption (Lankin et al., 2024). While preclinical studies demonstrate that antioxidants (e.g., vitamin E, N-acetylcysteine, polyphenols) mitigate OS and improve vascular function in HTN/DM (Black, 2022; He et al., 2023), human trials report inconsistent outcomes. Research indicates that high-dose vitamin E offers no cardiovascular benefits in individuals with DM (Kaye et al., 2025). In contrast, targeted therapies, such as mitochondria-specific antioxidants like MitoQ, show promising potential (Gioscia-Ryan et al., 2018). This disparity underscores the need for biomarker-guided personalized approaches. Consequently, the diagnosis of HTN and DM mediated by OS should consider an epigenetic perspective while factoring in the lifestyle of the target population.

This review evaluates: (i) the role of redox imbalance in HTN/DM progression; (ii) correlations between OS biomarkers and

clinical outcomes; and (iii) current evidence on antioxidant adjuvant therapies. We aim to identify gaps in translating OS biomarkers into actionable therapeutic strategies by integrating mechanistic insights and clinical data. Additionally, the criteria emphasize the design of clinical trials for antioxidant therapies and potential mechanistic approaches for addressing HTN-DM in clinical practice.

2 Data search

An extensive search was done on published reports from specialized data sources, including PubMed/MedLine, ScienceDirect, Google Scholar, SciFinder, Scopus, and the TRIP Database. The search methodology included terms as oxidative stress, reactive oxygen species, biomarkers, hypertension, endothelial dysfunction, mitochondrial dysfunction, diabetes, β -cell damage, vascular dysfunction, hyperglycemia, insulin resistance, vascular complications, diabetic neuropathies, disease progression, antioxidant therapy, clinical trials, and precision redox medicine. The search included *in vitro*, *in vivo*, and clinical studies published between 2014 and 2025. These studies are expected to provide comprehensive data on the relationship between ROS and lifestyle, as well as the metabolic pathways involved in HTN and DM. Published reports were downloaded and reviewed from specialized data sources. The number of records related to HTN and DM in Science Direct rose significantly from 1,453 in 2014 to 4,003 in 2024, indicating a growing interest in this topic within the scientific community. After refining the data across all databases, 3,336 records were registered. Out of these, 221 were included in the review, as shown in Figure 1.

3 Current diagnosis of oxidative stress

The most common approach to diagnose OS is the static measurement of OS biomarkers, but the effective management of OS relies on assessing oxidative burden accurately and implementing targeted therapies that extend beyond mere ROS scavenging. Direct and indirect biomarkers are used as follows:

- **Lipid peroxidation:** Malondialdehyde (MDA) is measured via the Thiobarbituric Acid-Reactive Substances (TBARS) assay, while F₂-isoprostanes (stable end-products of arachidonic acid oxidation) are quantified by gas or liquid chromatography-mass-mass spectrometry (HPLC/MS-MS) for high specificity (Sabitha et al., 2024).
- **Protein oxidation:** Protein carbonyl groups serve as reliable biomarkers of protein oxidation. The most widely used detection method involves the derivatization with 2,4-dinitrophenylhydrazine (DNPH)

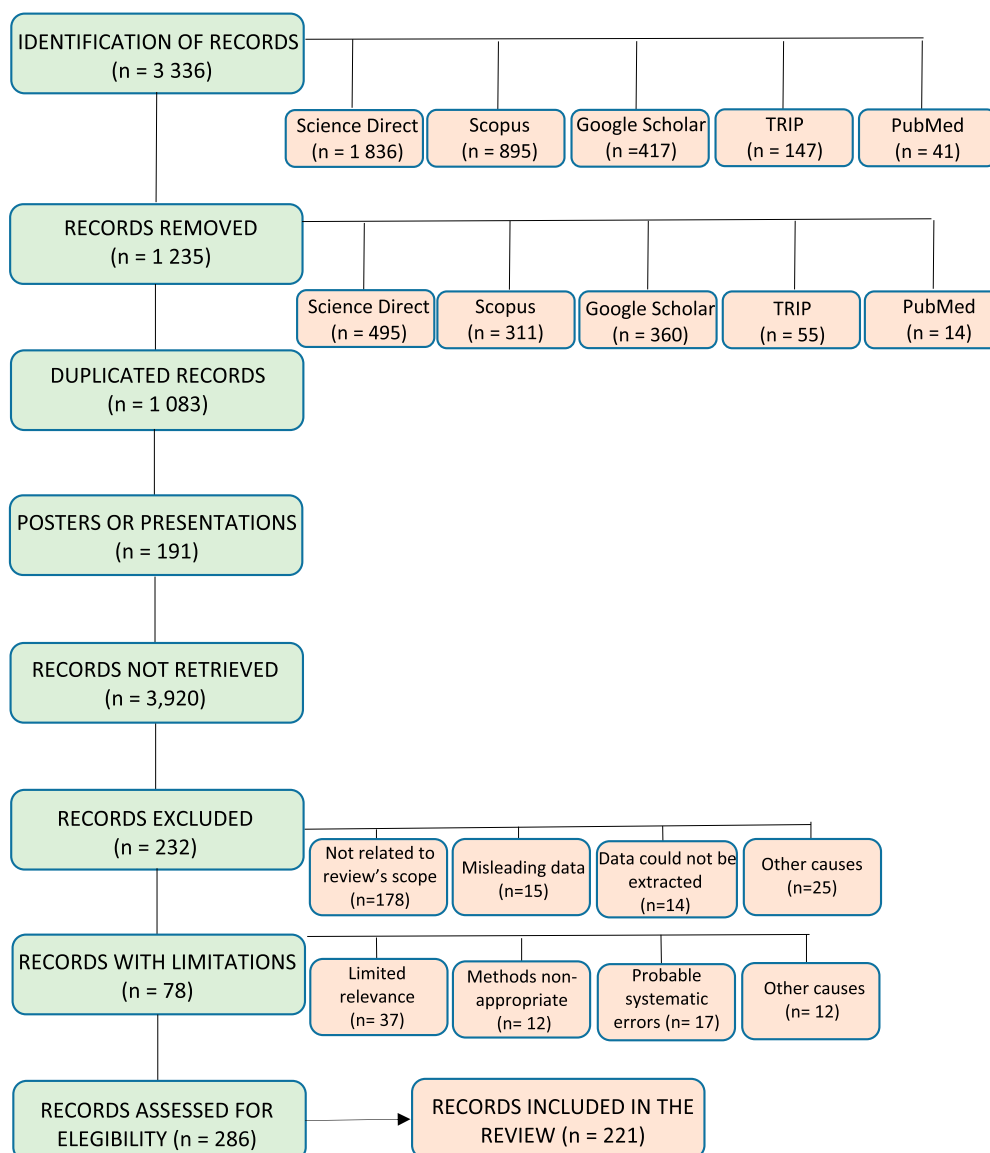


FIGURE 1
Flow chart diagram for the selection of records from databases.

and spectrophotometric quantification at 370–375 nm, with the absorbance being proportional to carbonyl content. Alternatively, immunoblotting with anti-DNP antibodies and ELISA-based approaches for higher throughput are applied (Kehm et al., 2021).

- DNA oxidation: 8-hydroxy-2'-deoxyguanosine (8-OHdG) is a well-established biomarker of OS for DNA oxidation, reflecting guanine base damage caused by ROS. Its levels are commonly quantified using high-performance liquid chromatography (HPLC) coupled with electrochemical or mass spectrometry detection (HPLC-ECD or HPLC-MS/MS), offering high sensitivity and specificity. ELISA kits provide a cost-effective and high-throughput alternative, though potential antibody cross-reactivity requires careful

validation. Elevated 8-OHdG levels correlate with aging, cancer, diabetes, and neurodegenerative diseases, making it a critical tool for assessing oxidative DNA damage in clinical and experimental research (Chiorcea-Paquim, 2022).

- Electron Paramagnetic Resonance Spectroscopy (EPR): It is a powerful tool to directly detect and quantify ROS in biological tissues. EPR spin trapping and fluorescent probes such as DCFH-DA enable dynamic monitoring of intracellular ROS (Ioannidou et al., 2025). EPR has also identified elevated superoxide anion ($O_2^{\bullet-}$) in glomeruli and tubules of diabetic rodents, correlating with albuminuria. (Epel et al., 2022). Moreover, vascular ROS (e.g., peroxynitrite) in resistant HTN models are quantified using NOX-specific probes. (Babić and Peyrot, 2019; Valaitienė and Laučytė-Cibulskienė, 2024).

- Omics Approaches: Redox proteomics and transcriptomics identify oxidative modifications and gene expression patterns, aiding personalized therapy (Dai and Shen, 2022)

All these assays, both traditional and advanced, single or combined, provide a comprehensive picture of oxidative damage across biomolecular targets. However, dynamic measurement of the OS state and progression has been developed recently, which will be discussed ahead with the focus on HTN and DM.

4 The role of lifestyle and the need for antioxidant supplementation in hypertension and diabetes

The interplay between lifestyle factors and OS is pivotal in the development and progression of HTN and DM. Modern sedentary behaviors, poor dietary habits, smoking, and chronic psychological stress exacerbate ROS production while impairing antioxidant defenses (Pizzino et al., 2017). Conversely, lifestyle modifications, including physical activity, Mediterranean or DASH (Dietary Approaches to Stop Hypertension) diets, smoking cessation, and stress management, have been shown to restore redox balance and reduce disease severity (Fogacci et al., 2019). The Mediterranean diet, rich in polyphenols (e.g., olive oil, nuts, berries), reduces lipid peroxidation (MDA) and enhances antioxidant enzymes (SOD, GPx) in diabetic patients (Ruiz-Pozo et al., 2024). The DASH diet reduces blood pressure (BP) and OS markers by enhancing nitric oxide (NO) bioavailability and decreasing NADPH oxidase (NOX) activity (Muszalska et al., 2025).

Moderate aerobic exercise upregulates endogenous antioxidants (e.g., SOD, CAT) and improves mitochondrial function in HTN and DM (Jiang et al., 2023). However, excessive exercise without proper recovery can paradoxically increase ROS, highlighting the need for balanced regimens (Margaritelis et al., 2018). Chronic sleep deprivation and stress elevate cortisol and pro-oxidant cytokines, worsening insulin resistance and endothelial dysfunction (Aboonabi and McCauley, 2024). Mindfulness and yoga have been shown to reduce OS biomarkers (e.g., F2-isoprostanes) in hypertensive patients (Lee et al., 2020). Considering these factors, is antioxidant supplementation necessary for those with HTN and DM?

The role of exogenous antioxidant supplementation remains controversial since meta-analyses have shown mixed results. Unlike endogenous antioxidants, synthetic supplements, such as vitamin C pills, may disrupt redox signaling by non-specifically scavenging ROS, including beneficial low-level oxidants that play a role in metabolic regulation (Forman and Zhang, 2023). Some targeted antioxidant supplementation has shown positive results, like vitamin D and Omega-3 Fatty Acids (OFAs), which have been promising in reducing inflammation and OS in HTN/DM when deficiency is confirmed (Mazidi et al., 2020). Recent studies suggested that fish oil may have beneficial effects in treating HTN, primarily due to various bioactive oxidation products formed by free radicals from OFAs. However, the specific molecular species responsible for these effects remains unidentified (Krupa et al., 2024). OFAs are linked to a reduced incidence of cardiovascular diseases. Evidence from randomized controlled trials strongly supports the

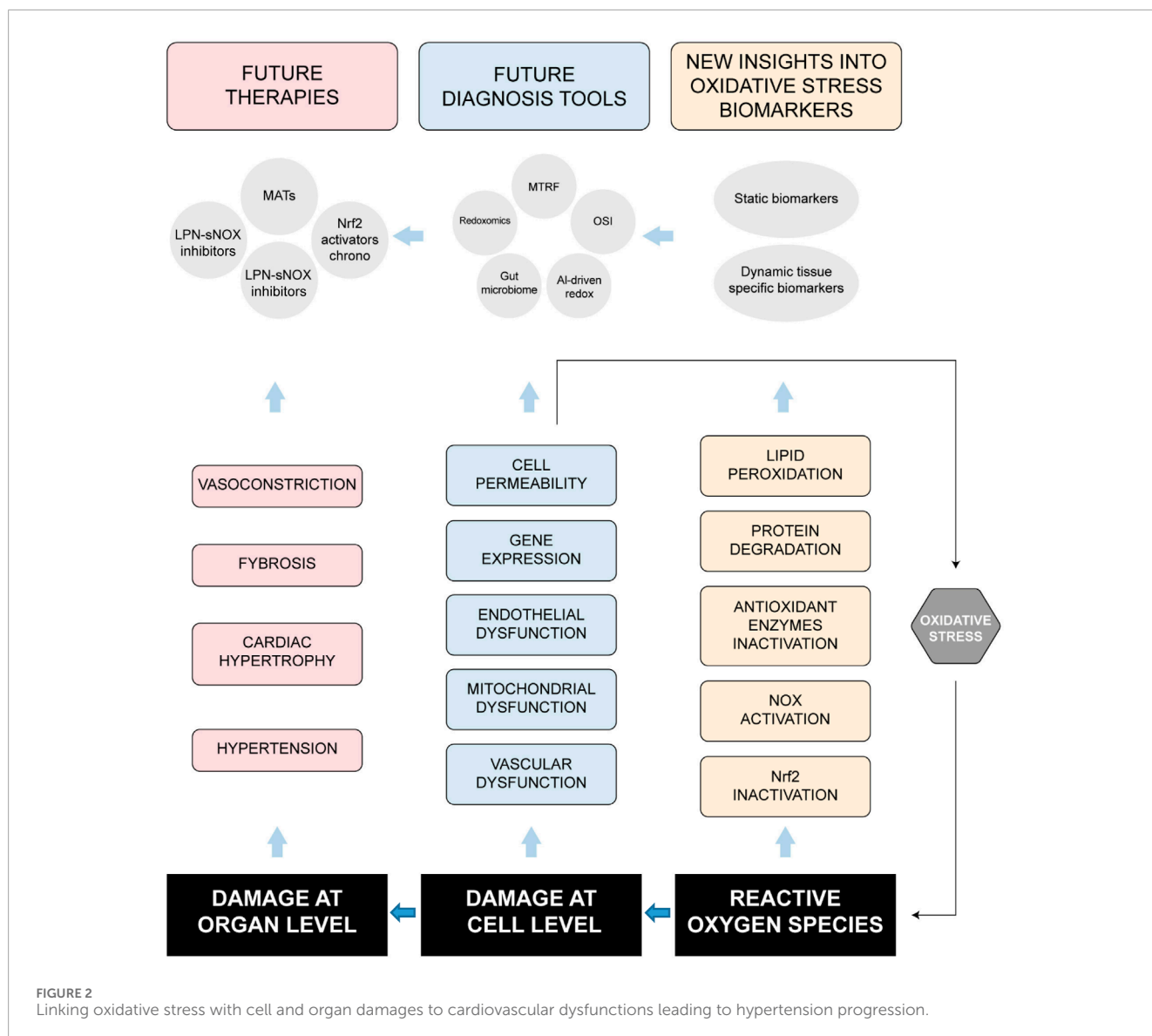
BP-lowering effects of fish oil, which is rich in eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) (Engler, 2017). Research has examined the impact of several doses of OFAs on HTN in clinical trials, ranging from 2 to 4 g per day. A meta-analysis of 36 randomized clinical trials found that supplementation with fish oil, providing an average of 3.7 g per day of EPA and DHA, resulted in a reduction of systolic BP (SBP) by 2.1 mm Hg and diastolic BP (DBP) by 1.6 mm Hg. (Miller et al., 2014). A recent review has connected various clinical events to redox factors and OS in cardiovascular pathophysiology, providing evidence for new pharmacological therapies such as OFAs, non-selective beta-blockers, and microRNAs (Fariás et al., 2017). Polyphenol-rich extracts (e.g., curcumin, resveratrol) improve endothelial function in early-stage diabetes, likely due to their pleiotropic effects beyond mere ROS scavenging (Mohd-Nor et al., 2022). N-acetylcysteine (NAC) may benefit patients with severe oxidative damage (e.g., diabetic nephropathy) by replenishing glutathione (Drózd et al., 2023).

Given the variability in individual redox status, future strategies should consider measuring OS biomarkers (e.g., MDA, SOD, GPx) to identify patients who may benefit from supplementation; combining antioxidants with lifestyle changes (e.g., Mediterranean diet + CoQ10) for synergistic effects (González-Guardia et al., 2015); and avoiding blanket supplementation in patients with adequate redox balance to prevent interference with physiological ROS signaling (Sies et al., 2024; Nuñez-Selles et al., 2019).

While lifestyle modifications remain the cornerstone of redox balance restoration in HTN and DM, targeted antioxidant supplementation may be justified in selected cases. Future research should focus on personalized antioxidant therapy guided by biomarker profiling to optimize clinical outcomes.

5 Oxidative stress and hypertension

Recent reviews have examined OS and the sources of ROS concerning HTN (Kreutzmann et al., 2023; Fountoulakis et al., 2025) as well as antioxidant therapeutic approaches (Młynarska et al., 2024; Kumar et al., 2023; Pari et al., 2024). Several *in vitro* and *in vivo* studies suggest that ROS activate specific molecular mechanisms that ultimately lead to elevated BP levels (Tain and Hsu, 2022). The role of OS in HTN remains a “chicken-or-egg” debate. Preclinical studies have shown a genetic deletion of antioxidant enzymes (e.g., SOD, GPx) inducing HTN in mice (Carlström et al., 2010), whereas human data led to elevated OS biomarkers (e.g., F2-isoprostanes) preceding HTN onset in normotensive individuals (Rodrigo et al., 2021). These findings suggest that OS may be a cause of HTN. However, there is also evidence indicating that OS can be a consequence of HTN, particularly due to mechanical stress from high BP activating reactive ROS-producing enzymes, such as NOXs (Camargo et al., 2025). Additionally, antioxidants like vitamin C have been shown to lower BP in individuals with pre-HTN, but they do not have the same effect in those with established HTN (Camargo et al., 2025). Therefore, OS is both a trigger and an amplifier of HTN, which creates a vicious cycle. A summary of how ROS are related to HTN at the cell and organ levels is shown in Figure 2.



Clinical studies have reported controversial outcomes despite support from basic research and pre-clinical studies (Table 1). This may be due to the complex pathophysiological nature of ROS signaling in humans with comorbidities. (Egea et al., 2017). The importance of antioxidant therapy in managing OS-induced HTN has been emphasized in a review of various preclinical studies and clinical trials (Amponsah-Offeh et al., 2023; Tenório et al., 2018). These studies highlight the significance of using antioxidants in HTN management (Ahmad et al., 2017). ROS are produced during normal cellular metabolic processes, while antioxidants work to eliminate these oxidants when they are in excess and repair the damage caused by ROS (Vakifahmetoglu-Norberg et al., 2017). Under normal physiological conditions, intracellular OS results from the abnormal production of ROS during typical mitochondrial respiration. It can also occur during the reperfusion of hypoxic tissue and concerning infection and inflammation (Sekhon et al., 2017). The excessive production of ROS has been associated with

endothelial injury and contributes to both extracellular and intracellular OS (Incalza et al., 2017).

The efficacy of antioxidant therapy in HTN depends on the HTN degree (I, II, or III). It has been demonstrated that OS was not present in HTN-I patients, and significantly, it was lower as compared to a healthy control group in an observational study with 355 subjects (Nuñez-Sellés et al., 2019). However, OS was moderate or severe for HTN-II and HTN-III, respectively. Most of the HTN-I patients had a combination treatment (ACE inhibitor + statin), which has been reported to have antioxidant effects (Profumo et al., 2014). In mild HTN, endogenous antioxidant enzymes were not decreased, and thus antioxidant therapy in early stages of HTN should not be a critical issue. Nevertheless, antioxidant supplementation would be recommended for HTN-II and HTN-III patients.

Certain medications used to treat HTN, such as ACE inhibitors and angiotensin (Ag II) receptor AT1 antagonists, have shown antioxidant properties. These antioxidant effects may partially

TABLE 1 Clinical trials of antioxidant therapies in hypertension (2014–2024).

Intervention	Trial	Population	Key findings	Limitations	References
Coenzyme Q10	Q-SYMBIO	Chronic HF + HTN (n = 420)	↓ SBP by 11 mmHg (p < 0.01)	Small sample size	Mortensen et al. (2014)
Vitamin C + E	HOPE-TOO	High-risk CVD (n = 7,030)	No reduction in BP or CVD events	Non-targeted approach. Broad spectrum	Młynarska et al. (2024)
Resveratrol	Meta-analysis	HTN (n = 407)	No reduction in BP or CVD events	No long-term follow-up	Fogacci et al. (2019)
Allopurinol	CKD-FIX	HTN + CKD (n = 369)	No BP change despite ↓ uric acid	Off-target effects	Badve et al. (2020)
Omega-3 FAs	STRENGTH	HTN + Hypertriglyceridemia	No BP benefit (4 g/day)	Confounded by lipid effects	Nicholls et al. (2020)
Melatonin	MEL-HT	Nocturnal HTN (n = 45)	↓ Nighttime SBP by 7 mmHg (p = 0.01)	Small, single-center trial	Ramos-Gonzalez et al. (2023)
N-Acetylcysteine	NAC-HYP	HTN-II (n = 120)	↓ MDA No BP change	Lack of hard endpoints	Gutziet et al. (2021)
MitoQ	MITO-HTN	Resistant HTN (n = 60)	Improved FMD ↑ 2.1% (p = 0.03)	Short duration (8 weeks)	Pang et al. (2025)
Sulforaphane	SFN-HTN	Diabetic HTN (n = 90)	↓ DBP by 5 mmHg ↑ Nrf2 activity (p < 0.05)	Biomarker-only focus	Mangla et al. (2021)
Polyphenol-rich olive oil	PREDIMED-Plus	Metabolic syndrome (n = 6,874)	↓ SBP by 3.2 mmHg (MedDiet + EVOO, p = 0.02)	Lifestyle co-interventions	Rosello et al. (2024)

HTN: hypertension, BP: blood pressure, SBP: systolic blood pressure, DBP: diastolic blood pressure, FMD: Flow-Mediated Dilation, CVD: cardiovascular, CKD: chronic kidney disease, MDA: Malonyl dialdehyde.

contribute to the overall benefits that these drugs provide in managing HTN. Ang II activates AT1 receptors, which can lead to various effects on vascular cells, including vasoconstriction, cell growth, and the induction of pro-inflammatory cytokines, as well as pro-fibrogenic actions (Silva-Velasco et al., 2024). Furthermore, Ang II stimulates the production of ROS within cells by activating NOXs. The production of ROS induced by Ang II is crucial in the development of HTN (Masi et al., 2019). As a result, Ang II is regarded as one of the most significant triggers of OS, both locally and systemically. Therefore, it is not surprising that both ACE inhibitors and AT1 antagonists are effective in reducing OS on various cardiovascular diseases (Ajoolabady et al., 2024).

5.1 Endothelial dysfunction

Endothelial dysfunction and OS are inextricably linked in the pathophysiology of HTN, leading to vascular damage and elevated BP. The vascular endothelium, a critical regulator of vascular tone, becomes dysfunctional when excessive ROS impairs nitric oxide (NO) bioavailability, a hallmark of HTN. Key sources of ROS include NOXs activation, uncoupled endothelial nitric oxide synthase (eNOS), and mitochondrial dysfunction, all of which are exacerbated by hypertensive stimuli such as Ang II and mechanical shear stress. When ROS (e.g., O₂•⁻) scavenges NO, vasodilation

is compromised, leading to increased peripheral resistance. Additionally, ROS promotes pro-inflammatory signaling (e.g., NF-κB activation) and endothelial adhesion molecule expression (e.g., VCAM-1, ICAM-1, TNF-α), further exacerbating vascular stiffness and atherosclerosis (Zeng and Yang, 2024). OS also reduces tetrahydrobiopterin (BH4), a critical cofactor for eNOS, causing it to produce more ROS instead of NO, thereby amplifying endothelial injury (Hernandez-Navarro et al., 2024). Clinically, this manifests as impaired flow-mediated dilation (FMD) and elevated biomarkers like asymmetric dimethylarginine (ADMA), an endogenous eNOS inhibitor (Kavurma et al., 2022). Therapeutic strategies targeting this axis (Figure 2), such as BH4 supplementation, NOX inhibitors, and lifestyle modifications (e.g., exercise, Mediterranean diet), aim to restore redox balance and endothelial function, though their efficacy varies by HTN stage and comorbidities. Thus, endothelial OS is both a driver and consequence of HTN, making it a pivotal target for interventions aimed at breaking the cycle of vascular dysfunction.

5.2 Mitochondrial dysfunction

Mitochondrial dysfunction plays a pivotal role in the pathogenesis of HTN-related OS, acting as both a major source and a vulnerable target of ROS in the cardiovascular system. In

hypertensive states, mitochondria generate excessive ROS due to electron transport chain (ETC) disruption, particularly at complexes I and III, coupled with reduced antioxidant defenses (e.g., Mn-SOD) (Grossini et al., 2025). This oxidative burden is exacerbated by Ang II, which promotes mitochondrial fission via dynamin-related protein 1 (Drp1) and inhibits mitophagy, leading to the accumulation of damaged, ROS-producing mitochondria (Tábara et al., 2025). Concurrently, mitochondrial ROS (mtROS) amplifies vascular dysfunction by oxidizing BH4, uncoupling endothelial eNOS, and reducing NO bioavailability while also activating pro-inflammatory pathways (e.g., NLRP3 inflammasome) and stimulating NOX activity, creating continuous oxidative damage (Penna and Pagliaro, 2025). mtROS further contributes to vascular remodeling by inducing smooth muscle cell proliferation and endothelial apoptosis via oxidative DNA damage (8-OHdG) and lipid peroxidation (MDA). Importantly, mitochondrial DNA (mtDNA) mutations, which accumulate with age and HTN, perpetuate this dysfunction, rendering the vasculature more susceptible to oxidative injury. Therapeutic strategies targeting mitochondrial OS, known as mitochondrial-targeted antioxidants (MTAs) such as MitoQ, are modulators and promoters of mitochondrial biogenesis (PGC-1 α activators), which have shown promise in preclinical HTN models (Halling et al., 2019).

Within the therapeutic tools proven effective, Elamipretide (SS-31) is one of the most promising. Elamipretide is a cell-permeable tetrapeptide that selectively accumulates in mitochondria, binds cardiolipin, stabilizes inner-membrane structure, inhibits excessive ROS production, and preserves ATP synthesis. In preclinical heart-failure models, elamipretide improved left ventricular function and attenuated remodeling. (Tung et al., 2025). Animal studies demonstrated cognitive protection and reduced inflammation in models of chronic sleep deprivation and DM2 (Zhang et al., 2024). Early clinical studies in Barth syndrome, primary mitochondrial myopathies, and age-related macular degeneration have shown favorable safety, tolerability, and signals of efficacy (Thompson et al., 2024). So far, clinical trial data for elamipretide and bardoxolone underscore the viability of mechanism-driven therapies in treating OS-related diseases (Karaa et al., 2024). These results justify ongoing Phase III trials exploring elamipretide's therapeutic potential.

Evidence about the role of mtROS has led to the development of MATs for therapeutic intervention in several diseases, including HTN -Figure 2 (Jiang et al., 2020; Colon-Hidalgo et al., 2022). MitoQ has shown promising preventive benefits for several metabolic disorders, including peripheral arterial HTN (Pool et al., 2021), whereas MitoTEMPO can scavenge mtROS, reduce oxidative damage to mitochondria, restore mitochondrial function, and help maintain normal cellular function (Chang et al., 2024). Clinical translation for using MTAs requires addressing challenges like tissue-specific delivery and long-term safety. Thus, mitochondrial dysfunction represents a critical hub in HTN-associated OS, linking metabolic, inflammatory, and vascular pathways, and its modulation offers a novel avenue for precision therapies in resistant HTN.

5.3 NADPH oxidases (NOXs)

NOXs are the only known enzymes specifically responsible for generating ROS (Bedard and Krause, 2007). There are seven human

NOX enzymes identified: NOX1, NOX2, and NOX3, which are regulated by various cellular proteins; NOX5 and DUOXs, which are activated by calcium due to additional calcium-binding domains; and NOX4, the only constitutively active member of this family (Chocry and Leloup, 2020). NOXs play a crucial role in coordinating cell damage, stress responses, and tissue regeneration. High levels of ROS, resulting from the hyperactivity of NOXs, can lead to genetic instability. This instability can cause excessive cellular proliferation, activation of the DNA damage response, proliferative senescence, and apoptosis (Ogrunc et al., 2014). Ang II plays a crucial role in the progression of HTN and is one of the most significant regulators of NOX activity in vascular cells. Consequently, researchers have extensively investigated the role of these enzymes in Ang II-dependent HTN (Pinheiro and Oliveira-Paula, 2020). Developing selective NOX inhibitors has been quite challenging due to the high degree of similarity in the core catalytic domains of these enzymes, which makes it difficult to achieve selectivity for specific isoforms. Current research trends in NOX inhibitors focus on creating compounds that are both highly effective and specific to individual isoforms, as shown in Figure 2 (Dustin et al., 2023).

5.4 The nuclear factor erythroid 2-related factor 2 (Nrf2)

The Nrf2 pathway serves as a critical endogenous defense mechanism against OS in HTN, orchestrating the transcription of antioxidant and cytoprotective genes that counteract vascular dysfunction (Lal et al., 2024). Under physiological conditions, Nrf2 remains sequestered in the cytoplasm by its inhibitor, the Kelch-like ETC-associated protein 1 (Keap1), but oxidative or electrophilic stress triggers Nrf2 dissociation, nuclear translocation, and binding to antioxidant response elements (AREs), upregulating genes such as heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQO1), and glutathione S-transferases (GSTs) (Cuadrado et al., 2019). In HTN, chronic activation of Ang II and NOXs overwhelms this system, leading to Nrf2 dysfunction or exhaustion, which exacerbates ROS accumulation, endothelial dysfunction, and vascular remodeling. Sulforaphane from cruciferous vegetables (e.g., broccoli), bardoxolone methyl, and dimethyl fumarate have demonstrated promise in preclinical models by restoring redox balance, reducing BP, and attenuating end-organ damage effects attributed to enhanced HO-1-mediated vasodilation, reduced NOX activity, and suppression of pro-inflammatory NF- κ B signaling (Alves et al., 2025; Simões e Silva, 2025; Jindam et al., 2024).

Unlike direct antioxidants, Nrf2 activators amplify endogenous defenses, offering sustained protection. The pivotal role of the Nrf2-Keap1 signaling pathway in maintaining redox, metabolic, and protein homeostasis, as well as regulating inflammation responses, has been discussed (Dinkova-Kostova and Copple, 2023). It was highlighted that inducible activation of Nrf2 promotes cytoprotective mechanisms against various immune, neurodegenerative, and metabolic disorders characterized by OS and inflammation. Novel prodrugs of monomethyl fumarate have achieved site-specific release at OS loci via Baeyer-Villiger oxidation, minimizing systemic side effects while activating Nrf2 locally (Avery et al., 2024).

However, the therapeutic potential of Nrf2 activation is tempered by paradoxical risks; prolonged overactivation may promote oncogenic pathways or, as seen in clinical trials with bardoxolone methyl, adverse renal outcomes (Pergola et al., 2021). Genetic polymorphisms in Keap1 or Nrf2 further influence individual susceptibility to HTN and response to therapy, highlighting the need for personalized approaches (Begum and Lakshmanan, 2023). While the Nrf2 pathway represents a compelling target for mitigating OS in HTN, its dual-edged nature requires careful modulation to harness its protective effects without triggering unintended consequences.

6 Oxidative stress and diabetes mellitus

OS in DM operates as both a causal driver and consequential amplifier of disease progression, accelerating metabolic dysfunction and tissue damage in a similar way as in HTN (Caturano et al., 2025). Chronic hyperglycemia fuels excessive ROS production through multiple pathways, including mitochondrial ETC leakage, NOX activation, and advanced glycation end products (AGE)-mediated RAGE signaling. These ROS directly impair pancreatic β -cell function by suppressing insulin gene expression (via PDX-1 inactivation) and triggering apoptosis through JNK and NF- κ B pathways, thereby reducing insulin secretion (Kaneto et al., 2022). Simultaneously, ROS disrupts insulin signaling in peripheral tissues by promoting IRS-1 serine phosphorylation and degrading phosphatidylinositol-3-kinase (PI3K)/Akt activation, leading to insulin resistance (Papaetis et al., 2025). Conversely, the resulting hyperglycemia and dyslipidemia further exacerbate OS through four key mechanisms: (1) the polyol pathway, where glucose conversion to sorbitol depletes NADPH and glutathione; (2) AGE-RAGE interactions that activate pro-inflammatory cascades and additional ROS generation; (3) protein kinase C (PKC) activation, which upregulates NOX isoforms; and (4) lipid peroxidation, where free fatty acids (FFAs) overload mitochondrial β -oxidation, generating cytotoxic aldehydes like 4-hydroxynonenal (4-HNE) (Młynarska et al., 2024). This bidirectional relationship is clinically evident in the early elevation of OS biomarkers (e.g., urinary 8-OHdG, serum F2-isoprostanes) preceding overt DM and their correlation with disease severity and complications (e.g., retinopathy, nephropathy). Therapeutic strategies targeting this cycle—such as Nrf2 activators (sulforaphane), mitochondrial antioxidants (MitoQ), and AGE inhibitors (aminoguanidine)—highlight the dual role of OS as interventions early in DM, which can improve β -cell function, while later-stage treatments primarily mitigate complications. Thus, OS in DM represents a paradigm of metabolic memory, where initial oxidative damage perpetuates dysfunction long after glycemic control is achieved, underscoring the need for early redox-targeted therapies.

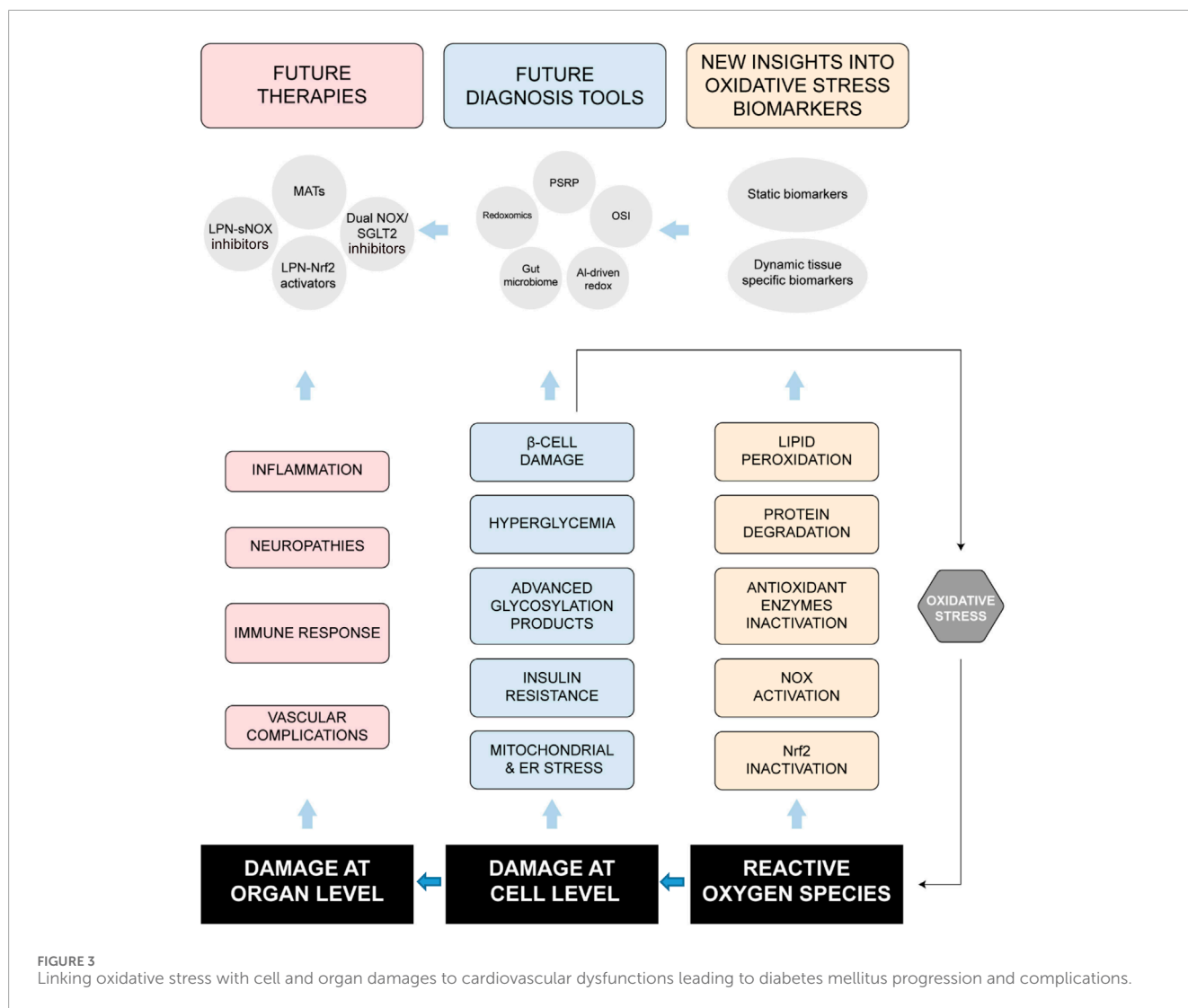
Some researchers suggest that acute spikes in plasma glucose trigger this stress (Papachristoforou et al., 2020), while others attribute it to glycemic variability (Rehman and Akash, 2017). If antioxidant levels are adequate, oxidative damage to key biomolecules in type 2 DM (DM2) may be minimized, potentially leading to improved outcomes. This has led to the proposal of investigating antioxidant supplementation as an adjunct therapy to

reduce oxidative stress and help to prevent vascular complications (Nuñez-Selles et al., 2019; Choi and Ho, 2018).

The effective management of blood glucose levels in individuals with DM2 relies on an integrative approach. This approach includes patient education, lifestyle modifications, pharmacotherapy, and vigilant monitoring for potential complications (Garber et al., 2021). Despite these guidelines, the burden of DM2 remains significant, posing considerable challenges for affected individuals and straining the healthcare system. As a result, developing new therapeutic strategies for preventing and managing DM2 continues to be a key area of research. One particular focus is the role of OS and inflammation. Research has shown that chronic hyperglycemia leads to OS and inflammation, which contribute to the onset and progression of DM2, as well as its associated complications, including renal and cardiovascular diseases (Pickering et al., 2018). One of the primary research challenges is reducing OS to alleviate diabetes symptoms. However, the insufficient evidence supporting the beneficial effects of antioxidants in preventing OS-related diseases has prompted the development of new strategies. One promising avenue involves the use of new inhibitors that target major ROS-producing systems, such as HO-1 expression. This approach offers an alternative to traditional antioxidant therapies for treating endothelial dysfunction (Arad et al., 2020). As a result, Nrf2 increases the expression of various well-known antioxidant markers, such as glutathione, HO-1, SOD, and NADPH quinone reductase (Paunkov et al., 2019). A summary of how ROS are related to DM is shown in Figure 3.

Clinical studies have reported controversial results despite the support from preclinical studies (Table 2). Several gene-therapy strategies aimed at countering OS in DM2. In an extended review, it was highlighted that some preclinical gene-therapy approaches delivering exogenous antioxidant enzymes, via viral vectors or nanozyme platforms, in animal models of hyperglycemic bone defects, significantly reduced inflammation, limited ROS-mediated damage, and improved healing outcomes (Li et al., 2024). A special gene therapy approach, that delivers treatment directly to heart and blood vessel cells, uses harmless viruses called AAVs (Adeno-Associated Viruses) as delivery vehicles to carry therapeutic genes into target cells (Weeks and Bernardo, 2025). Current research focuses on improving delivery precision to specific cell types, enhancing treatment effectiveness, and ensuring safety for clinical use. One main aspect is the choice of AAV serotype and capsid engineering to reach natural tropism. Serotypes such as AAV1, AAV6, AAV8, and especially AAV9 have demonstrated efficient cardiac transduction in small and large animals, enabling robust gene transfer to myocardium with intracoronary or intravenous delivery (Mazurek et al., 2024).

Another approach is the management of calcium-handling proteins, which have had variable results (Khan et al., 2025). A cardiac-bridging integrator 1 (cBIN1) delivered with AAV9 reversed chronic ischemic heart failure in canine models, improving survival and contractility. S100A1, another calcium-binding protein, has regulated cardiac contractility and calcium-handling. Its downregulation is linked to heart failure and hypertrophy. Anti-remodeling targets acting over regulating genes of the S100A1, like phospholamban-targeted shRNA, and antioxidant enzymes (e.g., CAT) are under preclinical evaluation for preventing pathological hypertrophy and fibrosis. A study highlighted



S100A1's ability to restore calcium cycling in cardiomyocytes, improving contractility in heart failure models, and gene therapy using AAV-S100A1 showed reduced fibrosis and hypertrophy in rodents (Rostami et al., 2025).

6.1 β-Cell damage by ROS

Pancreatic β-cells exhibit a unique vulnerability to OS due to their inherently low expression of antioxidant enzymes (SOD, CAT, and GPx) and their high metabolic activity, which generates substantial ROS under hyperglycemic conditions (Eguchi et al., 2021). Several conditions leading to ROS generation in β cells have been proposed, among which are hyperglycemia, hyperlipidemia, hypoxia, and endoplasmic reticulum (ER). Hyperglycemia, the defining diabetes, can be directly associated with increased ROS generation through a variety of mechanisms (Singh et al., 2025).

Chronic hyperglycemia drives mitochondrial overstimulation, leading to excessive electron leakage at complexes I and III of the ETC, producing superoxide anions ($O_2^{\bullet-}$). These ROS trigger

a cascade of detrimental effects: (1) transcriptional suppression of insulin via oxidative inactivation of PDX-1 and MafA, key regulators of insulin gene expression (Guo et al., 2023); (2) ER stress, where unfolded protein response (UPR) pathways (PERK, IRE1α, ATF6) shift from pro-survival to pro-apoptotic signaling (Eizirik et al., 2020); and (3) direct DNA damage, evidenced by elevated 8-OHdG in diabetic islets (Duni et al., 2017). Additionally, ROS activates stress-sensitive kinases (JNK, p38 MAPK) that phosphorylate IRS-2, accelerating its proteasomal degradation and further impairing β-cell survival (Liu et al., 2020). The resulting β-cell dysfunction manifests clinically as impaired glucose-stimulated insulin secretion (GSIS) and, ultimately, apoptosis—a process confirmed in human autopsies showing ca. 40% reduced β-cell mass in DM2 (Maheshvare et al., 2023).

Recent single-cell RNA sequencing has identified a subset of ROS-resilient β-cells that upregulate SIRT3 and FOXO1, suggesting potential therapeutic targets for preservation (Nie et al., 2024). Additionally, it has been reported that OS affects epigenetic regulatory mechanisms involved in the regulation of pancreatic β cell survival and insulin secretion, like DNA methylation, chromatin architectural modification, and non-coding RNA (Kowluru and

TABLE 2 Clinical trials of antioxidant therapies in diabetes mellitus (2014–2024).

Therapy	Trial	Population	Key outcomes	Limitations	References
Alpha-Lipoic Acid	ALA/Neuropathy	DM2 with neuropathy (n = 72)	Reduced pain and disability scores by more than 30% (p < 0.01)	Small sample size	Agathos et al. (2018)
MitoQ (Mitochondrial-targeted)	MitoQ	DM2 (n = 169)	Protective role on vasculature (inflammation markers and prevention of neuropathy)	Small sample size	Escribano-Lopez et al. (2016)
Sulforaphane (Nrf2 activator)	SFN-DM2	DM2 (n = 97)	↓ HbA1c by 0.5% (p = 0.04); ↑ Nrf2 activity	Short duration (12 weeks)	Axxelson et al. (2017)
Coenzyme Q10	CoQ10-DPP4	DM2 (+metformin) (n = 120)	Improved insulin sensitivity (HOMA-IR ↓18%, p < 0.05)	No CV outcome data	Dludla et al. (2020)
Bardoxolone Methyl	TSUBAKI	Diabetic CKD (n = 121)	eGFR ↑5.1 mL/min (p < 0.01); albuminuria ↓32%	Discontinued due to safety concerns	Pergola et al. (2021)
Resveratrol	RESDIAB	Prediabetes (n = 75)	↓ Fasting glucose by 0.8 mmol/L (p = 0.02)	No long-term follow-up	Sattarinezhad et al. (2019)

DM2: type 2 diabetes, ALA: alpha-lipoic acid, Nrf2: Nuclear Factor Erythroid 2-Related Factor 2, CKD: chronic kidney disease, HbA1c: Glycosylated Haemoglobin, HOMA-IR: homeostasis model assessment of insulin resistance.

Mishra, 2017). The application of CRISPR/Cas9 technology on the targeted epigenome would be able to differentiate cell types to improve insulin production (Dinić et al., 2022). These findings underscore OS as a central mediator of β-cell failure in diabetes, highlighting the need for targeted antioxidant strategies (see Figure 3) that mitigate ROS without disrupting physiological redox signaling (Zaher and Stephens, 2025).

6.2 Hyperglycemia-induced oxidative stress pathways

Glycated hemoglobin (HbA1c), which averages blood sugar levels over the previous two to 3 months within total red blood cell hemoglobin, is a measure of the degree of glucose’s natural binding to hemoglobin (glycation). In persons without diabetes, it is typically 5.7% or less. The percentage of HbA1c rises when blood sugar levels increase over time. Elevated HbA1c promotes the intraerythrocytic generation of highly reactive free radicals (AGEs), which alter membrane functions and those of different cellular systems by binding to RAGE and producing cytokines and intracellular glyco-OS (Núñez-Musa, 2024). By causing hyper-aggregation and hyper-viscosity, which ultimately lead to inflammation and atherogenic events, these results exacerbate endothelial damage (Nabi et al., 2019). Furthermore, changes in the metabolism and biodisposition of intracellular iron are brought about by the oxidative phenomena, resulting in the production of ferryl-hemoglobin. This more unstable substance subsequently triggers conversion processes into hemoglobin multimers. These multimers promote vascular intima injury, which increases endothelial permeability, monocyte adhesion, and macrophage accumulation, leading to plaque development (Bozza and Jeney, 2020). In general terms, the

pathophysiological cascade that arises from non-enzymatic protein glycosylation linked to persistent hyperglycemia determines the substrate for the cardiovascular effects of DM.

The polyol pathway consumes up to 30% of intracellular glucose under hyperglycemic conditions, with aldose reductase converting glucose to sorbitol in an NADPH-dependent reaction that depletes this critical cofactor required for glutathione reductase activity (Gupta, 2024). This glutathione depletion impairs cellular antioxidant capacity, leaving cells vulnerable to ROS accumulation. The formation of AGEs increases 3-5-fold in diabetes, as glucose-derived carbonyl groups nonenzymatically modify proteins and lipids; these AGEs then bind to RAGE receptors, activating NOX enzymes and generating O₂•⁻ while triggering pro-inflammatory NF-κB signaling (Yan et al., 2023). The PKC pathway becomes activated through hyperglycemia-induced increases in diacylglycerol (DAG), with PKC-β and PKC-δ isoforms particularly implicated in stimulating NOX activity while inhibiting AMPK-mediated antioxidant responses (Geraldese and King, 2018). Finally, excessive glucose flux through the hexosamine pathway leads to UDP-GlcNAc accumulation and subsequent O-GlcNAcylation of serine/threonine residues on antioxidant enzymes, like SOD, reducing their activity (Paneque et al., 2023). These pathways converge to create a state of chronic OS that further exacerbates insulin resistance and β-cell dysfunction, and plasma F2-isoprostanes (markers of lipid peroxidation) correlate strongly with HbA1c levels (Tang et al., 2018). This oxidative milieu persists even after glucose normalization, contributing to the phenomenon of “metabolic memory” observed in clinical trials, where early intensive glycemic control provides long-term protection against complications (Yang et al., 2024). Recent studies using isotopic tracer techniques have quantified the relative contributions of these pathways, revealing that mitochondrial O₂•⁻ production initiates

about 45% of hyperglycemia-induced damage, while PKC activation and AGE formation account for 30% and 25%, respectively (Chronic Complications of Diabetes Mellitus, 2024; Pardina et al., 2024). This quantitative understanding is now driving the development of pathway-specific inhibitors, such as PKC- β selective antagonists, SGLT2 inhibitors, and glyoxalase-1, which have shown promise in preclinical models for breaking this cycle without globally suppressing physiologically important ROS signaling -Figure 3 (Oliveira et al., 2023; Klen and Dolžan, 2023; Xing et al., 2024).

6.3 Oxidative stress and insulin resistance

The Insulin Resistance (IR) explains the failure of muscles, fat, and liver to respond well to insulin and their incapacity to take up glucose from the blood. To compensate, the pancreas increases insulin production, but cannot achieve positive results, and a progressive disorder of lipid and glucose metabolism takes place. The IR usually progresses in silence and manifests clinically years after being present in the form of hyperglycemia, favoring atherogenesis and plaque progression in a curvilinear relationship with vascular disease, even in the absence of hyperglycemia (Beverly and Budoff, 2020). OS is a critical link between metabolic overload and IR through multiple interconnected mechanisms that impair insulin signaling at various levels. At the cellular level, ROS directly modify key components of the insulin signaling cascade, including insulin receptor substrate (IRS) proteins and the insulin receptor itself. H_2O_2 and $O_2^{\bullet-}$ activate stress-sensitive serine/threonine kinases such as JNK, IKK β , and PKC θ , which phosphorylate IRS-1 at inhibitory serine residues (Ser307, Ser612), disrupting its interaction with the insulin receptor and promoting proteasomal degradation (Rodén et al., 2017). Lipid peroxidation products like 4-HNE form covalent adducts with cysteine residues in the insulin receptor β -subunit, reducing its tyrosine kinase activity by up to 60% (Furukawa et al., 2017). Mitochondrial dysfunction in skeletal muscle, characterized by reduced ATP synthesis rates and increased electron leak, generates excessive ROS that activates PKC ϵ , leading to impaired insulin-stimulated GLUT4 translocation (Regina and Tong, 2025). Adipose tissue contributes to systemic OS through NOX4 activation and reduces adiponectin secretion, creating a pro-inflammatory milieu that further exacerbates insulin resistance (Den Hartigh et al., 2017). OS also induces ER stress, activating the UPR and its downstream effector XBP-1, which suppresses insulin receptor expression (Zhang et al., 2025).

Sustained elevated glucose levels favor an imbalance between the availability of endothelial NO and the accumulation of ROS. This imbalance triggers a chain of pathophysiological events that lead to enzymatic dysfunction in endothelial homeostasis, inflammation, and cell proliferation. More particularly, a strong activation of PKC in the endothelium of diabetic patients is observed in the increased generation of ROS and microvascular OS (Nuñez-Musa, 2024), and vasoconstriction potentiates and insulin signaling peripherally impairs (Robson et al., 2018). With greater and more extensive experiences, PKC is a possible therapeutic target for the treatment of vascular complications in diabetics. Inflammatory factors, in addition to OS, play a significant role in the development of diabetes by promoting IR through the alteration of β -cell function and interference with insulin signaling (Oguntibeju, 2019). Given the

crucial role of interleukins in mediating inflammation through both pro- and anti-inflammatory properties, various interleukins have been investigated in DM2 patients (Alfadul et al., 2022; Yousef et al., 2023). OS and inflammation contribute to endothelial dysfunction through the inactivation of NO, which increases the risk of DM2, primarily through the renin-angiotensin-aldosterone system (Martyniak et al., 2024). These findings highlight OS and inflammation as both a cause and consequence of IR, creating a cycle that can be interrupted by targeted antioxidant approaches and/or lifestyle interventions that enhance endogenous antioxidant capacity (see Figure 3).

6.4 Oxidative stress and diabetic vascular complications

OS plays a central role in the pathogenesis of diabetic vascular complications through multiple interconnected pathways that promote endothelial dysfunction, vascular inflammation, and extracellular matrix remodeling. Chronic hyperglycemia-induced ROS overproduction, primarily derived from mitochondrial ETC leakage and NOX activation, leads to eNOS uncoupling due to BH4 oxidation, reducing NO bioavailability and impairing vasodilation (Giacco and Brownlee, 2010). Simultaneously, ROS activate PKC isoforms and the AGE-RAGE signaling cascade, which upregulate pro-inflammatory cytokines (TNF- α , IL-6) and adhesion molecules (VCAM-1, ICAM-1) through NF- κ B activation (González et al., 2023). In the macro-vasculature, OS accelerates atherosclerosis by promoting LDL oxidation, macrophage foam cell formation, and plaque instability via matrix metalloproteinase (MMP) activation (Caturano et al., 2025). Microvascular complications are equally driven by oxidative damage i.e., in diabetic retinopathy, ROS-induced VEGF overexpression through HIF-1 α stabilization leads to pathological angiogenesis (Kowluru and Mishra, 2017), while in diabetic nephropathy, podocyte injury results from NOX4-derived $O_2^{\bullet-}$ and mitochondrial ROS-mediated apoptosis (Jha et al., 2024). Clinical studies demonstrate strong correlations between OS markers and vascular complications including diabetic kidney disease (Forbes and Thorburn, 2018; Caturano et al., 2025).

Furthermore, due to a decrease in their destruction rate and/or a reduction in the production of CAT, SOD, and GPx, excess ROS promotes the deleterious changes associated with OS. Ultimately, tissues are more vulnerable to OS and, consequently, to diabetic complications, especially vascular problems, which are the main cause of comorbidity and mortality in DM2. Several challenges need to be considered while creating customized antioxidant treatments. These include gene therapy based on Mn-SOD used for systemic or organ-specific applications to protect tissues from OS-related damage (Greenberger et al., 2021) and antioxidant enzyme gene transfer, which has been investigated as a potential method to deliver antioxidant enzymes (Lombardi et al., 2017; Anwar et al., 2024).

Emerging therapeutic strategies aim to target these pathways specifically like MATs, NOX inhibitors, and Nrf2 activators, though clinical success has been limited by off-target effects and the dual role of ROS in physiological signaling (Pergola et al., 2021). New strategies include nanoparticle-based antioxidant delivery to vascular tissues and personalized redox therapies guided by genetic and biomarker profiling (Tang et al., 2024). These advances

emphasize the necessity for precise interventions in time and space that reduce harmful oxidative stress while maintaining beneficial redox signaling in diabetic blood vessels (Figure 3).

Pant et al. (2023) have reviewed the present status of long non-coding (lnc) RNA as a diagnostic biomarker and potential regulator of OS in vascular complications of DM2 and the recent advances in using MTAs in different animal models and clinical trials. They found that the expression level of lncRNA has shown a strong correlation with OS, indicating that they might modulate redox status in diabetic complications, and the emerging scientific evidence depicts that MTAs can mitigate mitochondrial OS caused by hyperglycemia in DM, providing promising OS-targeting therapeutic strategies. However, most studies to date investigating the effect of MTAs on OS have been conducted using animal models or cell cultures rather than diabetic patients. The epigenetic role in this context is not fully understood. Still, changes in DNA have been identified that could be meaningful in the identification and design of the next therapeutic strategies. It is considered that mitochondrial mutations can accumulate over time. Difficulties in this field arise due to the particularities encountered at the level of each individual. Large and complex studies are needed to identify and detail the changes at the mitochondrial level and the therapeutic approaches (Cojocaru et al., 2023).

Sodium-glucose cotransporter 2 inhibitors (SGLT2i) have demonstrated pleiotropic effects, including reducing mitochondrial ROS generation. In older patients with DM2 and HTN, an observational study correlated empagliflozin administration with decreased endothelial fragility and OS markers, attributable to reduced mitochondrial ROS production in endothelial cells (Mone et al., 2022). A 6-month controlled trial in diabetics with ischemic heart disease showed that empagliflozin attenuated both inflammation and systemic oxidative stress, resulting in clinically significant cardiovascular benefits (Gohari et al., 2022). Preclinical studies revealed that dapagliflozin activates the SIRT6–FOXO3 pathway to reduce myocardial fibrosis and oxidative damage in diabetic cardiomyopathy models (Ma et al., 2024). The use of compounds that stimulate Sirtuin and AMPK modulators SIRT1/SIRT6 (e.g., resveratrol, NMN) has shown potential to restore mitochondrial biogenesis and attenuate OS in insulin-resistant tissues (Wang et al., 2023), although robust clinical trials are lacking. Additionally, specific-purpose antioxidants introduced as potential treatment coadjutants, SOD (EUK-134) and CAT (manganese porphyrins) mimics demonstrated efficacy in animal models of diabetic nephropathy and resistant HTN opening the door to future human trials (Wilcox and Aslam, 2021).

6.5 Oxidative stress and diabetic neuropathies

Diabetic neuropathies encompass a heterogeneous group of disorders affecting multiple organ systems, all sharing OS as a common pathogenic mediator. These complications can be broadly categorized into microvascular, macrovascular, and neural manifestations. Each has distinct clinical features but shares overlapping molecular mechanisms driven by chronic hyperglycemia-induced ROS overproduction. The most prevalent form, Distal Symmetric Polyneuropathy (DSPN), results from

oxidative damage to Schwann cells and sensory neurons in peripheral nerves (Panou et al., 2025). Mitochondrial $O_2^{\bullet-}$ overproduction in dorsal root ganglia activates multiple injurious pathways, like the polyol pathway flux, which depletes NADPH, reducing glutathione regeneration (Mapanga and Essop, 2016); the AGE-RAGE signaling, which induces axonal atrophy through p38 MAPK activation (Sango et al., 2025), and the PKC- β activation, which impairs nerve blood flow via endothelin-1 overexpression (Chong and Souayah, 2025). Clinically, this manifests as progressive sensory loss (≥ 5.07 g monofilament testing) and neuropathic pain (DN4 score ≥ 4), correlating with serum 8-OHdG levels (Asim et al., 2024).

Hyperglycemia-induced mitochondrial dysfunction in neuronal and Schwann cells leads to excessive $O_2^{\bullet-}$ production at complex I and III of the ETC, which initiates a cascade of injury through four primary mechanisms (Wu L. et al., 2024). First, ROS directly modifies ion channels, oxidizing sulfhydryl groups on voltage-gated Na^+ channels, resulting in abnormal action potential propagation and contributing to the paradoxical coexistence of pain and numbness (Elafros et al., 2022). Second, OS activates the polyol pathway, depleting NADPH reserves and reducing glutathione levels, leaving nerves vulnerable to lipid peroxidation—a process evidenced by 3-fold higher levels of 4-HNE in sural nerve biopsies from diabetic patients with neuropathy compared to controls (Chong and Souayah, 2025). Third, ROS promotes microvascular insufficiency by oxidizing BH4, leading to eNOS uncoupling and reduced endoneurial blood flow, with clinical studies showing a strong inverse correlation between skin biopsy-measured intraepidermal nerve fiber density and serum 8-OHdG levels (Carnicer et al., 2021). Fourth, OS triggers inflammatory cascades through NF- κ B-mediated upregulation of TNF- α and IL-6, which further damage nerve fibers via Wallerian degeneration (Pop-Busui et al., 2016).

Cardiovascular Autonomic Neuropathy (CAN) is the most lethal form, involving oxidative damage to parasympathetic ganglia and sympathetic nerve terminals. In CAN, mROS in the sinoatrial node reduce heart rate variability, and NOX2-derived $O_2^{\bullet-}$ in vascular smooth muscle causes orthostatic hypotension (Singh et al., 2024). Late-stage CAN (Ewing score >7) carries a 5-year mortality of 50%, linked to plasma F2-isoprostanes >450 pg/mL (Pop-Busui et al., 2022). This classification underscores the need for phenotype-specific antioxidant approaches in diabetic neuropathies, moving beyond “one-size-fits-all” therapies. Future trials should incorporate redox biomarker stratification and advanced neuroimaging to match mechanisms with interventions (see Figure 3).

New therapeutic strategies focus on MATs approaches, including MitoQ, Mito-TEMPO, and nano-Mito-PBN (Pang et al., 2025; Pant et al., 2023; Wu et al., 2019); Nrf2 activators to reduce neuropathic pain, like sulforaphane (Aranda-Rivera et al., 2024), and PKC- β inhibitors, which preserved intraepidermal nerve fibers in clinical studies, like ruboxistaurin (Rendell, 2021). Mito-apocynin (Mito-Apo), a mitochondria-targeted derivative of apocynin, concentrates at the inner mitochondrial membrane to inhibit NOX2 directly, where ROS production is highest. In a lipopolysaccharide (LPS)-induced endotoxemia mouse model, oral Mito-Apo markedly reduced systemic oxidative markers (e.g., 4-HNE) and preserved mitochondrial function, while standard

apocynin showed minimal efficacy (Padhi et al., 2019). Another study has demonstrated that diapocynin exhibits a lower IC₅₀ for NOX2 inhibition (sub-micromolar) compared to parent apocynin, effectively blocking p47^{phox} translocation and attenuating endothelial superoxide generation in isolated vessel assays (Juric et al., 2023). In diabetic nephropathy models, GKT137831 reduced glomerular ROS (measured by dihydroethidium staining), decreased albuminuria by over 50%, and attenuated mesangial expansion—effects that correlated with diminished NOX4 expression in podocytes. Additionally, in bleomycin-challenged rodent lungs, setanaxib treatment cut lung hydroxyproline content by ~40%, suppressed peroxynitrite formation (assessed via EPR spin trapping), and improved compliance, demonstrating its capacity to interrupt NOX-driven fibrogenesis (Thannickal et al., 2023). Collectively, these results highlight the ability of highly engineered apocynin derivatives to specifically inhibit the NOX2 isoform at its mitochondrial source, while substances such as GKT137831 support the more general NOX inhibition strategy to reduce vascular and tissue-specific ROS in fibrotic diseases and diabetic nephropathy.

Recent advances in redox proteomics have identified novel biomarkers like peroxiredoxin-3 oxidation that predict neuropathy progression 5 years before clinical onset (Bourgonje et al., 2024), while nanoparticle-based delivery systems are being tested to selectively target antioxidants to damaged nerves without systemic side effects (De et al., 2025). These developments demonstrate the central role of OS in diabetic neuropathies and highlight promising avenues for mechanism-based therapies that could finally break the cycle of nerve injury in diabetes.

7 Clinical trial protocol design for antioxidant therapy in hypertension and diabetes

The selection of appropriate OS biomarkers is critical for assessing the efficacy of antioxidant therapies employed in the treatment of HTN and DM from the results of clinical trials. These biomarkers yield valuable insights regarding i) products of lipid peroxidation, ii) products of protein modification, iii) activities of antioxidant enzymes, and iv) DNA modifications that may influence gene expression (Mañon-Rossi et al., 2016). A significant portion of the clinical research concerning the application of antioxidants in HTN and DM has not fully incorporated information about OS biomarkers (Sies, 2020). Marrocco et al. (2017) proposed that biases resulting from independently applied methodologies could be alleviated by the utilization of OS indices that encompass multiple markers pertinent to the objectives of the clinical study and its implications for clinical practice.

Experimental evidence indicates that an accurate diagnosis of OS can enhance the efficacy of antioxidant therapies in patients suffering from HTN and DM. The Oxidative Stress Index (OSI) (Nuñez-Selles et al., 2019) offers valuable support within a rigorously designed clinical protocol aimed at optimizing the administration of antioxidant treatments. This methodology endeavors to augment the effectiveness of therapies while minimizing comorbidities when coupled with standard treatments currently utilized for these conditions. On the other hand, it is essential to focus on clinical trial endpoints related to disease progression and improvement

in antioxidant studies, as these are more meaningful than simply measuring variations in overall survival OS biomarkers or patient mortality. When designing antioxidant clinical trials in HTN and DM, it is important to demonstrate how antioxidant therapy (preferably used as an adjunct to standard treatment) can lead to improvements in BP for HTN and HbA1c levels for DM. The increasing trend of employing antioxidants in conjunction with standardized antihypertensive or antidiabetic medications appears to represent one of the most effective strategies for improving the prognosis of HTN and DM. This effectiveness must be correlated to the capacity to manage the molecular mechanisms underlying vascular function, metabolic equilibrium, and redox states through the intentional management of specific antioxidants (Sorriento et al., 2018). Additionally, implementing antioxidant therapies may play a pivotal role in addressing complications or deterioration stemming from physiological imbalances associated with AHT and DM.

Designing robust clinical trials to evaluate antioxidant therapies in HTN and DM requires addressing unique challenges, including patient heterogeneity, biomarker variability, and the dual role of OS as both cause and consequence of disease progression. An optimal protocol should incorporate stratified randomization based on OS biomarker profiles (e.g., plasma MDA, urinary 8-OHdG) to identify subgroups most likely to benefit. Control and/or placebo groups must be integrated according to the ethnic composition of the target population. One example in diabetic cohorts treated with MitoQ, the clinical protocol was designed combining DM endpoints (HbA1c and endothelial function) in a more preferable way than isolating redox-specific effects from glucose-lowering biomarkers. MitoQ improved flow-mediated dilation (FMD) by 3.1% in DM2 patients independent of glycemic changes (Carlini et al., 2024). Another study combined BP values with F2-isoprostanes plasma values as trial endpoints, leading to a correlation of these OS biomarkers with a BP reduction with sulforaphane as compared to the placebo group (Bai et al., 2015).

Dose-finding phases are critical, given the U-shaped efficacy curve of antioxidants. For example, a high dose of N-acetylcysteine (1,200 mg/day) paradoxically increased oxidative damage in hypertensive patients, while 600 mg/day reduced MDA by 27% (Szkudlinska et al., 2016). To mitigate placebo effects, crossover designs with washout periods (e.g., 4 weeks) are advantageous. Another example is flavonoid glycoside (rutin) administration to DM2 patients has improved BP, the levels of antioxidant enzymes, and quality of life (Bazyar et al., 2023), but the sample size (n = 19) acts as a handicap for its significance. The use of antioxidant phytochemicals has also been claimed to have potential benefits (Akpoveso et al., 2023; Arabshomali et al., 2023), but again, randomized clinical trials are scarce and contradictory. The low bioavailability and absorption of antioxidant phytochemicals currently limit their utility in the clinic, and further studies are needed to explore the antidiabetic potentials of phytochemicals, improve their absorption and bioavailability, and explore their potential side effects in clinical trials.

Okoduwa et al. (2015) investigated the correlation between age-related changes and the antioxidant defense system in patients with HTN and/or DM2. The cohort consisted of 200 patients divided into four groups, each containing 50 subjects: a diabetic group, a hypertensive group, a hypertensive-diabetic group, and a

control group of non-diabetic, normotensive individuals. The results indicated that several OS biomarkers increased with age. Specifically, lipid peroxidation potential rose by 90%, antioxidant enzyme levels increased by more than 10%, and serum selenium levels rose by over 50%. Based on these findings, it was recommended that elderly patients with HTN and DM2 should receive antioxidant supplementation.

Advanced monitoring techniques—such as electron paramagnetic resonance (EPR) for real-time ROS detection and mitochondrial DNA copy number assays—can enhance endpoint precision, as piloted in the MITO-HTN study (Rossman et al., 2018). Finally, long-term safety follow-up (≥ 12 months) is essential to assess risks of chronic Nrf2 activation (e.g., cancer progression) or antioxidant interference with physiological ROS signaling, highlighted by the bardoxolone methyl experience in diabetic kidney disease (Nangaku et al., 2020). Future trials should prioritize adaptive designs to evaluate combinations (e.g., NOX inhibitors + Nrf2 activators) and digital health tools (e.g., continuous glucose/BP monitoring) for dynamic OS assessment.

The application of antioxidant therapies aimed at lowering AGE could be a helpful tool because high AGE concentrations harm vascular smooth muscle and adversely affect NO function, which in turn impairs endothelial relaxation and prevents NO action against plaque formation from the oxidized form of LDL (Saleh, 2015). According to Csiha et al. (2025), alpha-lipoic acid reduced AGEs in diabetic neuropathy cases, suggesting that it may enhance endothelial function. Antioxidant treatments in patients with DM2 have yet to demonstrate beneficial results, primarily due to the insufficient understanding of the relationship between antioxidant functions and disease progression. One specific target regarding OS and DM2 is Nrf2, which regulates a range of detoxifying genes involved in the response to ROS increase (La Rosa et al., 2020). When Nrf2 is activated, it triggers the transcription of genes that detoxify reactive oxygen species (ROS) and eliminate damaged cellular components. Effective management of blood glucose levels in individuals with type 2 diabetes (DM2) involves a comprehensive approach. A study investigated the blood concentrations of various antioxidant defense biomarkers in a group of 80 patients with DM2 to evaluate the potential correlation between antioxidant defense, glycemic control, and the presence of diabetic complications (Gawlik et al., 2016). The researchers found that Ferric Reducing Ability of Plasma (FRAP), Glutathione Reductase (GSH), and gamma glutamyl transferase (GGT) levels were significantly higher in DM2 patients compared to the control group, while Glutathione Peroxidase (GPx) levels were significantly lower. Additionally, fasting serum glucose showed a correlation with GSH, GPx, FRAP, and GGT, whereas glycosylated hemoglobin (HbA1c) only correlated with GGT. No differences were observed in antioxidant defense biomarkers in patients with chronic diabetes complications and those without. The authors concluded that the overall antioxidant defense system in DM2 is unable to counteract the increased production of ROS. Therefore, they suggested that antioxidant supplementation should be considered as an adjuvant therapy in DM2 treatment. Interestingly, our results about OS biomarkers in DM2 and type 1 diabetes mellitus (DM1) showed that antioxidant therapy should not be recommended in DM1 (Nuñez-Selles et al., 2019).

8 Future directions

Conventional antioxidants face limitations in efficacy and specificity, but emerging strategies—including mitochondria-targeted compounds and Nrf2 activators—address these challenges through three transformative mechanisms:

- Precision targeting

Unlike broad-acting scavengers, these agents accumulate within mitochondria, the primary source of ROS. This subcellular localization enables the direct protection of mitochondrial lipids, proteins, and mtDNA from oxidative damage, a critical advantage in diseases like neurodegeneration and metabolic disorders.

- Endogenous defense amplification

Rather than transient ROS neutralization, these therapies enhance the cell's innate antioxidant capacity by upregulating glutathione synthesis, antioxidant enzymes, and phase II detoxification systems. This creates a sustained, adaptive response to OS.

- Multipathway disease modulation

By activating Nrf2 and interconnected pathways, they concurrently suppress pro-inflammatory signaling (e.g., NF- κ B), reduce apoptotic susceptibility, and improve metabolic efficiency via PGC-1 α regulation.

This dual approach-localized ROS interception coupled with systemic redox resilience, offers unparalleled promise for treating chronic conditions (e.g., diabetic complications, cardiovascular disease) where OS drives pathology, thus providing an advantageous therapeutic potential.

8.1 Hypertension

The development of dynamic, tissue-specific OS biomarkers represents a critical frontier in HTN management. Current limitations of static plasma/serum measurements (e.g., MDA, F2-isoprostanes) could be overcome through several innovative approaches. Real-time redox monitoring using implantable graphene-based electrochemical sensors is being developed for continuous measurement of vascular H₂O₂ and O₂^{•-} levels. Early prototypes have demonstrated 90% accuracy in correlating BP with endothelial dysfunction in animal models (Zhang et al., 2023). These could be paired with existing Continuous Glucose Monitoring-style wearable technology for simultaneous glucose and OS tracking in metabolic HTN. Mitochondrial redox fingerprinting with advanced mass spectrometry and nuclear magnetic resonance techniques is enabling the identification and quantification of OS biomarkers simultaneously at very low concentrations (Petrova and Guler, 2025). In this sense, important advances have been reported to define the mitochondrial profile metabolites in OS situations, such as cancer. These methods combined allow for the simultaneous identification and imaging of various free radicals, providing metabolic and biochemical data revealing significant

differences between whole-cell and mitochondrial metabolite profiles (Domingo-Ortí et al., 2023; Krishnamurthy et al., 2024). This approach provides insights into mitochondrial metabolic alterations, opening a gate to targeted therapeutic actions. Over 200 distinct mitochondrial redox compounds have been identified from a single blood draw for fingerprinting (Chen et al., 2019). Another study identified a distinct 12-metabolite signature that predicted progression to resistant HTN with 82% accuracy (Al-Ashmar et al., 2024).

Redoxomics is a novel type of omics based on knowledge about the role of redox homeostasis for the healthy state of cells and their pathogenesis in several diseases (Nakhod et al., 2024). The need of redox-sensitive fluorescent tags (e.g., roGFP2) coupled with microfluidic cell sorting will allow the profiling of OS heterogeneity within endothelial, vascular smooth muscle, and immune cell populations (Schwarzländer et al., 2016).

The next-generation of antioxidant interventions will focus on spatial and temporal precision, like the NOX isoform-specific nanotherapy based on lipid nanoparticles encapsulating NOX1 siRNA (Cartaya et al., 2019). Experimental results in primate models achieved a 70% knockdown in vascular smooth muscle cells without hepatic toxicity, and a first-in-human trial is planned to start in 2025.

Chronotherapeutic Nrf2 activation is being studied as an alternative for HTN treatment (Serrano and Medina, 2025). Some trials have reported that Nrf2 activators, such as sulforaphane, bardoxolone methyl, and dimethyl fumarate, have demonstrated promise in lowering OS and enhancing endothelial function thus far (Wu Q. et al., 2024). The majority of these studies, whether conducted on humans or animals, aimed to improve effectiveness by matching diurnal BP peaks as possible supplementary resources for antihypertensive medications (Hermida et al., 2016; Tanase et al., 2022). The Nrf2 transcription factor, which aids in OS defense, is regulated by the circadian rhythm (Pekovic-Vaughan et al., 2014). This control is crucial for avoiding fibrosis and tissue damage, and it might be relevant to the development of HTN.

Mitochondrial protonophore therapy is considered an attractive way of HTN treatment (Myszko et al., 2025). Since mitochondrial failure appears to be a major factor in the development of target organ damage linked to HTN, mitochondria have emerged as a possible target for treatment. For several years, researchers have been investigating the use of mitochondrial uncouplers, such as protonophores and UCP2, to modify mitochondrial activity to achieve modulatory action on this organelle. These uncouplers may lower ROS because they increase energy expenditure by moving protons across the inner mitochondrial membrane (Minanimo-Muta et al., 2018; Nesci and Rubattu, 2024). Controlled uncoupling with modified dinitrophenol analogs (e.g., DNP-Mito) reduced mtROS by 65% while maintaining ATP synthesis in hypertensive rat models (Kotova and Antonenko, 2022).

Experimental gene therapies delivering antioxidant enzyme genes (e.g., CAT, SOD, GPx) via viral vectors or nanoparticle carriers have shown promise in animal models by bolstering intrinsic defenses (García-González et al., 2024; Padmanaban et al., 2023). Some studies demonstrate the potential of nanoparticle-mediated delivery of antioxidant enzymes to protect neurons from OS. CAT was encapsulated within poly-lactic-co-glycolic

acid (PLGA) nanoparticles, which retained approximately 99% of the enzyme's activity. These nanoparticles provided sustained release of CAT over a month and effectively protected human neurons from H₂O₂-induced oxidative damage, preserving neuronal morphology and function (Ashok et al., 2022). A recent study developed human serum albumin (HSA)-based nanoparticles stabilized with polyethyleneimine (PEI) to co-deliver the SOD 1 gene and sulforaphane, a known Nrf2 activator. The nanocarriers demonstrated high biocompatibility and transfection efficiency (~66%) in human lung epithelial cells (L-132). The combined delivery system effectively enhanced antioxidant defenses, suggesting potential for treating diseases associated with oxidative stress (Naqvi et al., 2022).

Advancing OS Therapies in HTN should consider several alternatives. First, the optimization of the Nrf2 pathway through targeted drug delivery, e.g., lipid nanoparticles (LPNs) loaded with sulforaphane or bardoxolone methyl could enhance endothelial uptake while avoiding systemic side effects (Zhang et al., 2024), and LPNs conjugated to VCAM-1 antibodies for inflamed vasculature targeting (Gu et al., 2025). Translating all these advances mentioned above to clinical practice will require the construction of redox phenotyping panels with several biomarkers incorporating various sources of biomolecular and clinical data to guarantee a higher robustness and power of separation for a clinical test (Smith et al., 2021). A 10-parameter panel, including not only classical antioxidant tests (FRAC, Trolox, DPPH) but also endogenous antioxidant enzymes and protein degradation, has shown its advantages in stratifying OS status in different HTN degrees (Nuñez-Selles et al., 2019). The development of AI-driven treatment algorithms through supervised machine learning is successfully automating the process of evaluation and quantification of oxidative damage in biological samples, as well as extracting useful data from the abundance of experimental results (Pantic et al., 2022). Machine learning models may integrate redox biomarkers, pulse wave analysis, and gut microbiome data to predict optimal antioxidant regimens with high accuracy. Recently, microbiome redox modulation has appeared in the scenario. (Million and Raoult, 2018). Gut microbiota dysbiosis contributes to HTN through mechanisms involving OS and inflammation (Li et al., 2021). "Gut redox" focuses on the intricate redox equilibrium within the gastrointestinal tract, encompassing a dynamic interplay of ROS and antioxidants (Prasad et al., 2024). Akkermansia muciniphila supplementation has been shown to reduce BP through the reduction of OS markers, likely through increased propionate production, but its translation to the clinic is quite controversial (Lakshmanan et al., 2022). The impact of microbial metabolites on vascular function and its potential of targeting gut microbiota to modulate redox balance and BP is a promissory gate to bettering HTN syndrome (Li et al., 2021). Interventions targeting gut microbiota, such as probiotics and dietary modifications, show promise in managing OS and blood pressure.

These future directions collectively represent a transformative shift from reactive BP management to proactive redox-based prevention and personalized treatment of hypertensive vascular disease. The integration of advanced biomarkers, targeted therapies, and systems biology approaches promises to revolutionize our understanding and management of OS in HTN.

8.2 Diabetes

The growing recognition of OS as both a cause and consequence of diabetes pathogenesis has fundamentally reshaped the understanding of this metabolic disorder. By replicating glucose consumption and changes in oxidative phosphorylation, hyperglycemia and hyperlipidemia exacerbate inflammation. Cell migration causes the inflammatory response to expand into the vasculature, islets, and adipose tissue. Due in large part to endothelial damage and oxidative damage to the endoplasmic reticulum, glycolipotoxicity comprises the primary cause of the inflammatory response triggered in DM2 and ultimately resulting in IR. These arguments, which center on the molecular effects of OS precursors or modulators, imply that certain anti-inflammatory products might be taken into consideration as part of the antidiabetic treatment, just as they have been for myocardial infarction (Nuñez-Musa, 2024).

Mounting evidence positions redox imbalance not merely as a secondary phenomenon but as a primary driver of the disease continuum - from initial β -cell dysfunction to devastating micro- and macro-vascular complications. This paradigm shift has unveiled critical limitations in current antioxidant approaches while simultaneously opening unprecedented opportunities for mechanism-targeted interventions. The future of antioxidant therapy in DM lies in overcoming the limitations of broad-spectrum approaches by developing targeted, personalized, and mechanism-based interventions that address the nuanced role of OS in disease progression.

Next-generation interventions will focus on spatial and temporal precision protocols. β -Cell-specific redox modulators, as the conjugated glucagon-like peptide-1 (MitoTEMPO) by nanoparticles have achieved 80% β -cell targeting efficiency in primates, preserving insulin secretion under glucotoxic conditions (Marques et al., 2024). Dual NOX/SGLT2 inhibitors as the hybrid compound GKT137831 (inhibitor of NOX1 and NOX4) and empagliflozin (SGLT2 inhibitor) have shown synergistic effects, improving insulin sensitivity by 40% in diabetic rodent models (Karakasis et al., 2025). Additionally, cutting-edge metabolomic approaches are revolutionizing the ability to quantify mitochondrial oxidative stress in diabetes.

Emerging strategies focus on mitochondrial-specific antioxidants (e.g., MitoQ, SS-31), which have shown promise in preclinical and early-phase clinical trials by reducing mtROS and improving endothelial function in diabetic patients (Mason et al., 2022). Recent advances in Nrf2 activators, such as synthetic triterpenoids and natural compounds, highlight the need for temporal precision—activating Nrf2 early to preserve β -cell function but avoiding chronic overexpression, which may promote cancer (Mohamadi et al., 2023). Nanotechnology is poised to revolutionize delivery, with ROS-responsive nanoparticles encapsulating antioxidants (e.g., mangiferin, curcumin, resveratrol) for targeted release in pancreatic islets or atherosclerotic plaques (Cheng et al., 2024). Additionally, dual-target therapies that combine OS reduction with conventional antidiabetic effects—such as SGLT2 inhibitors with NOX4 inhibitors—are under investigation

to address multifactorial pathology (Balogh et al., 2023). Future clinical trials should integrate deep phenotyping techniques, such as redox proteomics and mitochondrial DNA damage assays, along with AI-driven biomarker panels. This approach will help identify patients most likely to benefit from interventions, advancing beyond just HbA1c levels to include dynamic OS metrics like real-time ROS sensors (Geraskevich et al., 2024). Lastly, lifestyle-integrated approaches, such as time-restricted eating (TRE) combined with low-dose antioxidants, may amplify endogenous defenses while minimizing pharmacological risks (Varady et al., 2022). The path forward requires a paradigm shift from reactive antioxidant supplementation to precision redox medicine, leveraging genomics, microbiome science, and targeted delivery systems to break the gap of OS in DM.

9 Conclusion

The management of OS has evolved from nonspecific free-radical scavenging to precision-driven interventions that address both the sources and consequences of redox imbalance. Traditional biomarkers, such as MDA, F_2 -isoprostanes, protein carbonyls, and 8-OHdG, combined with advanced techniques like EPR spectroscopy and multi-omics, now enable a comprehensive assessment of oxidative injury across lipids, proteins, DNA, and metabolites. Therapeutically, small-molecule antioxidants and nutraceuticals laid the groundwork, but their limited organelle specificity and bioavailability have driven the development of mechanism-based approaches. Mitochondria-targeted compounds directly neutralize ROS at its primary source, while Nrf2 activators amplify endogenous defense systems to sustain redox homeostasis. Gene- and enzyme-delivery platforms, ranging from AAV vectors encoding CAT/SOD to nanoparticle nanozymes, further bolster intracellular antioxidant capacity. In cardiometabolic contexts such as DM2 and HTN, agents like SGLT2 inhibitors and AMPK/Sirtuin modulators illustrate how metabolic correction can dovetail with oxidative protection. Finally, isoform-selective NOX inhibitors exemplify targeting of disease-relevant ROS sources. Looking ahead, integrating precise diagnostics with tailored antioxidant therapies promises to transform the treatment of chronic disorders driven by OS, like DM2 and HTN. Continued innovation in delivery technologies, patient stratification, and rigorous clinical validation will be essential to realize the full potential of these next-generation interventions.

Author contributions

AN-S: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Supervision, Visualization, Writing – original draft, Writing – review and editing. RN-M: Data curation, Formal Analysis, Investigation, Supervision, Writing – review and editing. RG-M: Methodology, Supervision, Visualization, Writing – review and editing.

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Conflict of interest

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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Generative AI statement

The author(s) declare that Generative AI was used in the creation of this manuscript. DeepSeek generative AI was used for manuscript writing, Google Grammarly was used for emglsh grammar revision.

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Mitochondrial mass and mitochondrial membrane potential of peripheral lymphocytes: promising biomarkers of systemic lupus erythematosus

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Background: Mitochondrial dysfunction is implicated in the pathogenesis of systemic lupus erythematosus (SLE). Single-cell mitochondrial mass (SCMM), low mitochondrial membrane potential (MMP-Low) in lymphocytes, and circulating mitochondrial DNA (mtDNA) can reflect mitochondrial impairment and may serve as potential novel biomarkers for SLE.

Purpose: We investigated the diagnostic utility of MMP-Low and SCMM in lymphocytes, as well as circulating mtDNA levels, in patients with SLE and examined their correlation with disease activity.

Methods: Flow cytometry was performed to detect MMP-Low and SCMM in peripheral lymphocytes from patients with SLE (n = 52) and healthy controls (HCs, n = 30). The level of circulating mtDNA was quantified using PCR.

Results: Patients with SLE exhibited significantly decreased MMP-Low in some peripheral lymphocyte subsets. Meanwhile, significantly increased SCMM in some lymphocyte subsets and circulating mtDNA were observed in patients with SLE. CD8⁺ T naïve (Tn) cell MMP-Low, CD8⁺ T effector memory cell MMP-Low, CD8⁺ T central memory (Tcm) cell MMP-Low, and SCMM-CD8⁺ Tn cells demonstrated a moderate diagnostic value for SLE, with an area under the curve (AUC) above 0.8. Both CD4⁺ Tcm MMP-Low and SCMM-CD3⁺CD4⁺ T cells were significantly associated with the SLE Disease Activity Index 2000 (SLEDAI-2K) and circulating mtDNA levels. These markers also showed significant alternations between inactive and active SLE.

Conclusion: Our data showed that patients with SLE exhibit mitochondrial dysfunction. Several MMP-Low and SCMM in CD8⁺T cell subsets could serve

as potential biomarkers for diagnosing SLE. Additionally, CD4⁺ Tcm MMP-Low and SCMM-CD3⁺CD4⁺ T cells were associated with SLE disease activity.

KEYWORDS

systemic lupus erythematosus, mitochondrial mass, mitochondrial membrane potential, mitochondrial DNA, lymphocytes

Background

Systemic lupus erythematosus (SLE), a prototypical autoimmune disease, is characterized by multiple organ involvement and varied clinical manifestations (Lazar and Kahlenberg, 2023; Caielli et al., 2023). It is more common in young female individuals. Due to the heterogeneity of the disease, early diagnosis of SLE still remains challenging (Dörner and Furie, 2019). Furthermore, the pathogenesis of SLE is complex. Numerous studies have revealed that the loss of immune tolerance to self-nucleic acids and self-antigens, immune cell hyperactivation, activation of the interferon system, and defects in apoptotic cell clearance contribute to SLE pathology (Owen et al., 2022). In addition, mitochondria also play an important role in SLE pathogenesis through various mechanisms (Caielli et al., 2023).

Mitochondria, critical organelles, play a key role in supplying energy for multiple cell activities and regulate cell activation and differentiation by generating adenosine triphosphate (ATP) through oxidative phosphorylation (Mills et al., 2017). Mitochondrial dysfunction contributes to SLE pathogenesis through excessive mitochondrial reactive oxygen species (ROS) production, mitochondrial DNA (mtDNA) damage, and impaired mitophagy (Zhao et al., 2022). In addition, mitochondrial components have also been proven to be the source of extracellular and intracellular danger-associated molecular patterns in SLE, leading to abnormal inflammatory response and immune cell activation (Caielli et al., 2023). Moreover, oxidative stress-mediated damage, including lipid peroxidation and protein modification, has been implicated in SLE. Specifically, 4-hydroxynonenal (HNE)-modified proteins contribute to autoimmune responses and serve as biomarkers of oxidative stress in lupus patients (Khan et al., 2016). Mitochondrial mass (MM) is a hallmark of mitochondrial fitness, and increased MM can induce abnormal activation of immune cells (Yu et al., 2020; Callender et al., 2020). Furthermore, impaired mitochondrial quality control has also been observed in SLE, and this change causes the dysfunction of immune cells in SLE (Caielli et al., 2023). However, the MM of immune cells in patients with SLE still remains unclear.

In this study, a new immunofluorescence technology was used to detect single-cell mitochondrial mass (SCMM) and low mitochondrial membrane potential (MMP-Low) in immune cells from patients with SLE (Puleston, 2015; Author Anonymous, 2021).

First, the distribution of immune cells, including CD3⁺ T cells, B cells, NK cells, CD4⁺ T cells, CD8⁺ T cells, CD4⁺ T naïve (Tn) cells, CD4⁺ T effector (Tef) cells, CD4⁺ T central memory (Tcm) cells, CD4⁺ T effector memory (Tem) cells, CD8⁺ Tn cells, CD8⁺ Tef cells, CD8⁺ Tcm cells, and CD8⁺ Tem cells, was analyzed. Furthermore, flow cytometry with Mito dye staining was used to detect the SCMM of peripheral immune cells. SCMM could more sensitively reflect the processes involved in mitochondrial quality control in immune cells, including mitophagy, mitochondrial dynamics, and mitochondrial biogenesis (Miwa et al., 2022; He et al., 2023; Pang et al., 2022). In addition, we analyzed the relationship between SCMM and MMP-Low in immune cells and mtDNA levels in patients with SLE. This analysis aimed to evaluate the diagnostic utility of MMP-Low and SCMM in lymphocytes, as well as circulating mtDNA levels, and examine their correlation with SLE disease activity.

Methods

Study participants

A total of 52 patients with SLE and 30 age- and sex-matched healthy controls (HCs) were enrolled between May 2022 and July 2022 at Peking Union Medical College Hospital (PUMCH). All patients with SLE fulfilled the 1997 classification criteria of the American College of Rheumatology (ACR) (Hochberg, 1997); individuals with other autoimmune diseases, infections, pregnancy, and malignant tumors were excluded from this study. Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K) was used to evaluate the disease activity of patients with SLE (Gladman et al., 2002). Patients with SLE whose SLEDAI-2K < 6 were regarded as inactive SLE, and those whose SLEDAI-2K ≥ 6 were regarded as active SLE. All of the HCs had a normal range in liver and kidney function tests and blood and urine routine tests, and they did not have any autoimmune diseases, infections, malignant tumors, pregnancy, or irrelevant chronic diseases. This cross-sectional study was approved by the Research Ethics Commission of PUMCH (JS-2156). Informed written consent was waived for the remaining routine test samples.

Blood sample collection and processing

Whole blood was collected from each individual in ethylenediaminetetraacetic acid (EDTA)-treated tubes. After receiving the sample, 200 µL of fresh whole blood was taken from an EDTA-treated tube for flow cytometric analysis. The remaining whole blood was centrifuged at 3,000 × g for 10 min, and the plasma

Abbreviations: ACR, American College of Rheumatology; ATP, adenosine triphosphate; EDTA, ethylenediaminetetraacetic acid; MM, mitochondrial mass; MMP-Low, low mitochondrial membrane potential; MtDNA, mitochondrial DNA; ROC, receiver operating characteristic; SCMM, single-cell mitochondrial mass; SLE, systemic lupus erythematosus; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; Tcm, T central memory; Tef, T effector; Tem, T effector memory; Tn, T naïve.

was collected into RNase/DNase-free Eppendorf tubes (MCT-150-C, Axygen, Wujiang, China). Plasma samples were then stored at -80°C for DNA analysis until mtDNA copy number was evaluated.

Flow cytometric analysis

Each whole blood sample was divided was added to two corresponding flow tubes (100 μL whole blood/tube), each containing flow antibodies. The tubes were gently mixed and incubated in the dark for 15 min at room temperature. Then, 2 mL of lysing solution (NH Lysis Solution, 10 $\times$) was added to the tubes, mixed again, and incubated in the dark for an additional 15 min at room temperature. The residual lysed specimens were removed following centrifugation at 300 g for a duration of 5 min. One flow antibody panel including PE-CD3, PE-CD56, FITC-CD8, FITC-CD19, and PE-CY7-CD4 (UBBIO, Zhejiang, China) was applied to detect CD3 $^{+}$ T cells, CD3 $^{+}$ CD4 $^{+}$ T cells, CD3 $^{+}$ CD8 $^{+}$ T cells, B cells, and NK cells, while another flow antibody panel with FITC-CD4, AC7-CD8, PC5.5-CD45RA, and PC7-CD62L was applied to classify CD4 $^{+}$ Tn cells, CD4 $^{+}$ Tef cells, CD4 $^{+}$ Tem cells, CD4 $^{+}$ Tcm cells, CD8 $^{+}$ Tn cells, CD8 $^{+}$ TefCD8 $^{+}$ Tem cells, and CD8 $^{+}$ Tcm cells. After centrifugation, the washed cells were resuspended in 200 μL of PBS and transferred to a 96-well plate containing a mitochondria-specific dye APC (UBBIO, Zhejiang, China) to assess SCMM and MMP-Low. The samples were incubated in a consistently controlled environment at 37°C for 30 min (protected from light). The MMP-Low proportion was recorded based on the group of cells with low APC fluorescence intensity, and MM was measured using the median fluorescence intensity (MFI) of APC (Puleston, 2015; Yu et al., 1999). SCMM was calculated using a built-in algorithm based on the MFI and the counts of cell subsets. All samples were tested using a Cytex[®] Aurora cytometer (Cytex Biosciences, 26 Fremont, CA).

Plasma total DNA extraction and quantification of circulating mtDNA copy numbers

Thawed plasma was centrifuged at 10,000 g for 10 min. Total DNA was extracted from 200 μL plasma using the M5 CWhipro Circulating Nucleic Acid Kit (MF063-plus-05, Mei5bio, Beijing, China), according to the manufacturer's protocol for plasma/serum. The extracted DNA was then eluted in 30 μL of elution buffer.

The circulating mtDNA level was measured using the 2X M5 HiPer SYBR Premix EsTaq (with Tli RNaseH) qPCR assay (MF787-01, Mei5bio, Beijing, China) with a Roche LightCycler 480 System. MT-ND2 (mitochondrial-encoded NADH: ubiquinone oxidoreductase core subunit 2) was amplified to reflect the levels of circulating mtDNA. The primer sequences were as follows: MT-ND2 (forward: 5'-CACAGAAGCTGCCATCAAGTA-3'; reverse 5'-CCGGAGAGTATATTGTTGAAGAG-3') (Sangon Biotech, Beijing, China). PCR standards for MT-ND2 (90 bp) were generated by cloning complementary DNA sequences in plasmid PUC57 (GenScript Co. Ltd., Nanjing, China). Concentrations were converted to copy numbers using the formula $\text{mol/g} \times \text{molecules/mol} = \text{molecules/g}$ via a DNA copy number

calculator online website (<http://cels.uri.edu/gsc/cndna.html>; University of Rhode Island Genomics and Sequencing Center) (Nga et al., 2010; Zozaya-Hinchliffe et al., 2008). Plasmid DNA (MT-ND2) solutions were diluted in 10-fold serial dilutions and used as standards.

All samples were analyzed in duplicate, and a no-template control was included in each analysis. The analyses of circulating mtDNA levels were expressed in copies per microliter of plasma based on the following calculation (Chiu et al., 2003):

$$c = Q \times V_{\text{DNA}} / V_{\text{PCR}} \times 1 / V_{\text{ext}}$$

where c is the concentration of DNA in plasma (copies/ μL plasma); Q is the quantity (copies) of DNA determined using the sequence detector in a PCR; V_{DNA} is the total volume of plasma DNA solution obtained after extraction, typically 200 mL per extraction; V_{PCR} is the volume of the plasma DNA solution used for PCR, typically 2 μL of a 10-fold diluted plasma DNA solution; and V_{ext} is the volume of plasma extracted, typically 30 μL .

Statistical analysis

SPSS 23.0 software and GraphPad Prism 9 were used for statistical analysis. Categorical variables were shown as percentages. The normality of data distribution was evaluated using the Shapiro–Wilk test. Normal distribution data were presented as mean $\pm$ SD, whereas non-normally distributed data were expressed as median (IQR). Student's t -tests or Mann–Whitney U -tests were performed to analyze the differences in continuous variables between patients and controls. In addition, the χ^2 test was used to detect the count data. Spearman's correlation test was used to assess the possible relationship between the level of mitochondrial damage in immune cells, the laboratory examination value, and the SLEDAI-2K score. The receiver operating characteristic (ROC) curves were evaluated for the diagnostic value of mitochondrial damage indicators. Two-tailed $P < 0.05$ was indicated as statistically significant. G*Power 3.1 software was used to conduct a *post hoc* power analysis for each significant parameter to determine whether the available sample sizes provided sufficient power to detect statistically significant effects. Hplot (<https://hiplot.org>) and GraphPad Prism 9 were used to visualize the data.

Results

Characteristics of study individuals

A total of 52 patients with SLE and 30 HCs were enrolled in our study. The detailed demographic and clinical characteristics of all individuals are shown in Table 1. Patients with SLE included 49 female individuals and 3 male individuals, whose median age was 34 years old (IQR: 27.25–44.00 years old). The HCs included 27 female individuals and 3 male individuals, with an age of 34.6 ± 8.6 years. The median SLEDAI-2K at the time of sample collection was 8. The levels of lymphocyte count, hemoglobin, and platelets were significantly decreased in patients with SLE compared to HCs, while the levels of blood urea nitrogen, blood urea acid, and C-reactive protein were significantly increased (Table 1). Seventeen

TABLE 1 Demographic and clinical characteristics of the study individuals.

Characteristic	SLE (n = 52)	HC (n = 30)	P value
Age, years	34.00 (27.25–44.00)	35.03 ± 9.46	0.821
WBC count, ×10 ⁹ /L	5.29 (4.22–7.49)	5.50 ± 1.41	0.897
Lymphocyte, ×10 ¹² /L	1.09 (0.79–1.80)	1.90 ± 0.51	<0.001
Hemoglobin, g/L	111.70 ± 23.75	133.67 ± 8.91	<0.001
Platelets, ×10 ⁹ /L	194.02 ± 83.19	260.27 ± 67.97	<0.001
Creatinine, μmol/L	66.00 (54.00–93.75)	63.77 ± 6.68	0.658
BUN, mmol/L	5.56 (3.74–9.29)	4.52 ± 0.82	0.035
UA, μmol/L	359.00 (299.25–459.50)	282.43 ± 48.13	<0.001
CRP, mg/L	1.05 (0.37–3.08)	0.39 (0.26–1.05)	0.003
C3, g/L	0.69 ± 0.29	NA	
C4, g/L	0.10 ± 0.07	NA	
IgG, g/L	12.55 (10.03–15.83)	NA	
IgA, g/L	2.46 ± 1.20	NA	
IgM, g/L	0.94 (0.66–1.25)	NA	
Anti-dsDNA, IU/mL	49.00 (10.60–106.25)	NA	
Urinary proteins, mg/L	0.92 (0.14–4.90)	NA	
ESR, mm/h	24.50 (8.75–45.00)	NA	
SLEDAI-2K	8 (4–17)	NA	

SLE, systemic lupus erythematosus; HCs, healthy controls; WBC, white blood cell; BUN, blood urea nitrogen; UA, blood urea acid; ESR, erythrocyte sedimentation rate; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000.

patients with SLE were grouped into inactive SLE, and 35 patients with SLE were grouped to active SLE according to SLEDAI-2K.

Differences in mitochondrial impairment in T cells, B cells, and NK cells between patients with SLE and HCs

The proportions of CD3⁺T cells ($P = 0.005$) and CD3⁺CD8⁺T cells ($P < 0.001$) were significantly higher in patients with SLE than in HCs, while the proportion of NK cells ($P < 0.001$) was significantly lower in patients with SLE than in HCs (Table 2). The gating strategy for the fluorescence intensity of mitochondrial staining of T cells, B cells, and NK cells is shown in Supplementary Figure S1. MMP[−]Low proportions were used to reflect the MM of lymphocytes. The percentages of CD3⁺T cell MMP[−]Low ($P < 0.001$, Figure 1A),

CD3⁺CD4⁺ T cell MMP[−]Low ($P < 0.001$, Figure 1A), CD3⁺CD8⁺ T cells ($P < 0.001$, Figure 1A), and B cells ($P = 0.006$, Figure 1A) were significantly lower in patients with SLE than in HCs. In addition, SCMM was calculated based on the MFI and the counts of cell subsets to reflect the MM of each single cell subset in their subsets. We observed that the SCMMs of CD3⁺ T cells ($P < 0.001$, Figure 1B), CD3⁺CD4⁺ T cells ($P = 0.001$, Figure 1B), and CD3⁺CD8⁺ T cells ($P < 0.001$, Figure 1B) were significantly higher in patients with SLE than in HCs, while no significant difference was detected in SCMMs of B cells ($P = 0.057$, Figure 1B) and NK cells ($P = 0.121$, Figure 1B) between patients with SLE and HCs. Meanwhile, the statistical power for SCMM-CD3⁺ T cells, SCMM-CD3⁺CD4⁺ T cells, and CD3⁺CD8⁺ T cells was found to be greater than 0.8 (Supplementary Table S1), indicating that the sample sizes for these results were sufficiently powered.

Differences in mitochondrial impairment in T-cell subsets between patients with SLE and HCs

Further analysis of mitochondrial impairment in T-cell subsets was detected in patients with SLE and HCs. First, the percentage of CD4⁺ Tn was significantly decreased in patients with SLE compared with HCs ($P = 0.04$, Table 2), while the percentage of CD4⁺ Tcm was significantly increased in patients with SLE and HCs ($P = 0.017$, Table 2). However, the power for the percentage of CD4⁺ Tn and CD4⁺ Tcm cells was found to be less than 0.8 (Supplementary Table S1), indicating an insufficiently powered sample size for these two parameters. No significant alternation of subsets in CD8⁺ T cells was observed between patients with SLE and HCs (Table 2). The gating strategy for the fluorescence intensity of mitochondrial staining of T-cell subsets is shown in Supplementary Figure S2. In CD4⁺ T cell subsets, the percentages of CD4⁺Tn MMP[−]Low ($P < 0.001$), CD4⁺Tem MMP[−]Low ($P = 0.002$), and CD4⁺ Tcm MMP[−]Low ($P < 0.001$) were significantly lower in patients with SLE than in HCs (Figure 2A). However, the SCMMs of CD4⁺Tn, CD4⁺Tem, and CD4⁺Tcm cells were significantly higher in patients with SLE than in HCs (Figure 2B). In CD8⁺T cell subsets, the percentages of CD8⁺Tn MMP[−]Low ($P < 0.001$), CD8⁺ Tef MMP[−]Low ($P < 0.001$), CD8⁺Tem MMP[−]Low ($P < 0.001$), and CD8⁺Tcm MMP[−]Low ($P < 0.001$) were significantly decreased in patients with SLE (Figure 2A). In addition, the SCMMs of CD8⁺Tn ($P < 0.001$), CD8⁺Tem ($P < 0.001$), and CD8⁺Tcm ($P = 0.003$) cells indicated a significant increase in patients with SLE compared with HCs (Figure 2B). The *post hoc* power analysis showed that the power for both the significant MMP[−]Low and SCMM of CD4⁺ and CD8⁺ T-cell subsets was sufficient to detect statistically significant effects given the available sample sizes (Supplementary Table S1).

Comparison of circulating mtDNA in patients with SLE and HCs

The circulating mtDNA (ND2) was detected in 49 patients with SLE and 30 healthy controls. The circulating mtDNA levels were significantly higher in patients with SLE than in HCs (median

TABLE 2 Comparison of parameters between patients with SLE and HCs.

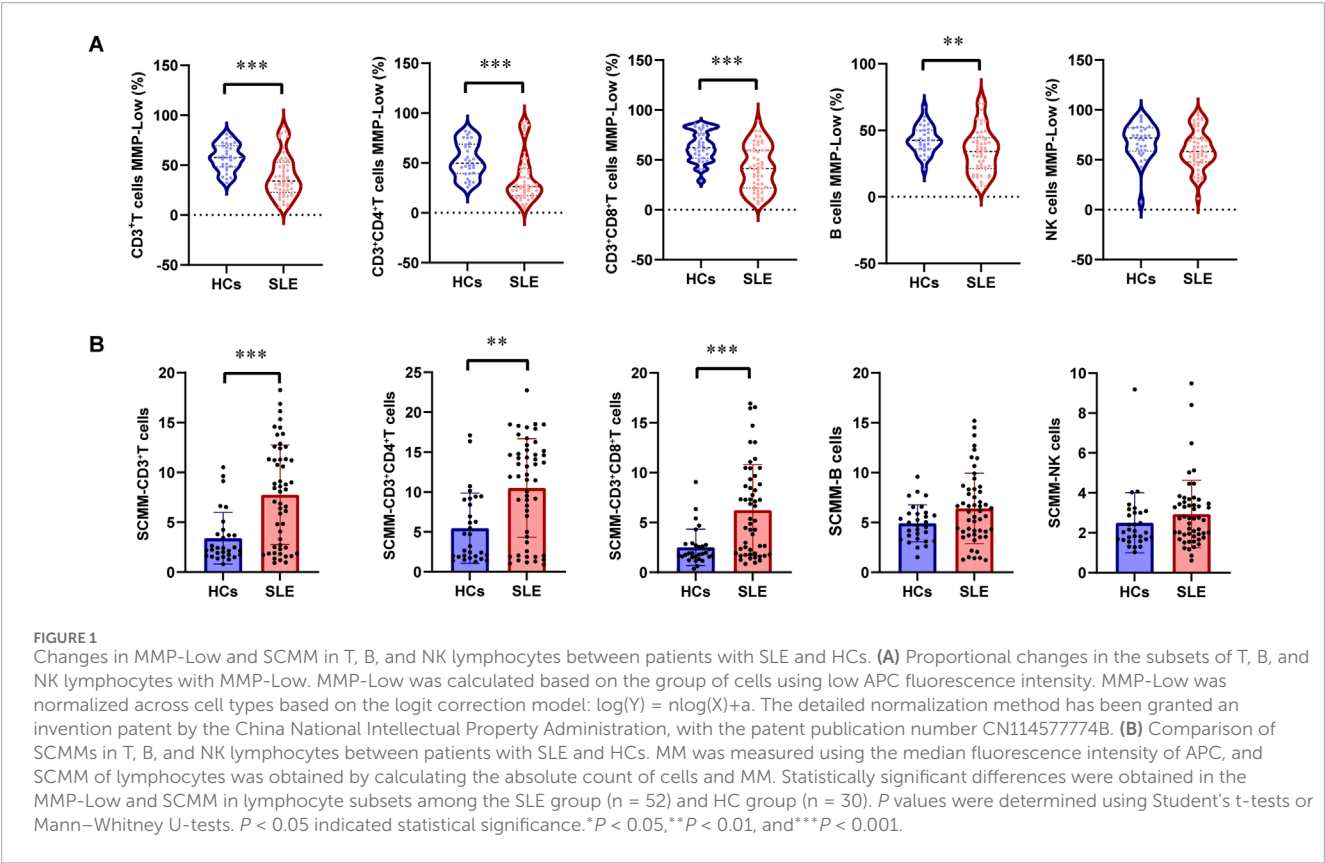
Parameter	SLE (n = 52)	HCs (n = 30)	P value
CD3 ⁺ T cells (% of lymphocytes)	72.64 (70.10–83.44)	68.52 ± 8.67	0.005
CD3 ⁺ CD4 ⁺ T cells (% of CD3 ⁺ T cells)	32.10 ± 9.16	34.12 (30.93–36.53)	0.214
CD3 ⁺ CD8 ⁺ T cells (% of CD3 ⁺ T cells)	38.93 ± 12.14	27.74 ± 5.18	<0.001
B cells (% of lymphocytes)	12.75 ± 7.50	10.65 ± 3.53	0.345
NK cells (% of lymphocytes)	7.84 (3.81–12.07)	17.21 ± 9.19	<0.001
CD4 ⁺ Tn (% of CD4 ⁺ T cells)	35.75 ± 15.09	42.72 ± 13.71	0.04
CD4 ⁺ Tef (% of CD4 ⁺ T cells)	2.99 (1.30 ± 6.95)	4.23 ± 2.99	0.658
CD4 ⁺ Tem (% of CD4 ⁺ T cells)	18.95 (18.35–31.38)	20.16 ± 9.75	0.386
CD4 ⁺ Tcm (% of CD4 ⁺ T cells)	38.93 ± 12.43	32.88 ± 8.41	0.017
CD8 ⁺ Tn (% of CD8 ⁺ T cells)	35.69 ± 17.63	36.57 ± 15.16	0.819
CD8 ⁺ Tef (% of CD8 ⁺ T cells)	22.87 ± 12.80	18.90 ± 8.28	0.256
CD8 ⁺ Tem (% of CD8 ⁺ T cells)	26.92 ± 13.17	30.25 ± 11.42	0.251
CD8 ⁺ Tcm (% of CD8 ⁺ T cells)	12.59 (9.01–17.89)	14.27 ± 6.13	0.832
MMP-Low CD3 ⁺ T cells (%)	38.90 ± 21.28	57.55 ± 13.45	<0.001
MMP-Low CD3 ⁺ CD4 ⁺ T cells (%)	26.18 (17.26–44.36)	52.98 ± 17.40	<0.001
MMP-Low CD3 ⁺ CD8 ⁺ T cells (%)	44.71 ± 22.05	63.24 ± 15.03	<0.001
MMP-Low B cells (%)	34.51 ± 17.00	43.21 ± 10.35	0.006
MMP-Low NK cells (%)	60.30 ± 19.44	68.51 ± 17.78	0.061
MMP-Low CD4 ⁺ Tn (%)	26.48 (8.35–53.35)	68.86 (42.26–88.71)	<0.001
MMP-Low CD4 ⁺ Tef (%)	44.61 ± 29.71	29.54 (8.27–56.46)	0.125
MMP-Low CD4 ⁺ Tem (%)	32.29 (23.16–51.78)	52.60 ± 15.31	0.002
MMP-Low CD4 ⁺ Tcm (%)	15.66 (9.58–38.37)	45.23 (23.62–74.86)	<0.001
MMP-Low CD8 ⁺ Tn (%)	47.01 ± 23.18	74.33 ± 13.65	<0.001
MMP-Low CD8 ⁺ Tef (%)	33.50 ± 22.76	50.12 ± 19.17	<0.001
MMP-Low CD8 ⁺ Tem (%)	22.99 (15.15–40.37)	55.78 ± 15.68	<0.001
MMP-Low CD8 ⁺ Tcm (%)	13.62 (7.90–27.98)	39.06 ± 14.06	<0.001
SCMM-CD3 ⁺ T cells	8.18 (2.47–11.33)	2.32 (1.70–3.90)	<0.001
SCMM-CD3 ⁺ CD4 ⁺ T cells	11.94 (3.87–15.06)	3.97 (1.95–9.10)	0.001
SCMM-CD3 ⁺ CD8 ⁺ T cells	5.27 (1.90–9.21)	1.89 (1.56–2.77)	<0.001
SCMM-B cells	6.41 ± 3.54	4.92 ± 1.86	0.057
SCMM-NK cells	2.71 (1.84–3.52)	2.06 (1.67–3.02)	0.121
SCMM-CD4 ⁺ Tn	9.52 (2.97–15.84)	2.32 (1.64–6.68)	<0.001

(Continued on the following page)

TABLE 2 (Continued) Comparison of parameters between patients with SLE and HCs.

Parameter	SLE (n = 52)	HCs (n = 30)	P value
SCMM-CD4 ⁺ Tef	15.79 (2.03–26.38)	22.24 (2.92–26.49)	0.544
SCMM-CD4 ⁺ Tem	9.71 (4.46–14.18)	3.14 (2.49–9.44)	0.006
SCMM-CD4 ⁺ Tcm	12.46 ± 6.80	5.76 (2.19–11.08)	<0.001
SCMM-CD8 ⁺ Tn	4.39 (2.62–8.92)	1.88 (1.48–2.45)	<0.001
SCMM-CD8 ⁺ Tef	9.51 ± 7.08	6.27 ± 4.19	0.066
SCMM-CD8 ⁺ Tem	9.73 ± 5.39	3.00 (1.91–6.51)	<0.001
SCMM-CD8 ⁺ Tcm	13.10 ± 6.45	8.92 ± 5.08	0.003

NK cells, natural killer cells; Tn, T naïve; Tef, T effector; Tem, T effector memory; Tcm, T central memory; MMP-Low, low mitochondrial membrane potential; SCMM, single-cell mitochondrial mass.



20,025 copies/ul versus 5,448.75 copies/μL, $P = 0.004$; Figure 3A). We further analyzed all 79 subjects to evaluate the diagnostic value of circulating mtDNA levels in distinguishing patients with SLE from HCs. The ROC curve analysis of circulating mtDNA revealed an area under the curve (AUC) of 0.700 ($P = 0.0048$, Figure 3B). This analysis exhibited a sensitivity of 87.8% and a specificity of 56.7%. Furthermore, we observed that the circulating mtDNA was significantly higher in active SLE than in inactive SLE (median 26,325 copies/μL versus 11,175 copies/μL, $P = 0.036$; Figure 3C).

Diagnostic value of mitochondrial impairment in lymphocytes for SLE

The ROC curve analysis was performed to evaluate the utility of mitochondrial impairment quantification in distinguishing patients with SLE from HCs. CD8⁺ Tn MMP-Low (AUC = 0.832, $P < 0.001$; Figure 4A), CD8⁺Tem MMP-Low (AUC = 0.835, $P < 0.001$, Figure 4B), CD8⁺ Tcm MMP-Low (AUC = 0.81, $P < 0.001$; Figure 4C), and SCMM-CD8⁺ Tn (AUC = 0.815, $P < 0.001$;

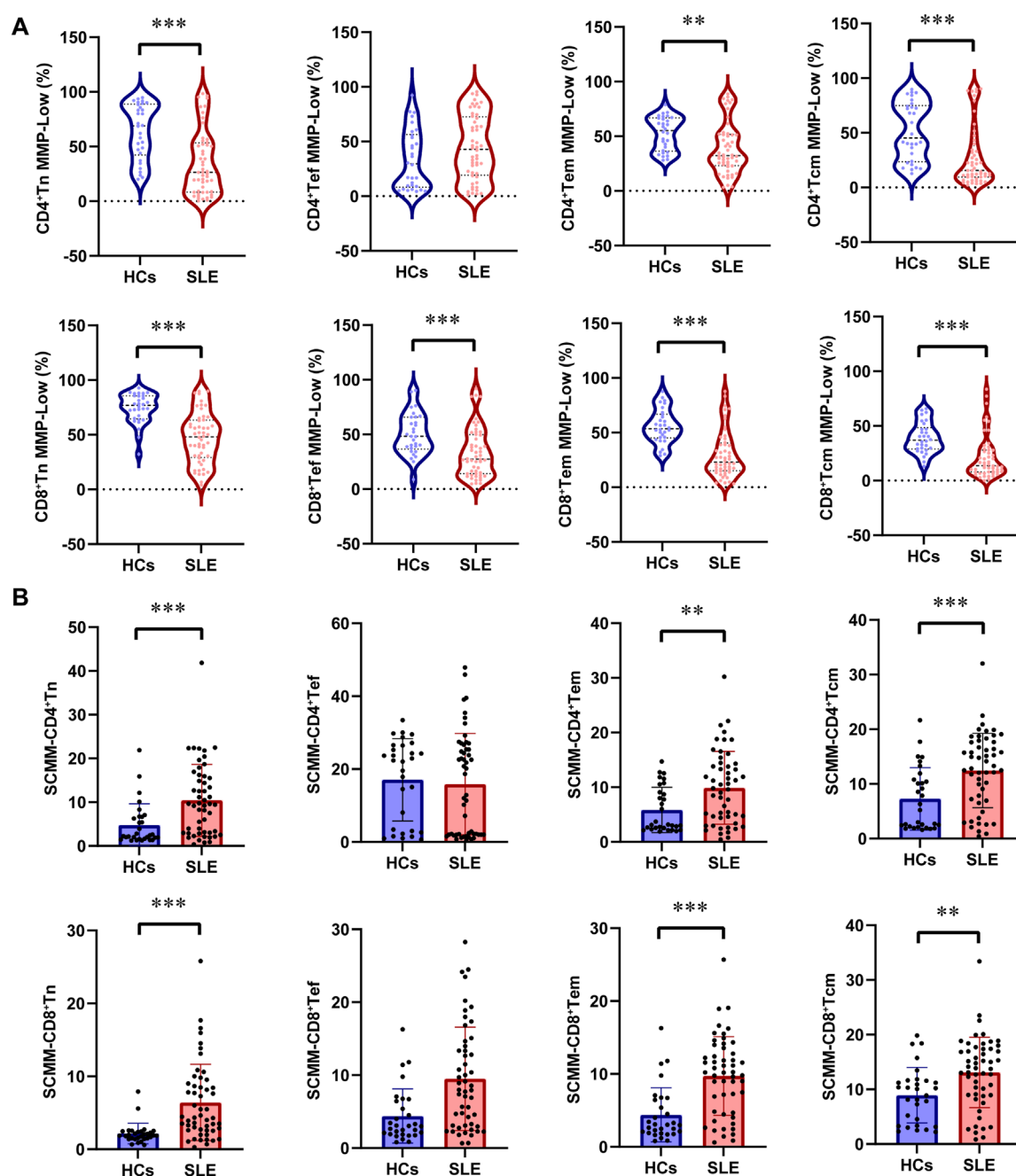


FIGURE 2

Changes in MMP-Low and SCMM in CD4 and CD8 lymphocyte subsets between patients with SLE and HCs. **(A)** Proportional changes in the subsets of CD4 and CD8 lymphocytes with MMP-Low. MMP-Low was calculated based on the group of cells using low APC fluorescence intensity. MMP-Low was normalized across cell types based on the logit correction model: $\log(Y) = \text{nlog}(X) + a$. The detailed normalization method has been granted an invention patent by the China National Intellectual Property Administration, with the patent publication number CN11457774B. **(B)** Comparison of SCMM in CD4 and CD8 lymphocyte subsets between patients with SLE and HCs. MM was measured using the median fluorescence intensity of APC, and SCMM of lymphocytes was obtained by calculating the absolute count of cells and MM. Statistically significant differences were obtained in the MMP-Low and SCMM in lymphocyte subsets among the SLE group ($n = 52$) and HC group ($n = 30$). P values were determined using Student's t -tests or Mann-Whitney U -tests. $P < 0.05$ indicated statistical significance. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Figure 4D) cells showed a moderate diagnostic value for SLE, as indicated by their AUC values exceeding 0.8 (Table 3). However, CD4⁺ TefMMP-Low (AUC = 0.602, $P = 0.124$), SCMM-B cells (AUC = 0.626, $P = 0.0579$), SCMM-NK cells (AUC = 0.603, $P = 0.121$), and SCMM-CD4⁺ Tef (AUC = 0.54, $P = 0.5422$) cells showed no diagnostic value for SLE (Table 3).

Correlation analysis of mitochondrial impairment in lymphocytes and disease activity in patients with SLE

Spearman's correlation test showed that NK cell MMP-Low was positively associated with C3 ($r = 0.401$, $P =$

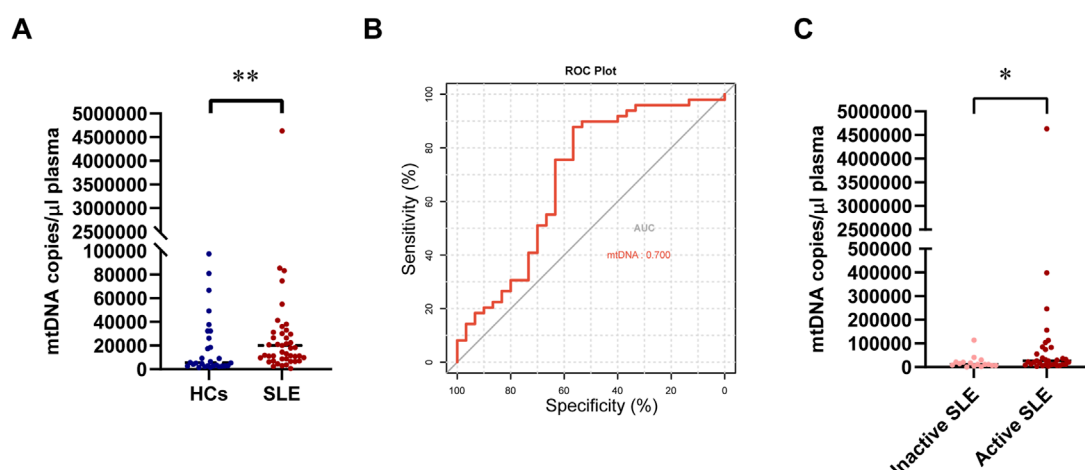


FIGURE 3

Changes in circulating mtDNA levels between patients with SLE and HCs. (A) Comparison of circulating mtDNA levels between patients with SLE and HCs. (B) Receiver operating characteristic curve for mtDNA plasma concentrations for distinguishing patients with SLE from HCs. (C) Comparison of circulating mtDNA levels between inactive and active SLE. Statistically significant differences were obtained in mtDNA levels between the SLE group ($n = 49$) and HC group ($n = 30$), as well as between the inactive SLE group ($n = 16$) and the active SLE group ($n = 33$). P values were determined using the Mann–Whitney U-tests. $P < 0.05$ indicated statistical significance. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

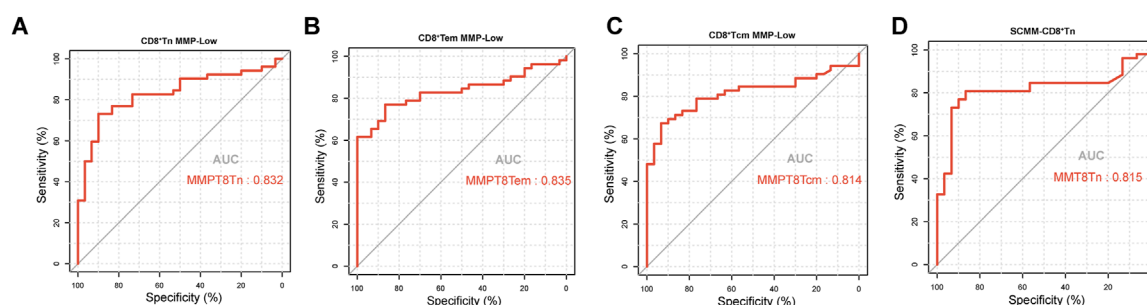


FIGURE 4

Receiver operating characteristic curve for CD8⁺Tn MMP-Low (A), CD8⁺Tem MMP-Low (B), CD8⁺Tcm MMP-Low (C), and SCMM-CD8⁺Tn (D) for distinguishing patients with SLE ($n = 52$) from HCs ($n = 30$).

0.003; [Supplementary Figure S3](#)) and C4 ($r = 0.301$, $P = 0.03$; [Supplementary Figure S3](#)) at the same time. In addition, CD3⁺ T cell MMP-Low ($r = 0.333$, $P = 0.016$; [Supplementary Figure S3](#)) and CD3⁺CD8⁺ T cell MMP-Low ($r = 0.328$, $P = 0.018$; [Supplementary Figure S3](#)) were positively associated with C3 in patients with SLE, while SCMM-CD3⁺ T cells ($r = -0.309$, $P = 0.026$; [Supplementary Figure S3](#)), SCMM-CD3⁺CD4⁺ T cells ($r = -0.339$, $P = 0.014$; [Supplementary Figure S3](#)), and SCMM-CD3⁺CD8⁺ T cells ($r = -0.324$, $P = 0.019$; [Supplementary Figure S3](#)) was negatively associated with C3. CD4⁺ Tcm MMP-Low (SLEDAI-2K: $r = -0.303$, $P = 0.029$, [Figure 5A](#); mtDNA: $r = -0.387$, $P = 0.006$, [Figure 5B](#)), CD8⁺ Tn MMP-Low (SLEDAI-2K: $r = -0.359$, $P = 0.009$, [Figure 5A](#); mtDNA: $r = -0.320$, $P = 0.025$, [Figure 5B](#)), and CD8⁺ Tcm MMP-Low (SLEDAI-2K: $r = -0.298$, $P = 0.032$, [Figure 5A](#); mtDNA: $r = -0.290$, $P = 0.043$, [Figure 5B](#)) were negatively associated with the SLEDAI-2K and circulating mtDNA in patients with SLE, while SCMM-CD3⁺ T cells (SLEDAI-2K: $r = 0.295$, $P = 0.034$, [Figure 5A](#); mtDNA: $r = 0.436$, $P = 0.002$, [Figure 5B](#)), SCMM-CD3⁺CD4⁺ T

cells (SLEDAI-2K: $r = 0.355$, $P = 0.01$, [Figure 5A](#); mtDNA: $r = 0.462$, $P = 0.001$, [Figure 5B](#)), and SCMM-NK cells (SLEDAI-2K: $r = 0.300$, $P = 0.031$, [Figure 5A](#); mtDNA: $r = 0.326$, $P = 0.022$, [Figure 5B](#)) was positively associated with the SLEDAI-2K and circulating mtDNA. Furthermore, we observed that CD4⁺ Tcm MMP-Low was significantly decreased in active SLE ($P = 0.0397$, [Figure 5C](#)), and SCMM-CD3⁺CD4⁺ T cells was significantly increased in active SLE compared with inactive SLE ($P = 0.025$, [Figure 5C](#)).

Discussion

This cross-sectional study revealed a higher alteration in MM in some subsets of lymphocyte subsets in patients with SLE, indicating increased mitochondrial biogenesis or heightened cellular activation in SLE. In addition, the level of circulating mtDNA was elevated in patients with SLE compared with HCs. Meanwhile, we observed that MM in some subsets of lymphocytes was positively associated with

TABLE 3 AUCs for the assessed variables in patients with SLE and HCs.

Parameter	Sensitivity (%)	Specificity (%)	AUC	P value
CD3 ⁺ T cells MMP-Low	67.3	83.3	0.781	<0.001
CD3 ⁺ CD4 ⁺ T cells MMP-Low	61.5	96.7	0.772	<0.001
CD3 ⁺ CD8 ⁺ T cells MMP-Low	63.5	90	0.775	<0.001
B cells MMP-Low	55.8	80	0.683	0.0059
NK cells MMP-Low	53.8	76.7	0.649	0.0249
CD4 ⁺ Tn MMP-Low	82.7	60	0.766	<0.001
CD4 ⁺ Tef MMP-Low	78.8	46.7	0.602	0.124
CD4 ⁺ Tem MMP-Low	53.8	86.7	0.705	0.0021
CD4 ⁺ Tcm MMP-Low	51.9	96.7	0.759	<0.001
CD8 ⁺ Tn MMP-Low	73.1	90	0.832	<0.001
CD8 ⁺ Tef MMP-Low	51.9	93.3	0.733	<0.001
CD8 ⁺ Tem MMP-Low	76.9	86.7	0.835	<0.001
CD8 ⁺ Tcm MMP-Low	67.3	93.3	0.814	<0.001
SCMM-CD3 ⁺ T cells	67.3	80	0.744	<0.001
SCMM-CD3 ⁺ CD4 ⁺ T cells	55.8	93.3	0.717	0.0011
SCMM-CD3 ⁺ CD8 ⁺ T cells	63.5	83.3	0.747	<0.001
SCMM-B cells	46.1	86.7	0.626	0.0579
SCMM-NK cells	40.3	86.7	0.603	0.121
SCMM-CD4 ⁺ Tn	61.5	83.3	0.74	<0.001
SCMM-CD4 ⁺ Tef	38.4	80	0.54	0.5442
SCMM-CD4 ⁺ Tem	76.9	56.7	0.685	0.006
SCMM-CD4 ⁺ Tcm	65.4	76.7	0.733	<0.001
SCMM-CD8 ⁺ Tn	80.8	86.7	0.815	<0.001
SCMM-CD8 ⁺ Tef	36.5	90	0.622	<0.001
SCMM-CD8 ⁺ Tem	71.2	86.7	0.789	<0.001
SCMM-CD8 ⁺ Tcm	63.5	83.3	0.699	0.003

AUC, area under the curve; NK cells, natural killer cells; Tn, T naïve; Tef, T effector; Tem, T effector memory; Tcm, T central memory; MMP-Low, low mitochondrial membrane potential; SCMM, single-cell mitochondrial mass.

the level of circulating mtDNA and SLEDAI-2K in patients with SLE. Our data established that abnormal MM in peripheral lymphocyte subsets might be involved in the pathogenesis of SLE.

MM is usually defined as the sum of the masses of all mitochondria and their contents within a cell (Yang et al., 2019). MM homeostasis contributes to maintaining normal mitochondrial function, which depends on the mitochondrial quality control

encompassing processes like mitophagy, mitochondrial biogenesis, and mitochondrial dynamics (Miwa et al., 2022). Increased MM suggests mitophagy dysfunction (Chourasia et al., 2015), enhanced mitochondrial biogenesis (Valero, 2014), and imbalanced mitochondrial fusion–fission.

Mitophagy could contribute to eliminating dysfunctional mitochondria and helps the cell respond to hypoxia and nutrient

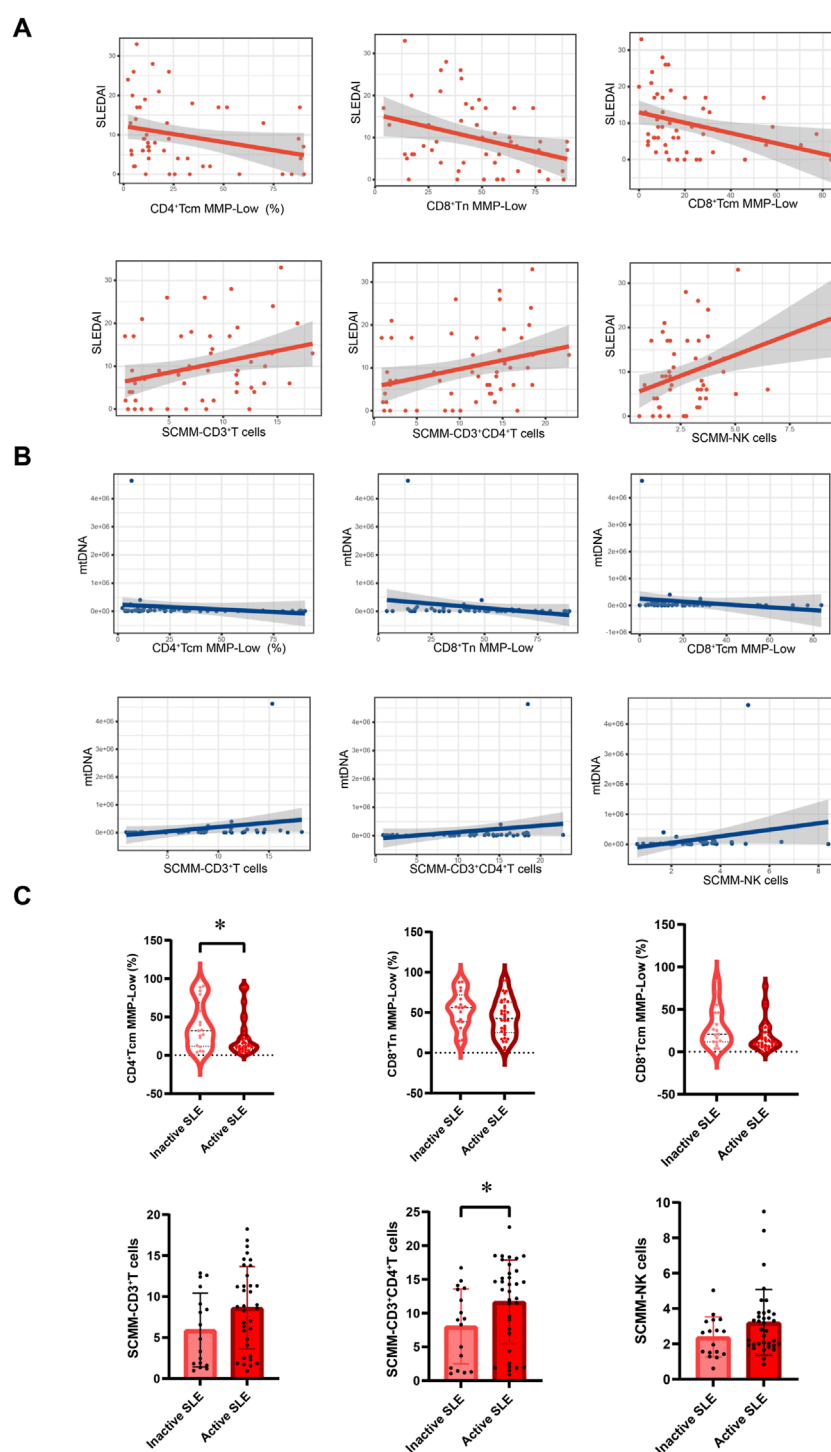


FIGURE 5

Association between mitochondrial impairment parameters and disease activity of SLE. **(A)** Association between CD4⁺Tcm cell MMP-Low, CD8⁺Tn cell MMP-Low, CD8⁺Tcm cell MMP-Low, SCMM-CD3⁺T cells, SCMM-CD3⁺CD4⁺T cells, SCMM-NK cells, and SLEDAI-2K scores. **(B)** Association between CD4⁺Tcm cell MMP-Low, CD8⁺Tn cell MMP-Low, CD8⁺Tcm cell MMP-Low, SCMM-CD3⁺T cells, SCMM-CD3⁺CD4⁺T cells, SCMM-NK cells, and circulating mtDNA levels. **(C)** Comparison of CD4⁺Tcm cell MMP-Low, CD8⁺Tn cell MMP-Low, CD8⁺Tcm cell MMP-Low, SCMM-CD3⁺T cells, SCMM-CD3⁺CD4⁺T cells, and SCMM-NK cells between inactive and active SLE groups. Statistically significant differences were obtained in CD4⁺Tcm cell MMP-Low, CD8⁺Tn cell MMP-Low, CD8⁺Tcm cell MMP-Low, SCMM-CD3⁺T cells, SCMM-CD3⁺CD4⁺T cells, and SCMM-NK cells between the inactive SLE group ($n = 17$) and active SLE group ($n = 35$). P values were determined using Student's t -tests, Mann-Whitney U -tests, and Spearman's correlation test. $P < 0.05$ indicated statistical significance. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

starvation by decreasing MM (Chourasia et al., 2015). Some studies showed that insufficient mitophagy could appear in T cells of SLE patients, resulting in increased MM and contributing to immune dysregulation in SLE (Caza et al., 2014; Xu et al., 2020). Dysregulated type I interferon (IFN) signaling is a hallmark of SLE (Caielli et al., 2016), contributing to the breakdown of immune tolerance and the maintenance of autoimmune reactions in SLE (Eloranta and Rönnblom, 2016). The loss of mitophagy can lead to the accumulation of ROS and mtDNA, which, in turn, promotes the release of type I IFNs (Halfon et al., 2024; Harris et al., 2018). Activated IFN α signaling in SLE monocytes leads to increased mitochondrial oxidative stress, characterized by elevated levels of ROS and mtDNA (Gkirtzimanaki et al., 2018). Additionally, another study has shown that SLE CD8⁺ T cells with a high type I IFN signature exhibit enlarged mitochondria and impaired mitochondrial metabolism, including reduced spare respiratory capacity (Buang et al., 2021). Therefore, mitochondrial dysfunction resulting from impaired mitophagy may be linked to the IFN signature observed in patients with SLE.

Mitochondrial biogenesis is the process of producing new mitochondrial offspring to maintain an adequate mitochondrial number and could increase MM and metabolic capacity in cells (Chang et al., 2022; Ding et al., 2021). Increased mitochondrial biogenesis can also be observed in T cells of SLE patients, contributing to the excessive production of reactive oxygen intermediates (ROIs) and the spread of oxidative stress (Perl, 2013). Oxidative stress can cause abnormal activation of cell-death signals and further trigger the release of nuclear debris from apoptotic and necrotic cells, thereby promoting the production of autoantibodies and causing immune system dysfunction, which participates in the pathogenesis of SLE (Perl, 2013; Perl et al., 2011).

Mitochondria constantly maintain a dynamic balance between local fission and fusion events to ensure structural stability and sufficient energy production for cellular metabolism (Ding et al., 2021). However, unbalanced fission and fusion events can alter MM and cause mitochondrial dysfunction (Yu et al., 2020). Dynamin-related protein 1 (Drp1) is a key molecule involved in mitochondrial fission and mitophagy (Halfon et al., 2024; Buang et al., 2021). The depletion of Drp1 can lead to increased mitochondrial fusion, impaired mitophagy, and increased MM (Caza et al., 2014). In peripheral blood lymphocytes of patients with SLE, Drp1 levels are significantly decreased compared to those in HCs. This reduction in Drp1 leads to mitochondrial hyperactivation, characterized by enlarged mitochondria, increased MM, and disrupted mitochondrial homeostasis (Caza et al., 2014).

Therefore, increased MM might reflect mitochondrial dysfunction and the alteration of cell function. In this study, SCMM was calculated by detecting the MFI of lymphocyte subsets using MitoTracker's fluorescent probe by flow cytometry (Pang et al., 2022). It was then divided by the count of the corresponding lymphocyte subsets to obtain the SCMM of each lymphocyte subset (Pang et al., 2022). MMP refers to the voltage difference between the inner and outer mitochondrial membranes, reflecting the ability to synthesize ATP (Lee et al., 2002). Increased MMP levels indicate mitochondrial hyperpolarization (MHP). Persistent MHP can occur in T cells of SLE patients, leading to increased ROI production, mitochondrial biogenesis, and MM (Gergely et al.,

2002; Nagy et al., 2004). Meanwhile, elevated MMP levels may inhibit mitophagy (Wan et al., 2022). Therefore, these two markers may reflect mitochondrial disturbance and mitochondrial energy metabolism in immune cells (Hu et al., 2022; Zhou et al., 2024).

It has been reported that the imbalanced distribution of circulating lymphocyte subsets has existed in patients with SLE (Lee et al., 2002; Torres-Ruiz et al., 2018; Picca et al., 2017). In our study, we observed that the proportion of CD3⁺CD8⁺T cells was increased in patients with SLE compared to HCs, while the proportion of NK cells was decreased. CD3⁺CD8⁺T cells and NK cells are important cytotoxic cells. However, Li et al. (2022) reported that the reduced cytolytic activity in CD3⁺CD8⁺T cells among patients with SLE results in higher rates of infection and the sustenance of autoimmunity. Patients with SLE exhibited elevated SCMM and decreased MMP-low in CD3⁺CD8⁺T cells in this study, indicating that mitochondrial dysfunction was observed in CD3⁺CD8⁺T cells of patients with SLE. Buang et al. (2021) also observed that CD8⁺ T cells with enlarged mitochondria and lower spare respiratory capacity in patients with SLE, which is associated with type I IFN exposure in SLE. Meanwhile, the cytotoxicity of NK cells is markedly suppressed in patients with SLE, contributing to immune system dysregulation in SLE (Park et al., 2009; Luo et al., 2022). However, no mitochondrial abnormalities were found in NK cells of patients with SLE because no significant differences in SCMM and MMP-Low of NK cells were observed in patients with SLE and HCs. Thus, the potential reason for NK cell impairment might not be related to mitochondrial dysfunction in patients with SLE. Although the distribution of CD3⁺CD4⁺T cells was not altered in patients with SLE, both SCMM and MMP-Low in CD3⁺CD4⁺T cells showed a significant difference between patients with SLE and HCs. Previous studies also reported that increased MM and hyperpolarization in CD4⁺T cells of patients with SLE lead to mitochondrial dysfunction (Caza et al., 2012).

CD3⁺CD4⁺T cells and CD3⁺CD8⁺T cells were further subdivided into subsets based on the expression of CD45RA and CD62L. The proportion of CD4⁺Tn cells was significantly decreased in patients with SLE compared to HCs, while the proportion of CD4⁺ Tcm cells was significantly increased in this study. This result is consistent with previous studies (Yuan et al., 2022; Sobel et al., 2011; Ugarte-Gil et al., 2015), indicating that the immune system was overactivated in SLE, leading to the process of proliferation and differentiation in naïve T cells. Furthermore, we also observed mitochondrial abnormalities in CD4⁺Tn cells, with higher SCMM and lower MMP-Low levels in patients with SLE compared with HCs, indicating that increased mitochondrial biogenesis and mitochondrial hyperpolarization may promote the activation of immune cells (Perl et al., 2012). The proportions of CD8⁺Tn, CD8⁺Tef, CD8⁺Tem, and CD8⁺Tcm cells showed no significant alteration between patients with SLE and HCs, in accordance with the data from Kajihara et al. (2023). However, CD8⁺ T cell subsets showed mitochondrial abnormalities in patients with SLE. Type I IFN is overproduced in patients with SLE, contributing to breaking the peripheral tolerance. Furthermore, type I IFN affects the mitochondrial function in immune cells by promoting the production of oxidative phosphorylation, especially in memory CD8⁺ T cells (van der Windt et al., 2012). Therefore, the overexpression of type I IFN affects the mitochondrial function of CD8⁺ T cell subsets in patients with SLE.

We observed a significantly higher level of circulating mtDNA in patients with SLE than in HCs, especially in active SLE. This result is consistent with the data from [Giaglis et al. \(2021\)](#). Human mtDNA is a double-stranded and closed-circular DNA molecule located in the mitochondrial matrix, which encodes electron transport chain proteins that are essential for ATP production ([Clayton, 1984](#)). The altered mtDNA level contributes to the process of mitochondrial dysfunction by enhancing the immune response ([Malik and Czajka, 2013](#)). Meanwhile, we found that MMP-Low and SCMM of some immune cells were associated with the level of circulating mtDNA. In addition, mtDNA also serves as self-antigens that can be recognized by DNA autoantibodies in patients with SLE, which contributes to SLE pathogenesis ([Chen and Tsokos, 2022](#)). The release of mtDNA results from defective mitophagy and increased mitochondrial ROS in SLE. Extracellular mtDNA can be recognized as a danger-associated molecular pattern (DAMP) due to its unmethylated CpG sequences, which structurally resemble bacterial DNA; this induces a type I IFN response via the activation of the cyclic GMP-AMP synthase (cGAS)–stimulator of interferon genes (STING) pathway or the endosomal TLR9 pathway ([Halfon et al., 2024](#); [Chen and Tsokos, 2022](#); [Lood et al., 2016](#); [West et al., 2015](#)). Type I IFN could further drive B cells to produce autoantibodies in SLE ([Hamilton et al., 2018](#)). Therefore, elevated levels of circulating mtDNA play a proinflammatory role in the pathogenesis of SLE. In this study, ROC analysis showed that circulating mtDNA could identify patients with SLE from HCs with high sensitivity, while the specificity and AUC were relatively poorer. Further studies with larger sample sizes are needed to confirm the diagnostic value of circulating mtDNA in SLE.

Previous studies have shown that some immune cell subsets or the co-stimulatory molecule expression on these subsets could be used to diagnose and evaluate disease activity in patients with SLE ([Park et al., 2020](#); [Men et al., 2019](#); [Miao et al., 2016](#); [Mortezagholi et al., 2016](#); [Ugarte-Gil et al., 2016](#)). In this study, several parameters demonstrated a good diagnostic value in patients with SLE, including CD8⁺ Tn MMP-Low, CD8⁺ Tem MMP-Low, CD8⁺ Tcm MMP-Low, and SCMM-CD8⁺ Tn. Thus, these indicators may serve as potential biomarkers for SLE diagnosis. Both MMP-Low and SCMM of CD4⁺ T cell subsets did not show good diagnostic performance due to AUC values below 0.8. However, we found that CD4⁺ Tcm MMP-Low and SCMM-CD3⁺CD4⁺ T cells showed a significant but weak correlation with the SLEDAI-2K and circulating mtDNA. This weak correlation suggests that CD4⁺ Tcm MMP-Low and SCMM-CD3⁺CD4⁺ T cells play a limited role in reflecting disease activity. Additionally, both markers showed significant alterations between inactive and active SLE. Therefore, future studies should enroll a larger sample size to validate whether CD4⁺ Tcm MMP-Low and SCMM-CD3⁺CD4⁺ T cells can serve as effective biomarkers for reflecting SLE disease activity.

There are several limitations to this study. First, mitochondrial parameters can be influenced by various biological and environmental factors, such as medication, stress, circadian rhythms, and infections. Our study excluded individuals with infections, but factors like treatment, stress, and circadian rhythms could still act as confounders that might affect mitochondrial parameter results. Future studies should account for these potential confounders to ensure more reliable findings. Second, the sample size was relatively small, and the number of individuals in the HC

and SLE groups was not equal, which may reduce the statistical power to detect significant relationships. The *post hoc* power analysis showed that the statistical power for the percentage of CD4⁺Tn, CD4⁺Tcm, and MMP-Low B cells was lower than 0.8, likely due to the limited sample size. This low power increases the risk of Type II errors and may prevent the detection of true difference in these parameters between HC and SLE groups. The small sample size may also be associated with higher variability in the estimates, making the sample statistics less likely to accurately reflect the population parameters. Additionally, the small sample size may introduce selection bias, where the characteristics of the included subjects do not accurately reflect those of the broader population. Therefore, more patients with SLE and HCs should be included in further investigation. Third, this study lacks a validation cohort, and we did not include disease controls to evaluate the utility of mitochondrial alterations in the diagnosis of patients with SLE. This omission indicates that whether these mitochondrial alterations are unique to SLE remains unclear. Meanwhile, we did not compare the diagnostic value of MMP-Low and SCMM in immune cells with existing SLE biomarkers in this study. More studies involving both patients with SLE and patients with other autoimmune diseases are needed to further validate the diagnostic value of MMP-Low and SCMM in immune cells and explore their performance compared to traditional biomarkers. Finally, our study applied a cross-sectional design, which could not reflect the predictive values of MMP-Low and SCMM in immune cells for SLE disease activity and their mechanistic causal roles in the pathogenesis of SLE. Future studies need to use mitochondrial inhibitors or Seahorse metabolic assays to investigate the alteration of the metabolic state in immune cells of SLE.

Conclusion

In conclusion, our findings indicate that patients with SLE exhibit mitochondrial dysfunction, as evidenced by detecting MMP-Low and SCMM in immune cells and circulating mtDNA levels. The level of mitochondrial impairment in T lymphocytes was significantly more severe in patients with SLE than in HCs. CD8⁺ Tn MMP-Low, CD8⁺ Tem MMP-Low, CD8⁺ Tcm MMP-Low, and SCMM-CD8⁺ Tn cells may serve as potential biomarkers for SLE diagnosis. Furthermore, CD4⁺ Tcm MMP-Low and SCMM-CD3⁺CD4⁺ T cells may reflect the disease activity of patients with SLE. These outcomes help provide novel mitochondrial-based insights into the pathogenesis of SLE.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by the Research Ethics Commission of Peking Union Medical College Hospital. The studies were conducted in accordance with the local legislation

and institutional requirements. The ethics committee/institutional review board waived the requirement for written informed consent for participation from the participants or the participants' legal guardians/next of kin as consent had already been waived for the remaining routine test samples.

Author contributions

GZ: Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft, and Writing – review and editing. HL: Formal analysis, Investigation, Software, Writing – original draft, and Writing – review and editing. YM: Investigation and Writing – review and editing. LC: Investigation and Writing – review and editing. YC: Investigation and Writing – review and editing. YH: Investigation and Writing – review and editing. HZ: Investigation and Writing – review and editing. YmL: Investigation and Writing – review and editing. YJ: Conceptualization, Writing – original draft. SW: Formal analysis, Writing – original draft. YzL: Conceptualization, Funding acquisition, Supervision, and Writing – review and editing. RZ: Conceptualization, Funding acquisition, Supervision, and Writing – review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

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Supplementary material

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Life's Crucial 9 score and chronic kidney disease: insights from NHANES 2005–2018 and the mediating role of systemic inflammation and oxidative stress

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Background: Chronic kidney disease (CKD) is a growing global health burden, closely linked to metabolic and cardiovascular risk factors. Life's Crucial 9 (LC9) is a novel health assessment tool that expands upon Life's Essential 8 (LE8) by incorporating mental health (depression) as a key component. This study aimed to investigate the association between LC9 and CKD, compare its predictive value with LE8, and explore potential mediating mechanisms.

Methods: This study analyzed data from 16,431 participants in the National Health and Nutrition Examination Survey (NHANES) 2005–2018. Logistic regression models were used to assess the association between LC9 and CKD, with comparisons to LE8. Restricted cubic spline models were applied to explore potential nonlinear relationships. Mediation analysis was conducted to evaluate whether systemic inflammation and oxidative stress mediated the association between LC9 and CKD. Receiver operating characteristic (ROC) analysis was performed to compare the predictive performance of LC9 and LE8 for CKD risk.

Results: Higher LC9 scores were significantly associated with a lower risk of CKD in both continuous and quartile-based analyses. A nonlinear relationship was observed between LC9 and CKD risk (P for nonlinearity < 0.001). Mediation analysis indicated that systemic immune-inflammation index (SII) and uric acid partially mediated the association between LC9 and CKD, with mediation proportions of 3.32 and 11.13%, respectively. ROC analysis showed that LC9 and LE8 had comparable predictive abilities for CKD.

Conclusion: Higher LC9 scores are associated with a reduced risk of CKD, with systemic inflammation and uric acid levels partially mediating this relationship. These findings highlight the importance of comprehensive lifestyle and mental health interventions in CKD prevention and management.

KEYWORDS

CKD, LC9, LE8, mediation analysis, NHANES

1 Introduction

Cardiovascular health (CVH) metrics have undergone significant evolution since the American Heart Association (AHA) first introduced Life's Simple 7 (LS7) in 2010 (1, 2). This framework, comprising seven modifiable factors—diet, physical activity (PA), smoking, body mass index (BMI), blood pressure, blood glucose, and total cholesterol—aimed to standardize cardiovascular risk assessment and promote preventive strategies (2). LS7 demonstrated robust associations with reduced risks of cardiovascular disease (CVD), diabetes, and mortality, establishing itself as a cornerstone in public health initiatives (2–4). However, emerging evidence highlighted gaps in its comprehensiveness, particularly regarding sleep health and refined scoring algorithms. In response, the AHA unveiled Life's Essential 8 (LE8) in 2020, integrating sleep health as an eighth component and revising scoring methodologies (5). While maintaining continuity with LS7's core principles, LE8 expanded the operationalization of CVH by incorporating sleep metrics, subsequently demonstrating associations with a broader spectrum of health outcomes—from nonalcoholic fatty liver disease to all-cause mortality—in population-based studies (6, 7).

The application of LE8 has extended beyond cardiovascular outcomes to chronic conditions such as chronic kidney disease (CKD), a condition intricately linked to cardiovascular pathology (8, 9). CKD and CVD share overlapping risk factors, including hypertension, atherosclerosis, diabetes, and systemic inflammation (10, 11). For example, atherosclerosis is one of the leading causes of morbidity and mortality in CKD and is also closely involved in the onset and progression of CVD (11, 12). Recent studies leveraging LE8 have demonstrated its inverse association with CKD incidence, suggesting that optimal CVH metrics may mitigate renal injury through shared pathways (8). For instance, adherence to LE8's dietary and PA guidelines may improve blood pressure regulation and glucose metabolism, both well-established contributors to CKD progression (13, 14). Despite these advances, the exclusion of psychological health from LE8 has sparked debate. Mental well-being, particularly depression, is increasingly recognized as an important influencing factor of cardiovascular and renal outcomes, yet its integration into CVH metrics remains unresolved (15, 16).

This gap prompted the conceptualization of Life's Crucial 9 (LC9), an exploratory framework combining LE8 with psychological health, operationalized through depression screening tools like the Patient Health Questionnaire-9 (PHQ-9) (17). Although not yet formally endorsed by the AHA, LC9 builds on accumulating evidence linking depression to adverse cardiometabolic outcomes. Depression affects over 300 million individuals globally and is associated with maladaptive health behaviors and physiological dysregulation (18). Notably, the prevalence of depression among patients with CKD is high and closely related to poor prognosis (19). Despite these insights, no studies have evaluated whether incorporating depression into CVH metrics enhances CKD risk prediction compared to LE8 alone.

Furthermore, the biological pathways connecting CVH metrics to CKD remain underexplored. Oxidative stress and inflammation are established mediators of renal injury, driving glomerulosclerosis and tubular atrophy (20, 21). At the same time, oxidative stress and inflammation also play critical roles in the pathogenesis and progression of CVD, and targeting these

pathways has been shown to be an effective strategy for the prevention and treatment of CVD (22, 23). LC9's components, such as healthy diet and PA, may attenuate these pathways by reducing oxidative stress and inflammation (24, 25). Similarly, depression is associated with elevated oxidative stress and systemic inflammatory, suggesting that LC9's inclusion of psychological health may offer a more holistic representation of CKD risk mechanisms (26, 27). However, whether LE8 or LC9 exerts protective effects on CKD through these mediators remains unexamined.

To address these knowledge gaps, this study utilized data from the National Health and Nutrition Examination Survey (NHANES) (2005–2018) to achieve three objectives: (1) compare predictive accuracy of LE8 and LC9 for CKD risk, (2) evaluate the incremental predictive value of depression beyond LE8 for CKD risk, and (3) investigate the mediating roles of oxidative stress (bilirubin; uric acid) and systemic inflammation (systemic immune-inflammation index, SII; systemic inflammation response index, SIRI) in these relationships. This study examines how oxidative stress and systemic inflammation mediate the link between LC9 scores and CKD risk, identifying modifiable pathways for prevention.

2 Methods

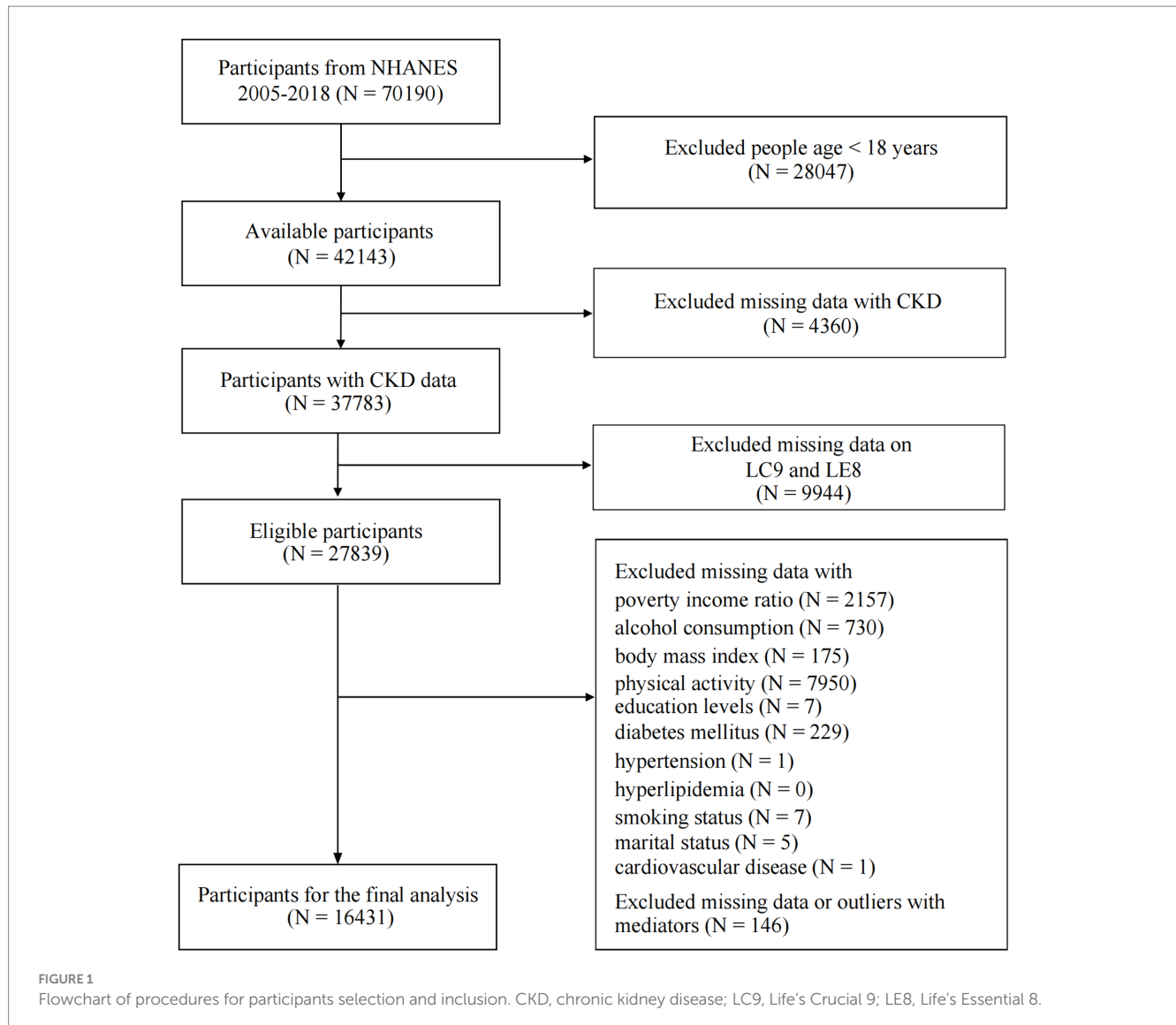
2.1 Study design and participants

This cross-sectional study utilized data from the NHANES, a nationally representative program conducted by the National Center for Health Statistics (NCHS) to assess the health status of the non-institutionalized U.S. residents.

We analyzed seven consecutive cycles from 2005 to 2018. The decision to focus on this period was based on the following considerations: (1) The necessary variables required for calculating LC9 became fully accessible starting in the 2005 cycle. (2) Excluding the 2019–2020 cycle ensured consistency in data collection protocols, avoiding potential biases introduced by disruptions related to the COVID-19 pandemic. Initially, 70,190 participants were included in the dataset. However, after excluding individuals who were under 18 years old, had missing data, or presented abnormal blood cell values, a final sample of 16,431 participants remained for analysis. [Supplementary Figure S1](#) presents box plots illustrating the distribution of lymphocytes, monocytes, neutrophils, and platelets, highlighting outlier identification and exclusion steps undertaken to improve data reliability. The detailed flowchart of the inclusion and exclusion process is presented in [Figure 1](#). By applying survey weights, our final study population represents an estimated 132,069,346 weighted individuals in the U.S. population.

2.2 Ethics statement

This study was carried out under the guidance of the NCHS and received ethical approval from the NCHS Institutional Review Board (IRB). Informed consent was obtained from all eligible participants before data collection and health assessments, ensuring adherence to ethical research principles and regulatory standards.



2.3 Definitions of LC9 and LE8

The LC9 score is a composite measure of cardiovascular and mental health, integrating eight key CVH metrics along with a depression assessment. It builds upon the LE8 framework while incorporating mental well-being as a crucial component of overall health.

The LC9 score consists of four health behaviors and four health factors, supplemented by a depression metric. Health behaviors include diet quality, nicotine exposure, PA and sleep duration. Health factors include BMI, blood pressure, blood glucose and blood lipids. The inclusion of depression is based on growing evidence linking mental health to cardiometabolic outcomes and chronic disease progression (5, 17). Diet quality was evaluated using the Healthy Eating Index (HEI) 2015, which measures adherence to dietary guidelines. Dietary data were obtained from two 24-h dietary recalls and analyzed using the U.S. Department of Agriculture's food pattern equivalents database. The HEI-2015 was computed using an established algorithm provided by the National Cancer Institute. The data of nicotine exposure, PA and sleep duration were assessed

through standardized self-reported questionnaires. BMI was calculated as weight (kg) divided by height (m²), with classifications based on standard BMI categories. Blood pressure was measured during standardized physical examinations. Blood glucose and blood lipids were assessed through blood samples analyzed in centralized laboratories. Depression score was assessed using the PHQ-9, a validated tool for screening depressive symptoms.

Each of the eight CVH metrics was scored on a continuous scale from 0 to 100 points, based on a modified Delphi approach that aligns with expert consensus on health risk stratification (28). The LC9 score was then calculated as the mean of the eight LE8 components plus the depression score, ensuring that mental health was integrated into the overall assessment (5). In contrast, the LE8 score was calculated without considering the depression component. This expanded framework allows LC9 to serve as a more holistic health index that captures both physical and psychological dimensions of chronic disease risk. While the LC9 score is conceptually grounded in the LE8 framework and supported by emerging literature on mental health integration, it remains an exploratory construct. To date, LC9 has not undergone formal independent validation. Nevertheless, a growing

body of research has demonstrated its potential utility in predicting a broad spectrum of health outcomes, including cardiovascular events, biological ageing, and all-cause mortality (5, 29). These findings suggest that LC9 may offer a more comprehensive and integrative approach to health risk assessment, particularly by capturing psychological dimensions that are not reflected in LE8 alone.

Notably, according to the referenced literature, both fasting and non-fasting blood samples can be used to assess blood glucose and blood lipids, with slight variations in calculation methods (28). In this study, we utilized non-fasting blood samples for these measurements.

2.4 Definitions of CKD

CKD is diagnosed when structural or functional kidney abnormalities persist for at least 3 months (30). This condition is identified by either a reduced estimated glomerular filtration rate (eGFR) of less than 60 mL/min/1.73m², an increased urinary albumin-to-creatinine ratio (UACR) of 30 mg/g or higher, or both (8). The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation, which relies on serum creatinine levels, is commonly used to estimate eGFR. Between 2005 and 2016, the NHANES study assessed serum and urinary creatinine levels using the Jaffe rate method. In contrast, for the 2017–2018 cycle, these measurements were conducted using enzymatic assays. Urinary albumin concentrations were determined through a fluorescence-based immunoassay.

2.5 Definition of potential mediators

In this study, we selected four potential mediators to represent two key biological processes: systemic inflammatory (SIRI and SII) and oxidative stress (bilirubin and uric acid). Each mediator is described in detail below. Both SIRI and SII serve as biomarkers for systemic inflammation (31). $SIRI = (\text{neutrophil count} \times \text{monocyte count}) / \text{lymphocyte count}$, and $SII = (\text{platelet count} \times \text{neutrophil count}) / \text{lymphocyte count}$. Bilirubin is a metabolic byproduct of hemoglobin breakdown and plays a dual role as both an antioxidant and a marker of liver function. Changes in bilirubin levels have been associated with increased oxidative stress and disturbances in metabolic homeostasis (32). Uric acid, another metabolic byproduct, originates from purine metabolism and serves as a key indicator of metabolic health (33).

2.6 Definition of covariates

Detailed descriptions of data collection and classification methods for hyperlipidemia, hypertension, and diabetes mellitus (DM) are provided in [Supplementary Table S1](#), while the definition of cardiovascular disease (CVD) follows criteria established in prior research (34). Participants completed a PA questionnaire that captured details of activities performed over the past 30 days. The questionnaire documented activity type, frequency, and intensity, classifying them into moderate and vigorous activity levels based on their physiological impact (35, 36). Moderate-intensity activities were those causing a slight increase in breathing and heart rate, whereas vigorous-intensity activities led to substantial elevations in both parameters. The total physical activity

volume (PA total MET) was estimated by summing the Metabolic Equivalent (MET) scores from activities related to work, recreation, and transportation. Age and Poverty-Income Ratio (PIR) were converted into categorical variables according to the classification scheme outlined in [Table 1](#). Further details regarding the definitions and classifications of covariates can be found in previously published studies (31).

2.7 Statistical analyses

For this study, we utilized data spanning the 2005 to 2018 NHANES survey cycles, incorporating a total of seven consecutive cycles. Following the analytical guidelines provided on the NHANES official website, appropriate survey weights were applied to ensure accurate statistical analyses. Descriptive statistics were used to summarize the characteristics of study participants. Continuous variables were expressed as means with standard deviations (SD), while categorical variables were presented as frequencies and percentages. To compare differences between groups based on CKD status, Chi-square tests were used for categorical variables, and analysis of variance (ANOVA) was applied for continuous variables. Before conducting regression analyses, collinearity diagnostics were performed on all covariates. Variance inflation factors (VIFs) were calculated to assess multicollinearity, with all covariates yielding VIFs values below 3, suggesting no significant collinearity issues. All statistical analyses were carried out in R Studio (version 4.3.1) using the *nhanesR* package (version 0.9.4.3), adhering to the STROBE guidelines.

The LC9 and LE8 scores were categorized into quartiles (Q1–Q4), ranging from the lowest to highest levels, as outlined in [Supplementary Table S2](#). To explore the relationship between LC9 and CKD, weighted logistic regression models were implemented. Associations were reported as odds ratios (ORs) with 95% confidence intervals (CIs). To verify the assumptions of logistic regression, the linearity between continuous independent variables and the logit(p) transformation was examined. Subgroup analyses were conducted to determine whether the relationship between LC9 and CKD varied across different population strata, such as age, sex, and BMI categories. This approach ensured that findings remained consistent across different demographic and clinical groups. Sensitivity analyses were also performed by conducting regressions without applying survey weights to assess the robustness of the results.

A histogram was used to visualize the distribution of LC9 ([Figure 2A](#)). To evaluate the potential nonlinear relationship between LC9 and CKD, restricted cubic spline (RCS) analysis was employed. *p*-values for nonlinearity were calculated to assess statistical significance. Due to the high collinearity between LC9 and LE8 (VIF = 26), we avoided including both variables in the same regression model for comparison. We used receiver operating characteristic (ROC) curve analysis to compare their predictive accuracy for CKD risk, as this method is not affected by multicollinearity. Z-tests were used to determine whether the predictive performance of LC9 and LE8 significantly differed.

A mediation analysis was performed using the “Mediation” package in R. As depicted in [Figure 3A](#), the analysis followed a two-step approach. (1) Path a: regression models were used to assess the effect of LC9 on the mediators. (2) Next, after accounting for the mediators, mediators’ impact on CKD (path b) was assessed, along with the effect of LC9 on CKD (path c’). Indirect effect = path a*path b. The mediation

TABLE 1 Descriptive characteristics of the study population stratified by CKD.

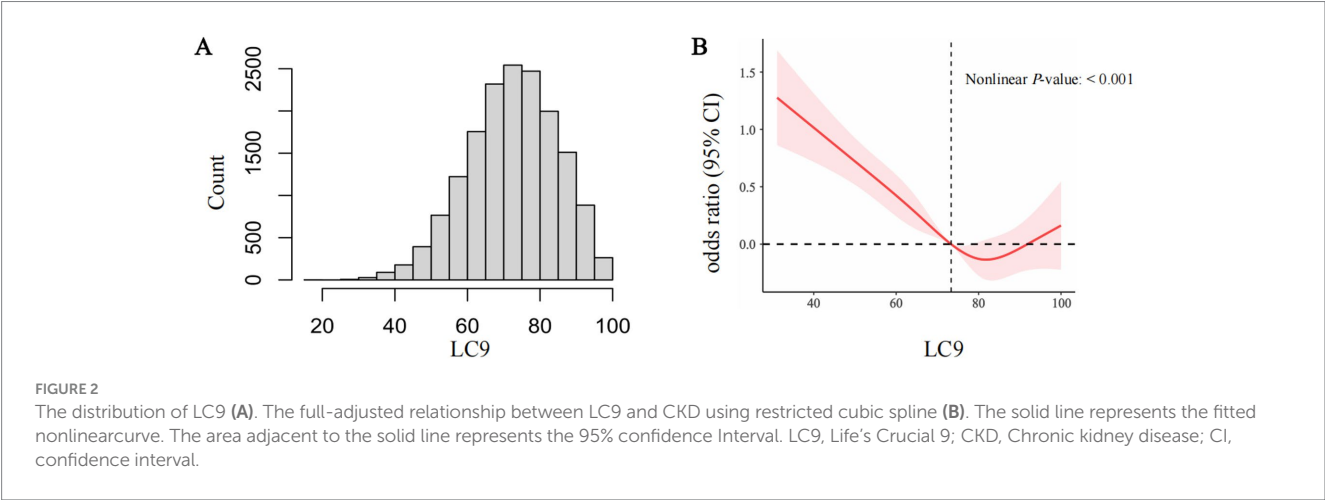
Characteristic	Total	Non-CKD	CKD	<i>p</i> -values
	(<i>N</i> = 16,431)	(<i>N</i> = 14,192)	(<i>N</i> = 2,239)	
LC9	74.05 ± 0.23	74.69 ± 0.23	68.73 ± 0.54	< 0.001
LE8	72.03 ± 0.24	72.70 ± 0.24	66.44 ± 0.55	< 0.001
Bilirubin (mg/dl)	0.67 ± 0.01	0.68 ± 0.01	0.65 ± 0.01	
Uric acid (mg/dl)	5.44 ± 0.02	5.39 ± 0.02	5.90 ± 0.06	< 0.001
SII	526.30 ± 4.18	519.74 ± 4.41	580.38 ± 9.91	< 0.001
SIRI	1.22 ± 0.01	1.19 ± 0.01	1.46 ± 0.04	< 0.001
PA total MET (MET)	4570.2 ± 95.9	4687.8 ± 104.9	3600.1 ± 176.3	< 0.001
Age, <i>n</i> (%)				< 0.001
18–29	3,037 (20.37)	2,886 (95.06)	151 (4.94)	
30–44	4,452 (28.15)	4,138 (93.58)	314 (6.42)	
45–59	4,198 (29.18)	3,749 (91.24)	449 (8.76)	
> =60	4,744 (22.31)	3,419 (75.62)	1,325 (24.38)	
Sex, <i>n</i> (%)				< 0.001
Female	7,867 (48.28)	6,760 (87.82)	1,107 (12.18)	
Male	8,564 (51.72)	7,432 (90.47)	1,132 (9.53)	
Race/Ethnicity, <i>n</i> (%)				< 0.001
Non-Hispanic white	7,649 (70.21)	6,588 (89.15)	1,061 (10.85)	
Non-Hispanic black	3,233 (9.63)	2,655 (85.87)	578 (14.13)	
Hispanic	3,780 (12.97)	3,363 (91.34)	417 (8.66)	
other race	1,769 (7.18)	1,586 (90.19)	183 (9.81)	
BMI, <i>n</i> (%)				< 0.001
Underweight/Normal	4,858 (31.24)	4,284 (90.74)	574 (9.26)	
Overweight	5,510 (33.79)	4,827 (90.60)	683 (9.40)	
Obese	6,063 (34.97)	5,081 (86.45)	982 (13.55)	
Marital status, <i>n</i> (%)				< 0.001
Married/Living with Partner	10,002 (63.46)	8,743 (89.81)	1,259 (10.19)	
Never married	3,208 (20.12)	2,930 (93.66)	278 (6.34)	
Widowed/Divorced/Separated	3,221 (16.43)	2,519 (81.32)	702 (18.68)	
PIR, <i>n</i> (%)				< 0.001
< 1.3	4,688 (19.51)	3,989 (87.51)	699 (12.49)	
1.3–3.5	6,089 (33.93)	5,159 (87.41)	930 (12.59)	
> 3.5	5,654 (46.57)	5,044 (91.19)	610 (8.81)	
Education levels, <i>n</i> (%)				< 0.001
Below high school	1,084 (3.22)	881 (82.40)	203 (17.60)	
High school	5,584 (30.27)	4,698 (86.88)	886 (13.12)	
Above high school	9,763 (66.51)	8,613 (90.57)	1,150 (9.43)	
Alcohol consumption, <i>n</i> (%)				< 0.001
Never	1,946 (9.46)	1,628 (86.97)	318 (13.03)	
Former	2,289 (11.05)	1,805 (83.20)	484 (16.80)	
Mild	5,959 (38.01)	5,174 (89.18)	785 (10.82)	
Moderate	2,784 (18.86)	2,477 (90.48)	307 (9.52)	
Heavy	3,453 (22.61)	3,108 (91.99)	345 (8.01)	
Smoking status, <i>n</i> (%)				

(Continued)

TABLE 1 (Continued)

Characteristic	Total	Non-CKD	CKD	<i>p</i> -values
	(<i>N</i> = 16,431)	(<i>N</i> = 14,192)	(<i>N</i> = 2,239)	
Never	9,192 (56.73)	8,068 (90.00)	1,124 (10.00)	
Former	4,001 (24.46)	3,291 (86.53)	710 (13.47)	
Now	3,238 (18.81)	2,833 (90.21)	405 (9.79)	
Hyperlipidemia, <i>n</i> (%)				< 0.001
No	4,935 (31.13)	4,516 (93.12)	419 (6.88)	
Yes	11,496 (68.87)	9,676 (87.42)	1,820 (12.58)	
Hypertension, <i>n</i> (%)				< 0.001
No	10,047 (66.35)	9,308 (93.80)	739 (6.20)	
Yes	6,384 (33.65)	4,884 (80.11)	1,500 (19.89)	
Diabetes mellitus, <i>n</i> (%)				< 0.001
No	12,518 (81.45)	11,319 (91.89)	1,199 (8.11)	
Prediabetes	1,369 (7.35)	1,151 (86.96)	218 (13.04)	
DM	2,544 (11.21)	1,722 (71.07)	822 (28.93)	
CVD, <i>n</i> (%)				< 0.001
No	15,024 (93.22)	13,280 (90.69)	1,744 (9.31)	
Yes	1,407 (6.78)	912 (68.57)	495 (31.43)	

CKD, Chronic kidney disease; LC9, Life's Crucial 9; LE8, Life's Essential 8; GGT, Serum gamma-glutamyltransferase; SII, Systemic immune-inflammation index; SIRI, Systemic inflammation response index; MET, Metabolic Equivalent; BMI, Body mass index; PIR, Poverty income ratio; CVD, Cardiovascular disease. Bold values indicate statistical significance at *P* < 0.05.



proportion = indirect effect/total effect. The total effect of LC9 on CKD was estimated without considering for mediators (path c). A bootstrap method with 500 iterations was applied to generate 95% confidence intervals for the mediation proportion, ensuring robust estimates.

3 Results

3.1 Descriptive characteristics

A total of 16,431 participants were included in this study (Table 1). The characteristics of the study population were stratified based on the presence or absence of CKD. The prevalence of CKD significantly increased with age (*p* < 0.001), with the highest prevalence observed

among participants aged ≥ 60 years (24.38%). Among the study population, 7,867 (48.28%) were female, and 8,564 (51.72%) were male. The prevalence of CKD was significantly higher among females (12.18%) compared to males (9.53%, *p* < 0.001). In terms of race, non-Hispanic Black individuals had the highest prevalence of CKD (14.13%), while Hispanics had the lowest prevalence (8.66%). BMI was also strongly associated with CKD prevalence (*p* < 0.001); CKD was more frequent in participants categorized as obese (13.55%), compared to those with overweight (9.40%) and underweight/normal (9.26%).

Socioeconomic factors were also significantly linked to CKD prevalence. The highest prevalence was found in participants who were widowed, divorced, or separated (18.68%), compared to those who were married/living with a partner (10.19%) or never married (6.34%) (*p* < 0.001). Education level was inversely associated with

CKD prevalence, with participants having below high school education showing the highest prevalence (17.60%, $p < 0.001$). Regarding lifestyle factors, former smokers (13.47%) had a higher prevalence of CKD compared to never smokers (10.00%) and current smokers (9.79%, $p < 0.001$). Former alcohol consumers had the highest CKD prevalence (16.80%).

Participants with hyperlipidemia (12.58%), hypertension (19.89%), diabetes mellitus (28.93%), and cardiovascular disease (31.43%) had significantly higher CKD prevalence compared to those without these conditions ($p < 0.001$ for all). In addition, individuals with CKD had lower LC9 and LE8 scores compared to those without CKD ($p < 0.001$), with mean LC9 scores of 68.73 ± 0.54 vs. 74.69 ± 0.23 , and mean LE8 scores of 66.44 ± 0.55 vs. 72.70 ± 0.24 . Participants with CKD also had higher levels of uric acid, SII, and

SIRI, along with lower physical activity total MET (PA total MET) values compared to those without CKD ($p < 0.001$ for all).

3.2 Binary logistic regression analysis

Binary logistic regression was performed to assess the association between LC9 score and CKD, with results summarized in Table 2. In the unadjusted model, LC9 (as a continuous variable) demonstrated a significant inverse association with CKD (OR: 0.96, 95% CI: 0.96–0.97, $p < 0.001$). This association remained robust in Model 1 (OR: 0.97, 95% CI: 0.96–0.97, $p < 0.001$) and persisted in Model 2 (OR: 0.98, 95% CI: 0.97–0.99, $p < 0.001$). When LC9 was analyzed in quartiles (Q1–Q4), in the fully adjusted Model 2, participants in the highest quartile

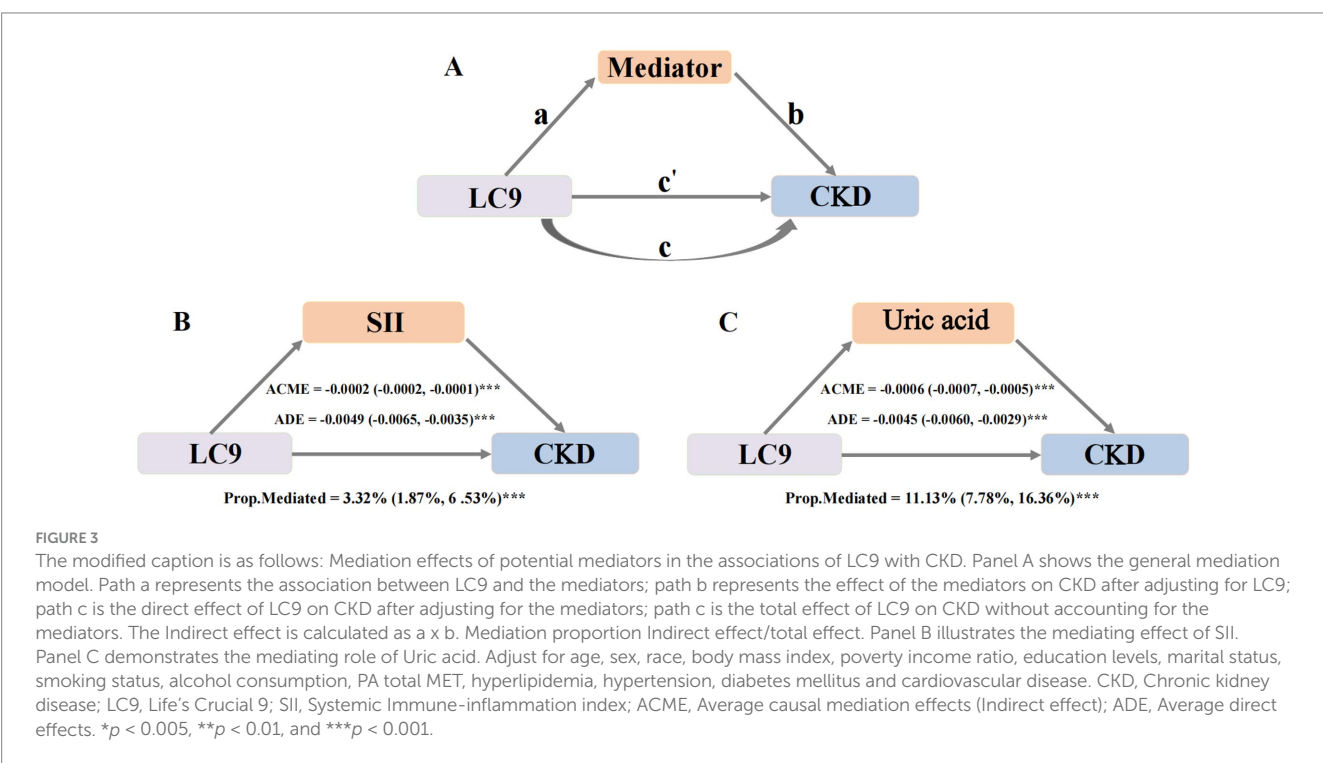


TABLE 2 Adjusted association of LC9 with CKD.

Exposure	Unadjusted model	Adjust 1	Adjust 2
	Odds ratio (95% CI) associated with CKD		
LC9 (continuous)	0.96 (0.96, 0.97); < 0.001	0.97 (0.96, 0.97); < 0.001	0.98 (0.97, 0.99); < 0.001
Quartile of LC9			
Q1	1 (Ref)	1 (Ref)	1 (Ref)
Q2	0.58 (0.50, 0.68); < 0.001	0.57 (0.48, 0.67); < 0.001	0.72 (0.59, 0.89); 0.003
Q3	0.39 (0.33, 0.46); < 0.001	0.40 (0.33, 0.49); < 0.001	0.58 (0.44, 0.77); < 0.001
Q4	0.29 (0.23, 0.36); < 0.001	0.34 (0.27, 0.44); < 0.001	0.57 (0.38, 0.85); 0.01
P for trend	< 0.001	< 0.001	0.002

Unadjusted model: non-adjusted model.

Adjust 1: Adjust for age, sex, race.

Adjust 2: Adjust for age, sex, race, body mass index, poverty income ratio, education levels, marital status, smoking status, alcohol consumption, PA total MET, hyperlipidemia, hypertension, diabetes mellitus and cardiovascular disease.

CKD, Chronic kidney disease; LC9, Life's Crucial 9; CI, Confidence interval. Bold values indicate statistical significance at $P < 0.05$.

TABLE 3 Adjusted association of LC9 with CKD for subgroup analyses.

Subgroups	Adjusted odds ratio (95% confidence interval); <i>p</i> *	<i>P</i> for interaction
Sex		0.044
Female	0.972 (0.957, 0.988); < 0.001	
Male	0.981 (0.966, 0.996); 0.015	
Age		0.008
18–29	0.984 (0.949, 1.019); 0.355	
30–44	0.965 (0.947, 0.984); < 0.001	
45–59	0.973 (0.950, 0.996); 0.025	
> =60	0.986 (0.972, 1.001); 0.070	
Body mass index		0.014
Underweight/Normal	0.987 (0.964, 1.010); 0.271	
Overweight	0.976 (0.958, 0.995); 0.013	
Obese	0.973 (0.959, 0.987); < 0.001	
Smoking status		0.576
Never	0.972 (0.958, 0.985); < 0.001	
Former	0.983 (0.963, 1.004); 0.104	
Now	0.986 (0.960, 1.014); 0.317	
Hyperlipidemia		0.084
No	0.976 (0.963, 0.989); < 0.001	
Yes	0.989 (0.965, 1.013); 0.366	
Hypertension		0.133
No	0.970 (0.955, 0.984); < 0.001	
Yes	0.989 (0.973, 1.005); 0.166	
Diabetes mellitus		0.066
No	0.983 (0.966, 1.001); 0.057	
Prediabetes	1.003 (0.975, 1.031); 0.850	
DM	0.955 (0.941, 0.971); < 0.001	
Cardiovascular disease		0.239
No	0.990 (0.960, 1.021); 0.509	
Yes	0.975 (0.962, 0.988); < 0.001	

*Adjust for age, sex, race, body mass index, poverty income ratio, education levels, marital status, smoking status, alcohol consumption, hyperlipidemia, hypertension, diabetes mellitus, triglyceride, high density lipoprotein and PA total MET, but not for the specific stratification variables of interest. Bold values indicate statistical significance at *P* < 0.05.

(Q4) had significantly lower odds of CKD compared to those in the lowest quartile (Q1) (OR: 0.57, 95% CI: 0.38–0.85, *p* = 0.01). Similarly, those in Q3 (OR: 0.58, 95% CI: 0.44–0.77, *p* < 0.001) and Q2 (OR: 0.72, 95% CI: 0.59–0.89, *p* = 0.003) also exhibited significantly lower odds of CKD relative to Q1. The *P* for trend across quartiles remained statistically significant in all models (*p* < 0.001 in the unadjusted and Model 1; *p* = 0.002 in Model 2), further reinforcing the graded relationship between higher LC9 scores and reduced CKD risk.

3.3 Subgroup analyses and sensitivity analysis

The results of the subgroup analyses are presented in Table 3. Overall, the inverse association between LC9 and CKD remained

consistent across most subgroups. However, significant interactions were observed for sex (*P* for interaction = 0.044), age (*p* = 0.008), and BMI categories (*p* = 0.014). These findings suggest that the association between LC9 and CKD may be more pronounced in certain groups, particularly females, middle-aged adults (30–59 years), and individuals with overweight or obesity.

Several possible explanations may underlie these subgroup differences. First, women may exhibit greater physiological responsiveness to cardiovascular health interventions due to sex-specific hormonal or inflammatory profiles, which may enhance the protective impact of LC9 (37). Second, middle-aged adults are often at a critical stage for both cardiovascular and renal risk accumulation, yet still within a window where lifestyle modifications could yield meaningful benefit. Third, individuals with overweight or obesity experience amplified metabolic stress and inflammation,

making them more susceptible to CKD but also more likely to benefit from improvements in LC9 components such as diet, PA, and mental health (38).

To test the robustness of the findings, sensitivity analyses were performed using an alternative model without applying survey weights (Supplementary Table S3). The results remained largely consistent with the primary analyses, confirming that higher LC9 scores were significantly associated with lower odds of CKD across all models.

In summary, subgroup and sensitivity analyses consistently supported the protective association between LC9 and CKD, with variations observed across specific subpopulations. These results highlight the potential role of LC9 in CKD prevention and underscore its applicability across diverse demographic and clinical groups.

3.4 Nonlinear relationships explore

The RCS analysis revealed a statistically significant nonlinear relationship (nonlinearity $p < 0.001$), suggesting that the association between LC9 and CKD does not follow a strictly linear pattern when using four knots (Figure 2). To further test the robustness of this finding, we conducted sensitivity analyses by varying the number of knots from 3 to 8 (Supplementary Table S4). Across all knot selections, the nonlinearity p -values remained significant (all $p < 0.001$), reinforcing the presence of a nonlinear association.

3.5 Mediation analysis

Mediation analysis was conducted to assess whether the relationship between LC9 and CKD was partially explained by systemic inflammation and oxidative stress. In this analysis, LC9 served as the independent variable, CKD as the dependent variable, and selected potential mediators as mediator variables.

First, we examined the association between LC9 and potential mediators (Supplementary Table S5). After full adjustment, SII, bilirubin and uric acid showed significant associations with LC9, suggesting that path a (the effect of LC9 on the mediator) was significant for these factors. Next, the association between potential mediators and CKD was analyzed while controlling for LC9 and all covariates (Supplementary Table S6). The results indicated that both SII, SIRI and uric acid were significantly linked to CKD, confirming the presence of path b (the effect of the mediator on CKD). However, bilirubin did not show a significant relationship with CKD and was therefore excluded from further mediation analysis.

As both path a and path b were statistically significant for SII and uric acid, these two mediators were selected for final mediation modeling (Figure 3). The analysis revealed significant indirect effects of LC9 on CKD through these mediators. SII mediated 3.32% (95% CI: 1.87–6.53%, $p < 0.001$) of the total effect. Uric acid mediated 11.13% (95% CI: 7.78–16.36%, $p < 0.001$). These findings indicate that systemic inflammation and oxidative stress contribute to the association between LC9 and CKD, reinforcing the role of systemic inflammation and uric acid levels in kidney health.

3.6 Comparing the LC9 and LE8

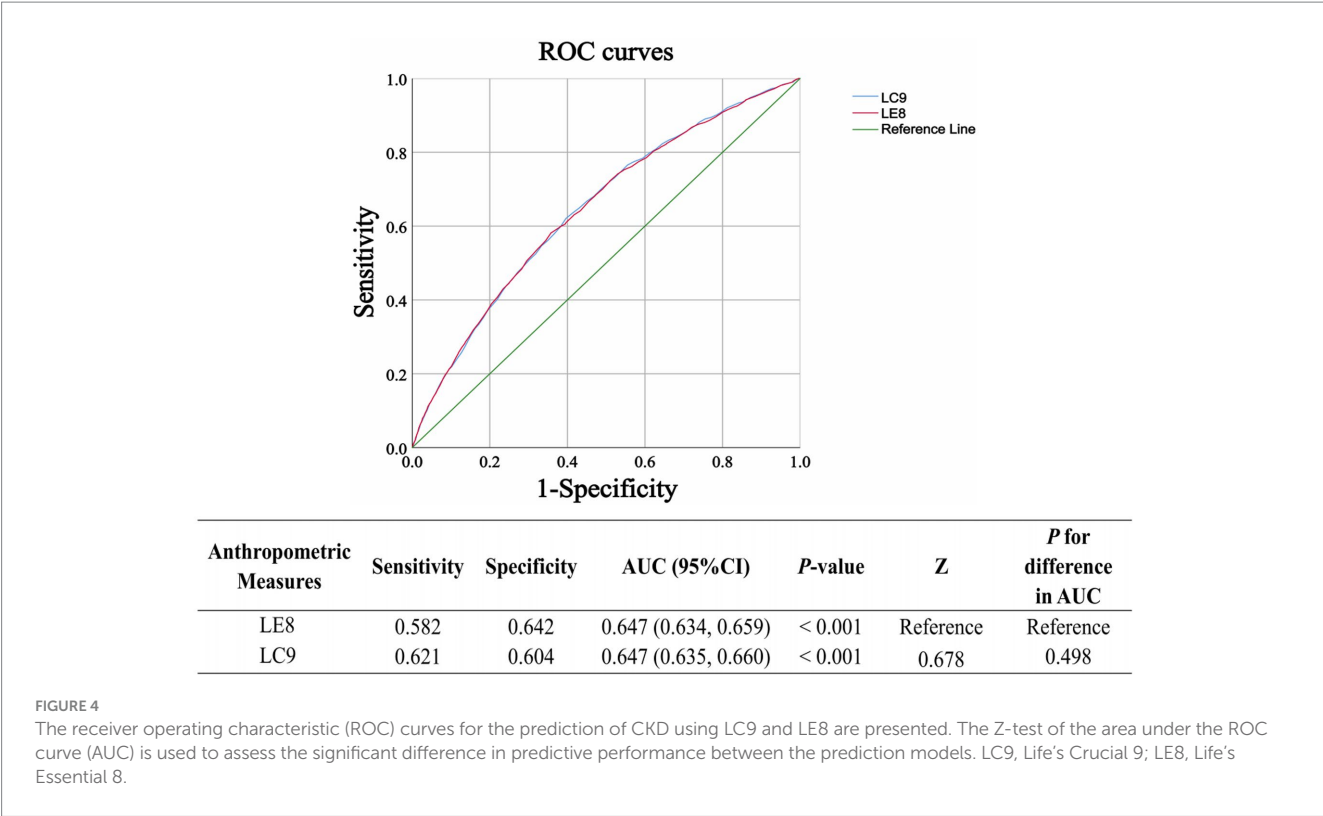
To assess the predictive performance of LC9 and LE8 for CKD, ROC curves were generated (Figure 4). The Z-test of the area under the curve (AUC) was used to compare the two models. The results indicated no statistically significant difference between the predictive abilities of LC9 and LE8 ($p = 0.498$), suggesting that both indices have similar performance in identifying individuals at risk for CKD.

4 Discussion

This study comprehensively investigated the association between LC9 and CKD, examining its predictive performance relative to LE8 and exploring potential mediating pathways. Our findings highlight the significant inverse association between LC9 and CKD, with a nonlinear relationship, and demonstrate that systemic inflammation and uric acid levels partially mediate this association. Additionally, we found that while LC9 and LE8 demonstrated similar predictive abilities for CKD.

Our analysis identified distinct patterns in CKD prevalence across socio-demographic groups. Older age, female sex, obesity, lower educational attainment, and lower socioeconomic status were associated with a higher prevalence of CKD. These findings align with previous studies analyzing national datasets, which have emphasized the influence of social determinants and health disparities on kidney disease risk (8, 30, 39). These results highlight the importance of considering these covariates when evaluating the association between LC9 and CKD. Moreover, our findings demonstrate that CKD is more prevalent in individuals with hyperlipidemia, hypertension, DM and CVD, conditions that are well-established risk factors for renal dysfunction and cardiovascular complications (10, 40–42). Hyperlipidemia has been strongly linked to glomerular injury, with studies showing that elevated lipid levels accelerate CKD progression and worsen renal outcomes (40). Similarly, hypertension is a major independent risk factor for CKD, as increased blood pressure contributes to glomerular hypertension and nephron loss, even in individuals without other metabolic disorders (43). DM and CKD are closely linked, with DM being the leading cause of kidney failure worldwide, driven by shared metabolic and cardiovascular risk factors (42). CKD and CVD are strongly interconnected, with shared common risk factors such as hypertension, diabetes, and dyslipidemia (10).

Discussing the relationship between depression and CKD is essential, as LC9 differs from LE8 by incorporating mental health factors, recognizing that depression not only coexists with CKD but also influences its progression through behavioral and biological mechanisms. Depression and CKD share a bidirectional relationship, with depressive symptoms increasing the risk of CKD onset and progression, while impaired renal function elevates the likelihood of developing depression (44). Individuals with depression often exhibit poor health behaviors such as physical inactivity, smoking, and unhealthy dietary patterns, which are also recognized risk factors for CKD (44–46). Additionally, chronic inflammation and dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis in depression may contribute to intrarenal microcirculatory dysfunction, endothelial damage, and accelerated renal decline (46–48). Conversely, CKD-associated systemic inflammation and oxidative stress may



impair neurotransmitter function and increase neuropsychiatric symptoms, fostering the onset of depression (48–51). These findings emphasize the need for early screening and management of depression in CKD patients and vice versa, as addressing both conditions concurrently may help mitigate their detrimental effects on overall health.

One of the novel contributions of this study is the identification of systemic inflammation and uric acid levels as mediators in the LC9-CKD relationship. Inflammation plays a critical role in the development and progression of CKD, contributing to renal fibrosis, endothelial dysfunction, and accelerated decline in kidney function (52). Persistent low-grade inflammation in CKD is driven by an imbalance between pro- and anti-inflammatory factors, resulting from immune system dysregulation, oxidative stress, gut dysbiosis, and impaired clearance of inflammatory mediators (52). Key inflammatory markers, including TNF- α , IL-6, and IL-1 β , are elevated in CKD and are associated with worsening renal outcomes and increased cardiovascular risk (52, 53). Furthermore, inflammasomes such as NLRP3 contribute to CKD progression by promoting pro-inflammatory cytokine release and renal injury (54). The inflammatory burden in CKD is further exacerbated by dialysis-related immune activation, highlighting the need for targeted interventions to mitigate inflammation and slow disease progression (55). Uric acid is increasingly recognized as a key player in CKD progression, contributing through multiple pathogenic mechanisms (33). Hyperuricemia has been linked to renal vasoconstriction, endothelial dysfunction, oxidative stress, and chronic inflammation, all of which accelerate glomerulosclerosis and tubulointerstitial fibrosis (33). Elevated uric acid levels promote the activation of the renin-angiotensin-aldosterone system (RAAS), leading to increased blood pressure and reduced renal perfusion, which further exacerbates

CKD progression (33, 56). Additionally, uric acid stimulates pro-inflammatory cytokine production and impairs nitric oxide bioavailability, contributing to vascular damage and kidney injury (57). The dual role of uric acid in oxidative stress is particularly relevant, as it can function as both an antioxidant extracellularly and a pro-oxidant intracellularly, with excessive uric acid levels favoring oxidative damage and renal impairment (33, 57, 58). These findings highlight the need for targeted strategies to manage uric acid levels in CKD patients, with emerging evidence suggesting that uric-acid-lowering therapies may help slow disease progression (33).

Our findings have several important implications for CKD prevention and management. Since LC9 encompasses both lifestyle behaviors and metabolic health indicators, it provides a holistic approach to CKD risk assessment. Interventions focusing on diet, physical activity, smoking cessation, metabolic health optimization and mental health may collectively reduce CKD burden. Given the mediation effects of systemic inflammation and uric acid, strategies that reduce chronic inflammation and improve metabolic health—such as anti-inflammatory dietary patterns and uric acid-lowering therapies—may be effective in preventing CKD progression. Future studies should refine risk prediction models by integrating LC9 components into CKD screening protocols.

This study has several methodological strengths: The use of a large, nationally representative dataset (NHANES) enhances generalizability to diverse U.S. populations. The incorporation of RCS analysis, mediation models, and ROC analysis provides robust analytical depth. The study comprehensively examined metabolic and inflammatory pathways, contributing to a more integrated understanding of CKD risk factors. However, several limitations should be acknowledged. First and most importantly, the cross-sectional design of this study limits the ability to establish causal

relationships. The reliance on self-reported data for lifestyle behaviors introduces potential recall bias. While LC9 and LE8 capture essential health components, additional risk factors (e.g., gut microbiota, genetic predisposition) were not included in this study and warrant further exploration (59, 60). Moreover, important covariates such as medication use and receipt of treatment for mental health conditions were not adjusted for in our models. Specifically, although NHANES includes data on antidepressant medication use, the complexity of extraction and potential for misclassification prevented its inclusion. Furthermore, psychotherapy status was not available in NHANES.

Future longitudinal cohort studies are needed to clarify the causal relationship between LC9 and CKD, and to assess whether changes in LC9 components over time are associated with kidney function outcomes. Given that LC9 primarily reflects cardiovascular health, it is particularly important to investigate CKD risk among individuals with existing cardiovascular risk factors. Future prospective research should explore whether targeted interventions aimed at improving LC9 scores in high-risk populations, such as those with hypertension, diabetes, or established cardiovascular disease, can improve cardiovascular outcomes while also reducing the onset or progression of CKD. In addition, well-designed intervention trials focusing on modifiable components of LC9, including diet, physical activity, and mental health, may help establish causal relationships and provide actionable evidence for CKD prevention.

5 Conclusion

In conclusion, this study highlights the inverse association between LC9 and CKD, with systemic inflammation and uric acid levels mediating part of this relationship. LC9 and LE8 exhibited similar predictive performance of CKD risk. These findings support the adoption of comprehensive lifestyle interventions targeting the nine components of LC9—diet quality, physical activity, nicotine exposure, sleep duration, body weight management, blood glucose control, blood pressure regulation, lipid profile, and depression—as well as addressing systemic inflammation and oxidative stress to reduce the burden of CKD. However, due to the cross-sectional design of this study, these findings should be interpreted as associations rather than causal relationships.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/[Supplementary material](#).

Ethics statement

The studies involving humans were approved by National Center for Health Statistics Ethics Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

SQ: Conceptualization, Writing – review & editing, Writing – original draft, Methodology, Visualization. JY: Writing – review & editing, Methodology. ZW: Writing – review & editing, Methodology. PH: Methodology, Writing – review & editing. TD: Writing – review & editing, Formal analysis, Data curation. ZR: Formal analysis, Data curation, Writing – review & editing. QL: Methodology, Writing – review & editing, Investigation, Conceptualization, Supervision.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The authors declare that Gen AI was used in the creation of this manuscript. During the preparation of this work the authors used ChatGPT 4.0 in order to improve language. After using this tool/service, the authors reviewed and edited the content as needed and takes full responsibility for the content of the publication.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmed.2025.1605931/full#supplementary-material>

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Association between hypertension and oxidative balance score: data from National Health and Nutrition Examination Survey 2005–2018

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Background: Hypertension, a prevalent worldwide public health issue, can result in a wide range of illnesses. The notably association between oxidative stress and the onset of hypertension has been corroborated through diverse animal models. The oxidative balance score (OBS) served as a tool to evaluate the overall systemic status of oxidative stress, indicating that higher OBS scores corresponded to greater exposure to antioxidants. However, the exact correlation between OBS and hypertension is unclear. Therefore, we aimed to investigate whether adult OBS is attached to hypertension.

Methods: There are 28,035 participants who were chosen from the National Health and Nutrition Examination Survey (NHANES) conducted between 2005 and 2018. The presence of hypertension was determined through a questionnaire. Twenty food and lifestyle parameters were used to score OBS. The connection between OBS and hypertension has been examined via weighted logistic regression and smoothing curves.

Results: The percentage of people with hypertension stood at 41.72%. In comparison to the first quartile of OBS, the adjusted odds ratios for the highest OBS quartile and hypertension were 0.81 (95% CI: 0.70–0.93), with a *p*-value for trend of 0.002. Age was the factor most strongly linked with both in stratified analysis.

Conclusions: OBS demonstrated a strong negative association with hypertension, particularly in the younger population (<60 years). These findings highlighted the importance of following an antioxidant-rich diet and lifestyle, which aids in hypertension prevention and appears to offer greater benefits to the younger age group.

KEYWORDS

hypertension, oxidative stress, oxidative balance score, NHANES, OBS

1 Introduction

Hypertension is a prevalent cardiovascular disease in older adults. Over the past few years, hypertension has progressively grown to be a global health concern (1). From 1990–2019, the prevalence of hypertension doubled, with a total of approximately 1.2 billion adults global having hypertension, most in countries with middle or low income (2). A global blood pressure screening study found that more than a third of people have high blood pressure and approximately a half are unaware they have it (3). Since high blood pressure rarely causes symptoms on its own, it is frequently disregarded. Hypertension, as a risk factor often leads to cerebral hemorrhage, heart failure, coronary heart disease and even illness (4). Recent studies also show that high blood pressure increases the incidence of breast cancer and Parkinson's disease (5, 6). Therefore, determining the best course of action for managing high blood pressure and reducing associated consequences has taken precedence.

The pathological mechanism of hypertension presents multi-dimensional interactive characteristics, involving multi-dimensional factors such as abnormal activation of the renin-angiotensin-aldosterone system (RAAS), imbalance of immune homeostasis, metabolic regulation disorders, vascular endothelial damage and genetic susceptibility (7, 8). Previous research (9) has connected the development of hypertension to oxidative stress, and the immune system plays a core regulatory role in it (8, 10). The evidence shows that hypertensive patients present with a persistent low-grade inflammatory state (11). The synergistic effect of innate and adaptive immune responses leads to abnormal activation and infiltration of monocytes/macrophages and lymphocytes, accompanied by enrichment of proinflammatory factors and chemokines, which aggravates oxidative stress. Oxidative stress reversely promotes the release of inflammatory mediators through signaling pathways (10). The two form a cascade amplification effect, jointly driving a vicious cycle of vascular remodeling and target organ damage. Besides, Oxidative stress occurs when there is an unbalanced concentration of oxidants and antioxidants in the body, with an emphasis on oxidants (12). This imbalance can cause disruptions to redox signaling, regulation, and molecular damage (12, 13). In addition to producing reactive oxygen species (ROS) by itself, which damages and kills endothelium, oxidative stress also interacts with the body's inflammatory proteins, hastening the death of cells (14, 15). Studies have shown that reducing oxidative stress in the body improves blood vessel stiffness and improves blood pressure (16).

However, several factors have a limited effect on the total oxidant/antioxidant system. Variations in dietary composition, obesity, exercise, smoking, and other lifestyle choices impact the body's degree of oxidative stress. Thus, we selected the oxidative balance score (OBS) as a measure to quantify the effect of diverse diets and lifestyles on the entire oxidant/antioxidant system (17). OBS is a composite index of 20 distinct dietary and lifestyle elements that accentuates the overall balance of antioxidants and oxidants at the dietary level. It's acknowledged that a higher OBS indicates superior antioxidant status. Previous

researches have demonstrated an adverse association between OBS and various diseases, including metabolic syndrome (18), depression (19), breast cancer (20), and type 2 diabetes (21). Here, we explored the connection between OBS and the incidence of hypertension. We investigated the potential effects of OBS on hypertension using data from the National Health and Nutrition Examination Survey (NHANES) from 2007–2018.

2 Method

2.1 Extraction of data and study population

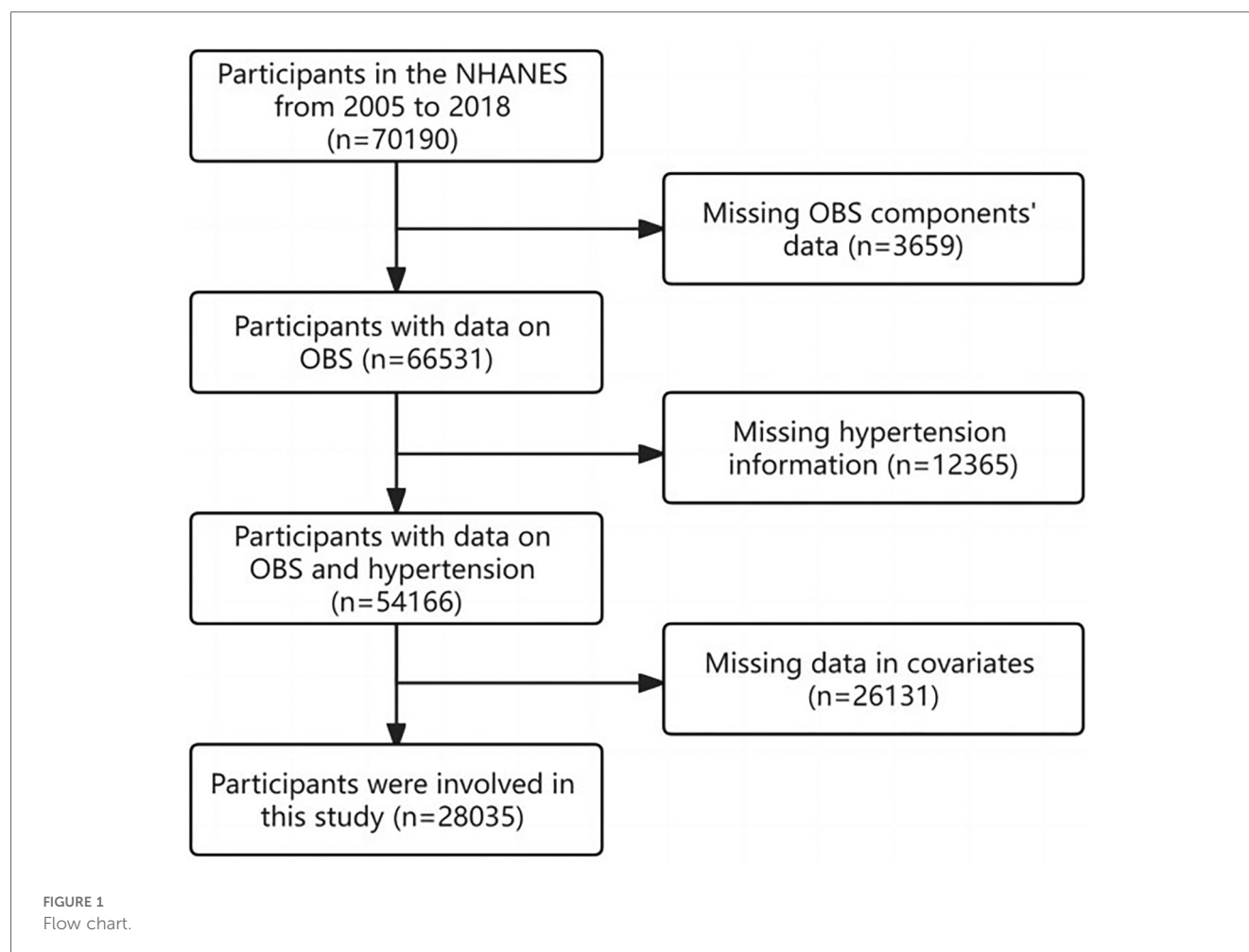
NHANES employs a multistage, stratified probability approach to pick a representative sample of US citizens. It has been approved by the Ethics Review Board of the National Center for Health Statistics, and all participants have provided written, informed consent. It comprises of interview and examination components. In this study, data from seven NHANES cycles spanning from 2005 to 2006 to 2017–2018 were utilized, resulting in an initial pool of 70,190 patients. After excluding patients lacking dietary or lifestyle data, hypertension data, and variables with missing values, 28,035 subjects were included in the study (Figure 1).

2.2 Evaluation of diabetes (outcomes)

To determine whether our patients had hypertension, we employed three different techniques. First, the presence of hypertension was confirmed if it was indicated in the medications prescribed to the patient. Second, Questionnaires have been employed to gather information about hypertension. “Has a doctor or other health professional ever told you that you have high blood pressure, also known as hypertension?” was the question that was used to measure self-reported hypertension. Prior studies have also confirmed the use of self-reported hypertension (22). Third, we computed the patient's average blood pressure, either systolic or diastolic. Patients were classified as hypertensive if their systolic blood pressure was equal to or greater than 140 mm Hg, or if their diastolic blood pressure was equal to or greater than 90 mm Hg. Therefore, hypertension was considered an outcome variable in our study.

2.3 Calculation of the oxidative balance score (exposure)

Based on a previous study (23), OBS was determined by screening 16 nutrients and 4 lifestyle factors, encompassing 15 antioxidants and 5 pro-oxidants. There was a favorable correlation between participants' antioxidant activity and higher OBS. Dietary intake of 16 nutrients, including dietary fiber, carotene, riboflavin, niacin, vitamin B6, total folate, vitamin B12, vitamin C, vitamin E, calcium, magnesium, zinc, copper, selenium, total fat, and iron, was obtained from the first dietary review interview. The four lifestyle factors are physical activity, body mass index (BMI),



alcohol consumption, and smoking. The amount of smoking is measured in terms of cotinine. The remaining factors were classified as antioxidants, while total fat, iron, BMI, alcohol use, and smoking were classified as pro-oxidants.

2.4 Covariates

We incorporated potential covariates that could impact the relationship between OBS and hypertension, such as sociodemographic variables, dietary quality, lipid-related indicators, lifestyle, and co-morbidities. Sociodemographic variables encompassed age, gender (male/female), race (black, Mexican, white, and other), education (<high school, high school, college), BMI, and poverty income ratio (PIR). Total calorie intake and the 2015 Healthy Eating Index (HEI) have been employed to judge overall dietary quality (24). Total cholesterol, and triglycerides are examples of lipid-related markers. Lifestyle primarily involved smoking and alcohol consumption. Co-morbidities included diabetes and hyperlipidemia. Furthermore, we employed stratified variables for age (below/above 60), sex (male/female), hyperlipidemia (yes/no), diabetes (DM, IFG, IGT, NO), and pre-specified effect modifiers to assess the interaction effect.

2.5 Statistical analysis

Due to the complexity of stratified sampling, weighted statistics are used in this paper. We applied the frequency (percentage) for representation and chi-square tests for categorical variables and the mean (standard deviation) for representation and *t*-tests for continuous variables. To validate the correlation between OBS and hypertension and explore the potential nonlinear relationship, OBS was converted from a continuous variable into a categorical variable by utilizing quartiles (Q1, Q2, Q3, and Q4), and the *P*-value for trend was determined.

Multivariable logistic regression models (from crude model to model 2) were utilized to examine the relationship between OBS and hypertension while adjusting for various potential confounders. The only covariate in the crude model that was changed was OBSQ. Model 1 was corrected for age, gender, ethnicity, and OBSQ. Model 2 further adjusted for education, PIR, BMI, hyperlipidemia, DM, kcal, HEI, smoke, triglycerides, total cholesterol, and HDL. Heterogeneity between OBS and hypertension was further assessed through subgroup analysis based on variables such as age groups, gender, hyperlipidemia, and diabetes. Smooth curves were also plotted to display the association between OBS and hypertension.

All statistical analyses were conducted using R software (version 4.2) or Empowerstats (version 4.1). A significance threshold of $\alpha < 0.05$ was set, and all analyses were two-sided. A p -value of 0.05 or less on both sides was deemed statistically significant.

3 Results

3.1 Baseline characteristics

The study reported 28,035 samples in total, with a median age of 47.03, and 41.72% of those samples had hypertension (Table 1). The distribution of genders was fairly equal. The majority of the participants (12,662, 45.16%) were white, followed by black (5,687, 20.29%), individuals of other races (5,349, 19.08%), and Mexican individuals (4,337, 15.47%). The proportion of highly educated individuals in the population is greater than 50% ($n = 14,939$, 53.29%). Participants had lower BMI and smoking rates, but higher age, PIR, calorie, HEI, and HLD values, as well as a higher percentage of men and higher levels of education, as compared to the lowest OBS quartile.

3.2 Association between OBS and hypertension

Weighted logistic regression analysis revealed a correlation between OBS and hypertension, as indicated in Table 2. Irrespective of covariate adjustments, a substantial inverse correlation was noted between the highest OBS quartile and the lowest OBS quartile with the likelihood of developing hypertension. In the crude model, however, there was a positive association between the second quartile of OBS and hypertension. Further sensitivity analysis confirmed this pattern (p for trend < 0.0001). Smooth curve (Figure 2) fitting revealed a declining probability of hypertension occurrence with increasing OBS values, consistent with the logistic regression outcomes. Additionally, after taking the logarithm of the odds ratio for hypertension, it was evident that higher OBS values were associated with a reduced risk of developing hypertension.

3.3 Subgroup analysis

Table 3 displays the results of the subgroup analysis split down by age, gender, diabetes, and hyperlipidemia. The age subgroup of hypertension showed statistical significance (p for interaction = 0.041). Within the age subgroup, individuals under 60 years old were found to be more responsive to higher OBS compared to those over 60. In other words, the population of lower 60 years had an 17.9% lower risk of developing hypertension in the top quartile compared with the first quartile (all p for trend < 0.05). Men in the fourth quartile of the OBS population who do not have diabetes mellitus or concomitant

hyperlipidemia can also be observed to have a strong negative connection with hypertension.

4 Discussion

28,035 samples from seven NHANES cycles between 2005 and 2018 have been included in this study's analysis of the connection between OBS and hypertension. We discovered in this study that OBS was considerably higher in non-hypertension individuals than in hypertensive individuals. Additionally, a higher OBS demonstrated a negative relationship with the likelihood of developing hypertension. Upon accounting for potential influencing factors, the findings indicated that the impact of OBS on hypertension was significantly influenced by age. People under 60 were more likely to benefit from the trial and had a lower risk of developing hypertension than people over 60.

Numerous studies on animals and in humans have tied oxidative stress to the onset and development of hypertension. When the quantity of ROS surpasses the antioxidants' ability to scavenge them, oxidative stress results. In addition to directly affecting cellular macromolecules, which may result in cell damage and death (25), oxidative stress also influences inflammatory factors, which can accelerate blood vessel aging and elevate blood pressure (15). The probability of cardiovascular disease climbs with age, indicating that age is an important factor in cardiovascular disease (26). A cohort study revealed a significant link between age and hypertension, particularly hastening the onset of hypertension in middle age (27). In aged hypertensive mice, Angela Wirth et al. (28) discovered the development of accelerated endothelial cell dysfunction in conjunction with elevated ROS in the vasculature. Besides, as we age, blood vessels begin to stiffen, and when the endothelial cells in the blood vessels are attacked by oxidants or inflammatory substances, the blood vessels stiffen rapidly, increasing blood flow (29). Aged cardiomyocytes have been shown to have increased free radicals and a chronic proinflammatory state too (30). The middle intima of medium-sized arteries thickens as a result of endothelial cell failure brought on by high levels of oxidative stress and inflammation *in vivo* (31). The renewal rate of cardiomyocytes is highest at around 20 years of age, declining steadily thereafter to less than 0.5% per year in the elderly (32). Based on these findings, it is believed that younger patients have better vascular conditions and are more likely to benefit from antioxidants.

It has been proven that the NOX family plays a substantial part in the development of hypertension (33). The production of oxides by the NOX family during the redox process in the population is the main reason for the increase in blood pressure (34, 35). In experiments with hypertensive mice, blocking the NOX pathway with a drug led to an improvement in blood vessel function and normalization of blood pressure (36). Consequently, NOX isoforms (NOX2) may be expressed in immune cells, producing high levels of oxygen free radicals and causing damage to the vascular endothelium (37).

TABLE 1 The distribution of baseline characteristics by quartiles of the OBS, national health and nutrition examination survey 2005–2018.

Characteristic	Total <i>n</i> = 28,035	Q1 (0,12) <i>n</i> = 7,358	Q2 (12,19) <i>n</i> = 7,028	Q3 (19,25) <i>n</i> = 6,863	Q4 (25,37) <i>n</i> = 6,786	<i>p</i> -value
Age	47.03 (0.26)	45.07 (0.33)	48.21 (0.34)	47.44 (0.33)	47.29 (0.40)	<0.0001
PIR	3.03 (0.03)	2.56 (0.04)	2.89 (0.04)	3.14 (0.04)	3.44 (0.04)	<0.0001
BMI	29.01 (0.09)	29.59 (0.12)	29.69 (0.13)	29.31 (0.13)	27.71 (0.12)	<0.0001
Total calorie intake	2,167.05 (8.90)	1,815.30 (17.09)	1,862.32 (13.64)	2,205.96 (14.68)	2,666.99 (19.53)	<0.0001
Healthy eating index	50.88 (0.23)	45.85 (0.30)	47.87 (0.24)	51.20 (0.24)	57.14 (0.30)	<0.0001
Triglycerides	155.15 (1.27)	156.05 (1.82)	160.95 (2.42)	158.07 (2.45)	147.02 (1.88)	< 0.0001
Total cholesterol	195.23 (0.50)	193.63 (0.68)	196.35 (0.86)	195.35 (0.78)	195.47 (0.86)	0.03
HDL	53.37 (0.21)	51.55 (0.34)	52.39 (0.28)	53.29 (0.33)	55.72 (0.30)	<0.0001
Sex						<0.001
Female	13,970 (49.83)	3,336 (47.65)	3,558 (51.29)	3,588 (53.29)	3,488 (50.63)	
Male	14,065 (50.17)	4,022 (52.35)	3,470 (48.71)	3,275 (46.71)	3,298 (49.37)	
Ethnic						<0.0001
Black	5,687 (20.29)	1,950 (15.19)	1,642 (12.44)	1,218 (8.88)	877 (5.62)	
Mexican	4,337 (15.47)	1,162 (9.37)	1,054 (8.25)	1,084 (8.27)	1,037 (7.27)	
Other	5,349 (19.08)	1,374 (13.31)	1,253 (11.63)	1,309 (11.69)	1,413 (11.79)	
White	12,662 (45.16)	2,872 (62.13)	3,079 (67.67)	3,252 (71.16)	3,459 (75.32)	
Education						<0.0001
College and high	14,939 (53.29)	3,004 (49.34)	3,467 (56.21)	3,926 (63.47)	4,542 (74.52)	
High school	6,574 (23.45)	1,978 (28.60)	1,763 (26.70)	1,577 (23.75)	1,256 (16.97)	
Less than high school	6,522 (23.26)	2,376 (22.06)	1,798 (17.09)	1,360 (12.77)	988 (8.52)	
Hyperlipidemia						<0.0001
No	8,117 (28.95)	2,084 (29.12)	1,858 (26.00)	1,915 (28.07)	2,260 (33.24)	
Yes	19,918 (71.05)	5,274 (70.88)	5,170 (74.00)	4,948 (71.93)	4,526 (66.76)	
Diabetes						<0.0001
DM	5,092 (18.16)	1,563 (15.86)	1,461 (16.51)	1,191 (13.65)	877 (9.90)	
IFG	1,300 (4.64)	358 (4.77)	338 (4.66)	330 (4.83)	274 (3.81)	
IGT	1,126 (4.02)	281 (3.15)	285 (3.60)	297 (3.94)	263 (3.29)	
No	20,517 (73.18)	5,156 (76.22)	4,944 (75.23)	5,045 (77.58)	5,372 (83.00)	
Smoke						<0.0001
Former	6,822 (24.33)	1,610 (20.52)	1,767 (25.50)	1,725 (25.53)	1,720 (26.67)	
Never	15,456 (55.13)	3,544 (46.88)	3,730 (51.48)	3,890 (56.87)	4,292 (62.72)	
Now	5,757 (20.54)	2,204 (32.60)	1,531 (23.02)	1,248 (17.60)	774 (10.61)	
Alcohol						<0.0001
Former	4,513 (16.1)	1,324 (14.95)	1,262 (14.32)	989 (11.53)	938 (11.99)	
Heavy	5,750 (20.51)	1,735 (27.49)	1,419 (23.07)	1,390 (21.24)	1,206 (17.48)	
Mild	9,438 (33.67)	2,062 (28.55)	2,320 (35.44)	2,395 (36.64)	2,661 (43.44)	
Moderate	4,392 (15.67)	1,106 (17.26)	1,016 (16.20)	1,159 (19.29)	1,111 (18.00)	
Never	3,942 (14.06)	1,131 (11.74)	1,011 (10.98)	930 (11.30)	870 (9.09)	
Hypertension						<0.0001
No	16,338(58.28)	4,097(61.10)	3,798(58.33)	4,070(62.16)	4,373(67.22)	
Yes	11,697(41.72)	3,261(38.90)	3,230(41.67)	2,793(37.84)	2,413(32.78)	

Mean (SD) for continuous variables; the *P*-value was calculated by the t-tests. Frequency (percentage) for categorical variables; the *P*-value was calculated by the chi-square tests. Q, quartile; PIR, ratio of family income to poverty; BMI, body mass index; DM, diabetes; IFG, impaired fasting glucose; IGT, impaired glucose tolerance.

TABLE 2 Weighted logistic regression analysis models showing the relationship between OBS and hypertension.

nhs—Hypertension	OBSQ					
	Crude model		Model 1		Model 2	
Character	95% CI	<i>P</i>	95% CI	<i>P</i>	95% CI	<i>P</i>
Q1	ref		ref		ref	
Q2	1.12 (1.03,1.23)	0.01	0.95 (0.86,1.06)	0.34	0.97 (0.87,1.09)	0.61
Q3	0.96 (0.86,1.06)	0.39	0.85 (0.76,0.95)	0.003	0.90 (0.79,1.01)	0.08
Q4	0.77 (0.69,0.85)	<0.0001	0.67 (0.60,0.75)	<0.0001	0.81 (0.70,0.93)	0.003
<i>p</i> for trend (character2 integer)		<0.0001		<0.0001		0.002
<i>p</i> for trend (Median value)		<0.0001		<0.0001		0.002

Crude model: OBSQ; Model 1: OBSQ, age, sex, ethnic; Model 2: OBSQ, age, sex, ethnic, education, ratio of family income to poverty, body mass index, hyperlipidemia, diabetes, total calorie intake, healthy eating index, smoke, triglycerides, total cholesterol, HDL. The specific range for the quartiles is consistent with Table 1.

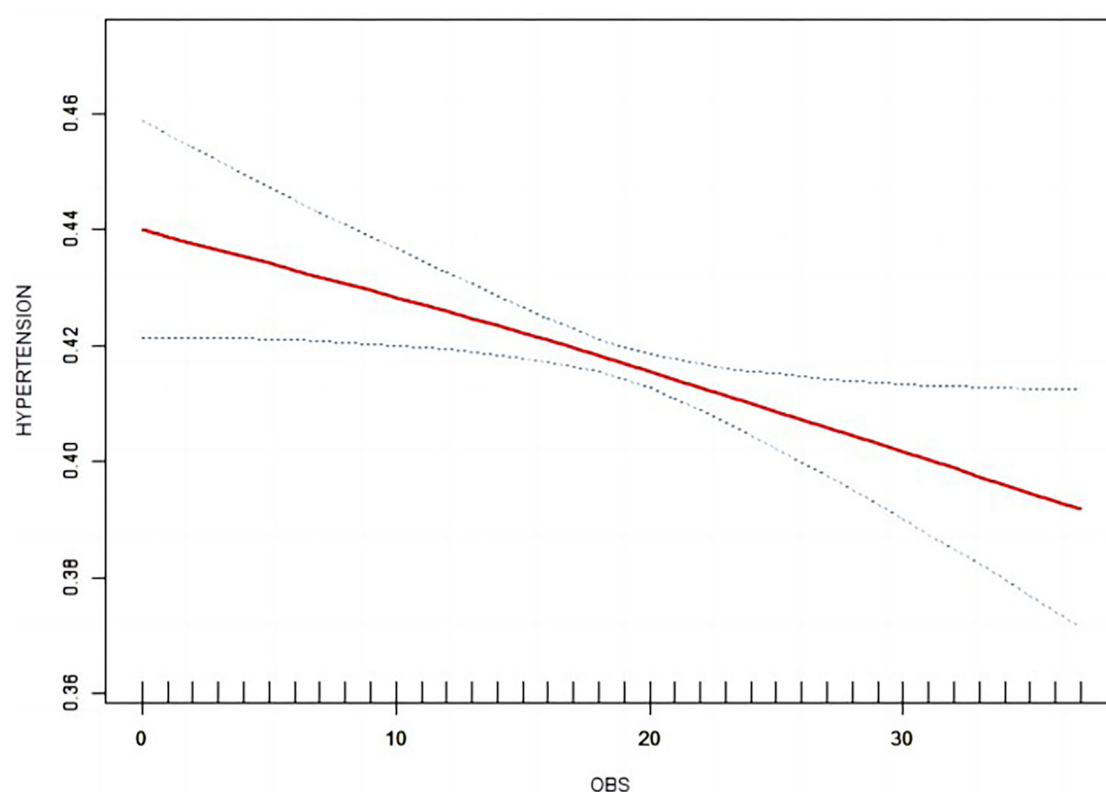


FIGURE 2
Smooth curve between OBS and hypertension.

Several research have also verified the positive correlation between antioxidants and a decreased risk of hypertension. A retrospective study discovered that eating foods high in antioxidants, such as lycopene, α -carotene, β -carotene, lutein with zeaxanthin, and total carotenoids, reduced the incidence of high blood pressure (38). Besides, in a dietary study aimed at elderly patients with metabolic syndrome (MetS) in Korea, when patients ate foods rich in antioxidants for 4 weeks, changes in the state of oxidative stress and MetS involving hypertension, arteriosclerosis and dyslipidemia (39). Additionally, *in vitro* experiments conducted on mice demonstrated that resveratrol plays a role in lowering blood pressure, improving oxidative stress, and enhancing cellular endothelial function (40). Furthermore, a recent study on mice demonstrated that raising the levels of the mitochondrial deacetylase sirt3 decreased oxidative stress, prevented endothelial dysfunction and vascular inflammation, and slowed down hypertension and vascular aging (41). Patients with hypertension who received the physiological oxidant melatonin1 for that year exhibited improvements in arterial stiffness and a drop in serum TAC (a measure of oxidative stress), according to randomized controlled research (42).

According to this research, an antioxidant-rich diet and way of life are good for mental health. In addition, there was age dimorphism in these trends, with younger people seeing a higher

protective impact from antioxidant diet and lifestyle choices. The results emphasize the need of maintaining a diet and way of life high in antioxidants, as they can aid in the prevention and treatment of hypertension.

This study revealed the dual value of antioxidant dietary patterns and lifestyle interventions in the management of hypertension. Data from a cohort study confirmed that higher OBS in hypertensive patients was significantly associated with reduced all-cause mortality (43). Coincidentally, a large-scale cross-sectional study conducted in China also found that a healthy lifestyle and a diet rich in antioxidants can prevent hypertension (44). These findings reinforce the pivotal role of the balance of the oxidation/antioxidant system in blood pressure regulation from an epidemiological perspective. It is worth noting that OBS, as a quantitative assessment tool, can provide individuals with a visual oxidative stress risk assessment by integrating dietary components and lifestyle parameters, thereby guiding precise nutritional counseling.

Our research advantages lie in the following aspects. First, we used seven waves of high-quality, representative data from the NHANES, which are large and multi-stage. Second, the logistic regression model was adjusted to eliminate some potential confounding factors, including demographic, socioeconomic, health and lifestyle information, to obtain more accurate results.

TABLE 3 Subgroup analysis.

Characteristic	Q1 (0,12) n = 7,358	Q2 (12,19) n = 7,028	p	Q3 (19,25) n = 6,863	p	Q4 (25,37) n = 6,786	p	p for trend (character2 integer)	p for trend (Median value)	p for interaction
Age										0.041
Age < 60	Ref	0.978 (0.859,1.113)	0.734	0.932 (0.810,1.073)	0.322	0.821 (0.693,0.972)	0.022	0.026	0.026	
Age ≥ 60	Ref	1.138 (0.944,1.371)	0.173	0.994 (0.824,1.197)	0.945	0.966 (0.769,1.213)	0.762	0.461	0.565	
Sex										0.581
1	Ref	1.008 (0.869,1.168)	0.916	0.951 (0.806,1.121)	0.544	0.783 (0.667,0.919)	0.003	0.004	0.005	
0	Ref	1.159 (0.988,1.359)	0.070	1.029 (0.879,1.204)	0.719	0.990 (0.820,1.195)	0.914	0.641	0.799	
Hypertlipidemia										0.135
Yes	Ref	1.088 (0.965,1.227)	0.167	0.991 (0.882,1.115)	0.883	0.873 (0.756,1.007)	0.062	0.039	0.06	
No	Ref	0.957 (0.763,1.201)	0.702	0.778 (0.622,0.972)	0.027	0.677 (0.531,0.864)	0.002	<0.001	<0.001	
DM										0.428
No	Ref	1.058 (0.937,1.195)	0.361	0.940 (0.825,1.072)	0.352	0.805 (0.699,0.926)	0.003	0.001	0.002	
DM	Ref	1.047 (0.794,1.380)	0.743	0.904 (0.696,1.175)	0.448	1.023 (0.685,1.527)	0.911	0.823	0.859	
IFG	Ref	1.384 (0.836,2.288)	0.203	1.162 (0.676,1.999)	0.582	1.090 (0.625,1.902)	0.758	0.823	0.73	
IGT	Ref	0.918 (0.554,1.522)	0.738	0.948 (0.561,1.601)	0.840	0.994 (0.587,1.682)	0.981	0.967	0.999	

DM, diabetes; IFG, impaired fasting glucose; IGT, impaired glucose tolerance.

There are a few other restrictions on the current investigation. First, because the study is cross-sectional, it is challenging to determine a causal association between OBS and hypertension. Thus, more research with a prospective design is required to demonstrate the effectiveness of OBS. In the future, prospective study designs will be used to track the association between dynamic changes in OBS and blood pressure trajectories and to use bidirectional Mendelian methods to explore the causal relationship between OBS and hypertension. Second, it is difficult to integrate all OS-related nutrition and lifestyle exposures into OBS; flavonoids, for example, were one of the components with restricted availability. Subsequent studies need to integrate food composition databases to more accurately quantify dietary antioxidant exposure. Third, all pro-oxidants and antioxidants are assumed to have a linear correlation with oxidative stress, ignoring the threshold effects of antioxidants. Finally, the limited data did not account for the effect of hypertensive medications on hypertensive patients, making it impossible to determine whether there was a differential effect of OBS between hypertensive patients taking or not taking hypertensive medications. It is anticipated that this problem will be addressed in later clinical research.

5 Conclusion

In conclusion, our analysis suggested that there was a substantial reverse correlation among OBS and hypertension. The study revealed that higher OBS, which signifies greater antioxidant exposure compared to prooxidant exposure in diet and lifestyle, is linked to decreased likelihood of hypertension. The precise mechanism behind the association between OBS and hypertension as well as its causal link warrant further investigation.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: <https://www.cdc.gov/nchs/nhanes/index.htm>.

Author contributions

LT: Conceptualization, Writing – review & editing. YZ: Writing – original draft, Software. LW: Methodology, Supervision, Writing – review & editing. YZ: Validation, Visualization, Writing – original draft. JL: Formal analysis, Visualization, Writing – original draft. CL: Conceptualization, Supervision, Writing – review & editing. YP: Validation, Writing – review & editing. JL: Funding acquisition, Resources, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Oxidative stress-related genes in uveal melanoma: the role of CALM1 in modulating oxidative stress and apoptosis and its prognostic significance

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Background: Uveal melanoma (UVM) is a rare yet aggressive form of ocular cancer with a poor prognosis. This study aims to investigate the role of oxidative stress-related genes (OSGs) in UVM, focusing on their involvement in key signaling pathways and immune infiltration and their potential as prognostic biomarkers and therapeutic targets.

Method: Differential gene expression analysis was conducted using 175 samples of normal retinal pigmented epithelium-choroid complex samples and 63 samples from UVM. Protein-protein interaction (PPI) networks were constructed to identify hub genes, and machine learning algorithms were utilized to screen for diagnostic genes, employing methods such as least absolute shrinkage and selection operator (LASSO) regression, random forest, support vector machine (SVM), gradient boosting machine (GBM), neural network algorithm (NNET), and eXtreme gradient boosting (XGBoost). A risk signature model was developed using data from The Cancer Genome Atlas (TCGA) cohort and validated using the International Cancer Genome Consortium (ICGC), GSE84976 dataset. Clinical samples were used to validate the diagnostic value. Experimental validation encompassed H₂O₂-induced oxidative stress assays and CALM1 overexpression analysis in UVM cells to evaluate its protective effects.

Results: A total of 2,576 differentially expressed genes (DEGs) were identified, with 185 overlapping OSGs enriched in pathways such as HIF-1, FoxO, PI3K-Akt, and apoptosis. Prognostic hub OSGs, including ACACA, CALM1, and DNM2, were associated with poor survival outcomes in the training set and multiple validation data. Revalidation using clinically collected samples confirmed that CALM1 exhibits superior diagnostic value. The risk signature model demonstrated strong predictive accuracy for a 5-year overall survival (AUC = 0.844). Immune infiltration analysis revealed increased CD4⁺ memory-activated T cells and mast resting cells in the high-risk group. Additionally, CALM1 overexpression attenuated H₂O₂-induced oxidative stress and apoptosis in UVM cells. CALM1 upregulation also mitigated the inhibitory effects of H₂O₂ on key cellular processes, including proliferation, migration, and invasion.

Conclusion: This study underscores the critical role of OSGs in the progression of UVM and their potential as prognostic biomarkers and therapeutic targets. The identified risk signature model and the protective role of CALM1 offer valuable insights for developing targeted therapies and enhancing patient clinical outcomes in UVM.

KEYWORDS

uveal melanoma, oxidative stress, CALM1, risk signature, machine learning algorithms

Introduction

Uveal melanoma (UVM) is the most common primary intraocular malignancy in adults, accounting for approximately 85% of all ocular melanomas (1). Despite its rarity, UVM presents significant clinical challenges due to its high metastatic potential and poor prognosis, particularly when metastasis occurs—most commonly in the liver (2–4). Unlike cutaneous melanoma, UVM originates from melanocytes within the uveal tract, which includes the iris, ciliary body, and choroid (5, 6). Although early diagnosis and treatment of primary UVM have improved local disease control, effective systemic therapies for metastatic UVM remain lacking (7, 8), underscoring the need for a deeper understanding of its underlying biology and the development of targeted therapeutic approaches. This review aims to provide an overview of the current knowledge on UVM, with a focus on its molecular mechanisms, diagnostic advancements, and emerging treatment strategies.

Oxidative stress—defined by an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense system—plays a dual role in tumorigenesis and cancer progression (9, 10). On the one hand, excessive ROS can induce DNA damage, genomic instability, and mutations, contributing to cancer initiation and promotion (11). ROS are also capable of triggering cell death through oxidative stress in various cancers (12). On the other hand, cancer cells often exploit elevated ROS levels to promote proliferation (13), survival (11, 14), and metastasis (15) by activating oncogenic signaling pathways such as PI3K/AKT (14), MAPK (16), and NF- κ B (17, 18). Studies have shown that elevated oxidative stress contributes to the anticancer activity of UVM cell lines (19). Recent research has also emphasized the complex interaction between oxidative stress and the tumor microenvironment (TME), wherein ROS modulate immune cell function, angiogenesis, and extracellular matrix remodeling, thereby influencing tumor progression and resistance to therapy. For instance, studies have demonstrated that reactive oxygen species play critical roles in enhancing antigen presentation, regulating immune responses, and preventing immune escape in gastric cancer (20). Targeting oxidative stress pathways—whether by antioxidants or ROS-inducing agents—has thus emerged as a promising therapeutic strategy. However, the context-dependent roles of ROS in cancer demand a nuanced approach to leveraging oxidative stress therapeutically without inadvertently promoting tumorigenesis.

In the present study, we hypothesized that a comprehensive oxidative stress-related gene signature could serve as a prognostic biomarker for patients with UVM. Therefore, differentially expressed genes and a protein–protein interaction (PPI) network were analyzed, followed by machine learning approaches to identify oxidative stress-related prognostic genes. The prognostic efficacy of these genes was then evaluated through a risk signature model. The correlation between risk scores and immune cell infiltration, as well as immune score, was assessed using the Cell-type Identification By Estimating Relative Subsets Of RNA Transcripts (CIBERSORT) algorithm. Additionally, the roles of CALM1 in oxidative stress and apoptosis in UVM cells were validated through cytological experiments.

Methods

Data acquisition and performance

RNA sequencing data from 80 UVM patients, along with corresponding clinical information, were collected as the training cohort from The Cancer Genome Atlas (TCGA) database (<https://www.cancer.gov/tcga>). Dysregulated gene expression was analyzed using datasets from the Gene Expression Omnibus (GEO), including GSE22138 with 63 UVM samples and GSE29801 with 175 normal retinal pigmented epithelium-choroid complex samples. A validation cohort consisting of 370 UVM patients was obtained from the International Cancer Genome Consortium (ICGC) database (<https://www.icgc-argo.org>). Another validation GSE84976 database was further used to evaluate the prognostic analysis of individual genes, including 28 UVM patients.

Additionally, a total of three UVM tumor samples and matched adjacent normal tissues were collected as clinical validation samples. RNA sequencing (RNA-seq) of tissue samples was performed on an Illumina Nova X Plus (Novogene, Beijing, China) using a paired-end approach with 150-bp reads. The fragments per kilobase of transcript per million mapped reads (FPKM) of genes in clinical samples, including three UVM tumor samples and matched adjacent normal tissues, were provided in [Supplementary Table 1](#). All human samples used in this study were obtained with written informed consent from participating patients, in accordance with the ethical guidelines of The First Affiliated Hospital of Anhui

Medical University. Specimens were anonymized and handled in compliance with the Declaration of Helsinki.

All data analyses were performed using R software (version 4.1.0) and associated Bioconductor packages. Additionally, 1,065 oxidative stress-related genes (OSGs) were retrieved from previous literature for further investigation (see [Supplementary Table 2](#)).

Identification of dysregulated OSGs

A total of 63 UVM samples from the GSE22138 dataset and 175 normal retinal pigmented epithelium-choroid complex samples from GSE29801 (21) were included in the analysis. To minimize batch effects in the combined RNA sequencing data from GSE22138 and GSE29801, the “normalizeBetweenArrays” function from the limma R package and the “ComBat” function from the sva R package were used. Differentially expressed genes (DEGs) between UVM and normal samples were identified using limma, with a significance threshold of $P < 0.01$ and $|\log_2(\text{fold change})| > 1.5$. Dysregulated OSGs were visualized in a Venn plot using the VennDiagram R package. Furthermore, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed using the KEGG Orthology Based Annotation System (KOBAS) database (<http://bioinfo.org/kobas/>).

Network of protein–protein interactions

A PPI network was constructed using the STRING database (<http://string-db.org/>) (22). Subclusters within the PPI network of OSGs were created to identify candidate hub genes for further analysis, using a median degree threshold (degree cutoff > 61). The subclusters were visualized using the Cytoscape software (version 3.8.2).

Prognostic risk signature model

Multivariate Cox regression analysis was conducted using the survminer R package to refine the set of OSGs with the best predictive performance. The formula for the risk signature model was defined as follows: $\text{risk score} = \text{Exp}^{\text{DNM2}} \times 0.133679 + \text{Exp}^{\text{POMC}} \times 0.591935 + \text{Exp}^{\text{HSP90B1}} \times \text{Exp}^{\text{POMC}} \times 0.591935 + \text{Exp}^{\text{CALM1}} \times 0.221127 + \text{Exp}^{\text{ACACA}} \times 0.889051$. All UVM patients were classified into high-risk and low-risk groups based on the median risk score (0.960617). Kaplan–Meier survival curves were generated using the survminer package. Additionally, a receiver operating characteristic (ROC) curve was plotted using the survivalROC package to assess the predictive accuracy of the prognostic model.

Clinical relevance and nomogram development

Next, we investigated the relationship between OSG-related risk signature and clinicopathological characteristics, including age,

gender, race, and stage. The “rms” R package was used to develop the nomogram to illustrate each patient’s 1-, 3-, and 5-year overall survival probability, integrating OSG-related risk signature and clinicopathological features. Calibration curves were used to confirm the consistency between the predicted and the actual overall survival.

Immune cell infiltration

To explore the mechanisms underlying the prognostic impact of OSGs in UVM, immune cell infiltration analysis was conducted using the Cell-type Identification By Estimating Relative Subsets Of RNA Transcripts (CIBERSORT) algorithm. Furthermore, the Estimation of STromal and Immune cells in MAlignant Tumor tissues using Expression data (ESTIMATE) algorithm was employed to compute immune and stromal scores based on gene expression data, using the estimate R package. Correlations between these scores and either individual risk scores or specific hub genes were then assessed.

Machine learning analysis

Six machine learning algorithms were applied to identify key genes with significant predictive power. The least absolute shrinkage and selection operator (LASSO) method, implemented via the glmnet R package, performed sparse regularization to select crucial genes. Random forest analysis was carried out using the randomForest package to determine gene importance via ensemble learning with decision trees. Support vector machine (SVM) regression, implemented with the e1071 package, contributed to gene selection. Gradient boosting machine (GBM), using the gbm package, applied a sequential ensemble method to screen for predictive genes. Extreme gradient boosting (XGBoost), accessed through the xgboost package, employed advanced tree penalization to refine gene selection. A neural network (NNET) model was constructed using the nnet package. All models were built using 10-fold cross-validation. Model performance on the validation set was evaluated using the mlr3 package, with predictive accuracy quantified by the area under the ROC curve (AUC), calculated using the pROC R package.

Cell culture and transfection

The UVM cell lines (MP65, MM28) were provided by the Stem Cell Bank, Chinese Academy of Sciences. All cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 20% fetal bovine serum (FBS, #C0226, Beyotime Biotechnology, China) and 1% penicillin/streptomycin (#C0222, Beyotime Biotechnology, China) at 37°C in a humidified atmosphere containing 5% CO₂. UVM cells were treated with hydrogen peroxide (H₂O₂) at concentrations of 50, 100, and 200 μmol/L (Merck KGaA, Darmstadt, Germany) in serum-free

RPMI-1640 medium for 24 h. The CALM1 overexpression vector (oeCALM1) and a negative control vector were synthesized by GenePharma (Shanghai, China). Transfection was performed using Lipofectamine™ 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The medium was replaced three times per week, and cells were passaged upon reaching confluence.

Protein extraction and Western blot

Cells were harvested and washed twice with ice-cold phosphate-buffered saline (PBS) to remove residual medium. They were then lysed in radioimmunoprecipitation assay (RIPA) buffer containing 1% protease inhibitor cocktail. The supernatant containing total protein was collected, and protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (#P0012, Beyotime Biotechnology, China). Equal amounts of protein (20 µg) were mixed with 4× Laemmli buffer and loaded onto a 10% SDS-polyacrylamide gel (SDS-PAGE). After electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using a wet transfer system. The membrane was blocked in 5% non-fat dry milk diluted in Tris-buffered saline with Tween-20 (TBST; 20 mM of Tris, 150 mM of NaCl, 0.1% Tween-20, pH 7.6) for 1 h at room temperature. The membrane was then incubated overnight at 4°C with primary antibodies diluted in blocking buffer at the following concentrations: 1:1,000 for anti-GAPDH, 1:2,000 for anti-SOD2, 1:2,000 for anti-CAT, 1:3,000 for anti-CASP3, 1:3,000 for anti-BAX, and 1:2,000 for anti-CALM1. Afterward, the membrane was washed three times for 10 min each with TBST to remove unbound primary antibodies. It was then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit IgG, #A0208, Beyotime Biotechnology, China) diluted 1:200 in blocking buffer for 1 h at room temperature. The membrane was washed three times again with TBST, and protein bands were visualized using an enhanced chemiluminescence (ECL) substrate (#P0018S, Beyotime Biotechnology, China) and detected with a GeneGnome XRQ chemiluminescence imaging system.

Cell viability assay and ELISA

Cell viability was assessed using the CCK-8 assay (#C0038, Beyotime Biotechnology, China). All UVM cells (MP65 or MM28) were seeded at a density of 5×10^4 cells per well in 96-well plates and transfected with oeCALM1 or the control vector for 24 h. After transfection, 10 µL of CCK-8 reagent was added to each well, and the plates were incubated for 2 h at 37°C in 5% CO₂. The optical density (OD) at 450 nm was measured using a microplate reader (Thermo Fisher Scientific, USA).

Additionally, the concentrations of superoxide dismutase (SOD, #S0101S), malondialdehyde (MDA, #S0131S), and lactate dehydrogenase (LDH, #P0393S) were measured using ELISA kits (all from Beyotime Biotechnology, China), following the manufacturer's protocols.

Transwell for migration and invasion

For migration, we obtained harvest cells (e.g., MP65 or MM28) and resuspended them in serum-free medium and seeded 2×10^5 cells in the upper chamber. The complete medium (with 10% FBS) was added to the lower chamber as a chemoattractant. For oxidative stress conditions, cells were treated with 200 µmol/L of H₂O₂ in the upper chamber. For CALM1 overexpression groups, the overexpressing vector and the control negative vector were transfected into cells prior to seeding. Then, all cells were incubated for 48 h at 37°C with 5% CO₂. Next, we removed non-migrated cells from the upper chamber with a cotton swab. The migrated cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The images were captured under a microscope, and cell counts were determined by analyzing three random fields per insert. For the cell invasion assay, the same steps as above were followed, but the transwell membrane was precoated with Matrigel (50 µg/mL, (BD Biosciences, San Jose, USA)) to simulate extracellular matrix barriers. All the cells were solidified by Matrigel for 4 h at 37°C before cell seeding. After incubation for 48 h, a microscope was used to capture the image.

Apoptosis assays

Apoptosis was assessed using Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) flow cytometry, as described in previous studies (23). Cells were stained with an Annexin V-FITC/PI Apoptosis Detection Kit (MedChemExpress, USA, #HY-K1073), and apoptosis was analyzed using a flow cytometer.

Statistical methods

All results were expressed as mean ± standard deviation (SD). Statistical analyses were conducted using R software (version 4.1.0). Continuous variables were compared using the Wilcoxon rank-sum test. One-way analysis of variance (ANOVA) was applied to determine statistical significance among experimental groups. A *P*-value <0.05 was considered statistically significant unless otherwise indicated. A flowchart of the study design is presented in Figure 1.

Results

The associated DEGs are implicated in UVM

To analyze the differential expression of OSGs in UVM, 175 normal retinal pigmented epithelium-choroid complex samples from the GSE29801 dataset and 63 UVM samples from the GSE22138 dataset were included in the present study after removing batch effects. Principal component analysis (PCA) was conducted to assess batch effect correction (Figures 2A, B). A total of 2,576 DEGs were identified, consisting of 1,164 upregulated and



1,412 downregulated genes, using a threshold of $P < 0.01$ and $|\log_2(\text{fold change})| > 1.5$ (Supplementary Table 3). A Venn diagram showed that 185 genes overlapped between the DEGs and OSGs (Figure 2C). These intersection genes were subsequently visualized in a hierarchical clustering heatmap (Figure 2D). Enrichment analysis revealed that these dysregulated OSGs were significantly enriched in pathways related to melanoma, cancer, HIF-1 signaling, FoxO signaling, PI3K-Akt signaling, T-cell receptor signaling, PD-L1 expression and the PD-1 checkpoint pathway, and apoptosis (Figure 2E).

Identification of prognostic hub OSGs in UVM

To further investigate the interactions among OSGs, the dysregulated OSGs identified above were analyzed using the STRING database to construct a PPI network. The resulting

network revealed extensive interactions among dysregulated OSGs (Figure 3A). To identify hub genes, subclusters of the PPI network were generated using a median degree cutoff (>61), resulting in 42 hub OSGs for subsequent analysis (Figure 3B). In addition, 54 prognostic genes were identified based on Kaplan–Meier survival curves. The intersection of hub genes from the PPI network and prognostic genes yielded 15 common genes (Figure 3C). Kaplan–Meier survival analysis demonstrated significant associations between gene expression levels and patient prognosis (Figures 3D–R). High expression was strongly correlated with poor survival, including ACACA (Figure 3D), CALM1 (Figure 3F), CALR (Figure 3G), CXCR4 (Figure 3I), DNM2 (Figure 3J), EDN1 (Figure 3K), HMOX1 (Figure 3L), HSP90B1 (Figure 3M), IL6 (Figure 3N), POMC (Figure 3O), TEK (Figure 3Q), and TNFSF10 (Figure 3R). Conversely, low expression of AKT2 (Figure 3E), CDK2 (Figure 3H), and SPP1 (Figure 3P) was associated with poor prognosis.

To identify novel genes with diagnostic potential, six machine learning algorithms were applied: LASSO regression, random forest,

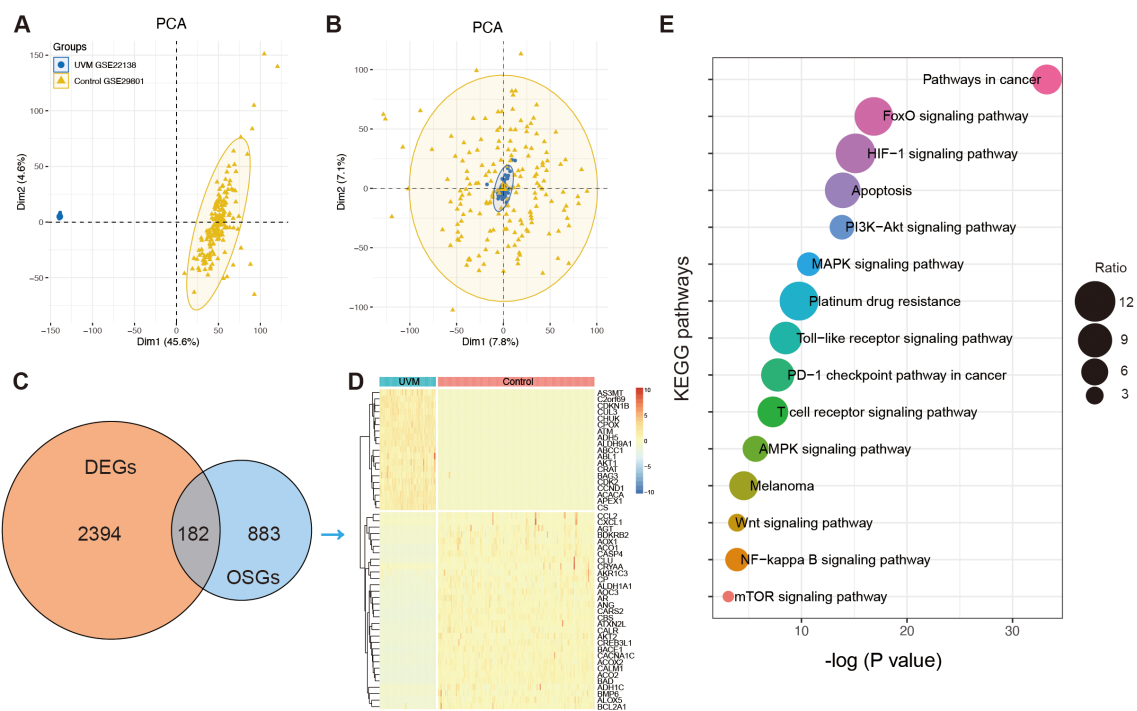


FIGURE 2

The identification of oxidative stress-related genes (OSGs). (A) Principal component analysis (PCA) before batch effects in merging the RNA sequencing data of GSE22138 and GSE29801. (B) PCA analysis after batch effects. (C) The Venn diagram showing the intersection of differentially expressed genes (DEGs) and OSGs. (D) Heatmap showing the differential expression of the intersection of OSGs between normal and UVM samples. (E) The enrichment function of Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis.

SVM, NNET, GBM, and XGBoost. ROC curve analysis showed that all models achieved area under the curve (AUC) values exceeding 0.85 (Figures 4A–F). Compared with random forest (Figure 4B), higher diagnostic potential was observed in the models including LASSO (Figure 4A), SVM (Figure 4C), NNET (Figure 4D), GBM (Figure 4E), and XGBoost (Figure 4F). Based on feature importance rankings, the top gene identified by each algorithm was selected for further analysis: ACACA (LASSO), CALM1 (random forest), HSP90B1 (SVM), DNM2 (NNET), ACACA (GBM), and POMC (XGBoost) (Figure 4G).

Construction and verification of the OSG-related risk signature

Using multivariate Cox regression analysis, a risk signature model was constructed based on the coefficients and expression levels of key genes identified through machine learning (Figure 5A). Patients were stratified into high- and low-risk groups according to the median risk score (0.960617). The high-risk group exhibited significantly poorer overall survival compared to the low-risk group (Figure 5B), suggesting that the risk score may serve as an independent prognostic factor in UVM. Risk score distribution (Figure 5C), survival status (Figure 5D), and gene expression profiles (Figure 5E) for each patient in the TCGA cohort were also visualized. ROC analysis demonstrated

that the model had the highest sensitivity in predicting 5-year overall survival (AUC = 0.844), followed by 3-year (AUC = 0.759) and 1-year (AUC = 0.691) predictions (Figure 5F). To validate the model, the ICGC dataset was used in this study. Similar to the training set, the high-risk group in the validation cohort also exhibited significantly worse prognosis ($P = 6.67 \times 10^{-3}$; Figure 6A). Distributions of risk scores, survival outcomes, and gene expression profiles are shown in Figures 6B–D. ROC curves indicated moderate predictive sensitivity for 1-, 3-, and 5-year overall survival (Figures 6E–G).

Association between risk score and immune landscape

To investigate the association between risk score and immune infiltration, the CIBERSORT algorithm was applied. The analysis of immune-related functions revealed that the OSG high-risk group exhibited elevated infiltration scores for CD4⁺ memory-activated T cells, activated NK cells, and resting mast cells (Figure 7A). In contrast, the OSG low-risk group showed higher infiltration scores for CD4⁺ memory resting T cells, resting NK cells, and monocytes (Figure 7A). Additionally, the correlations between novel OSGs in the risk signature model and immune cell infiltration scores were assessed. The results showed that these OSGs were positively correlated with CD4⁺ memory-activated T cells and resting mast cells (Figure 7B).

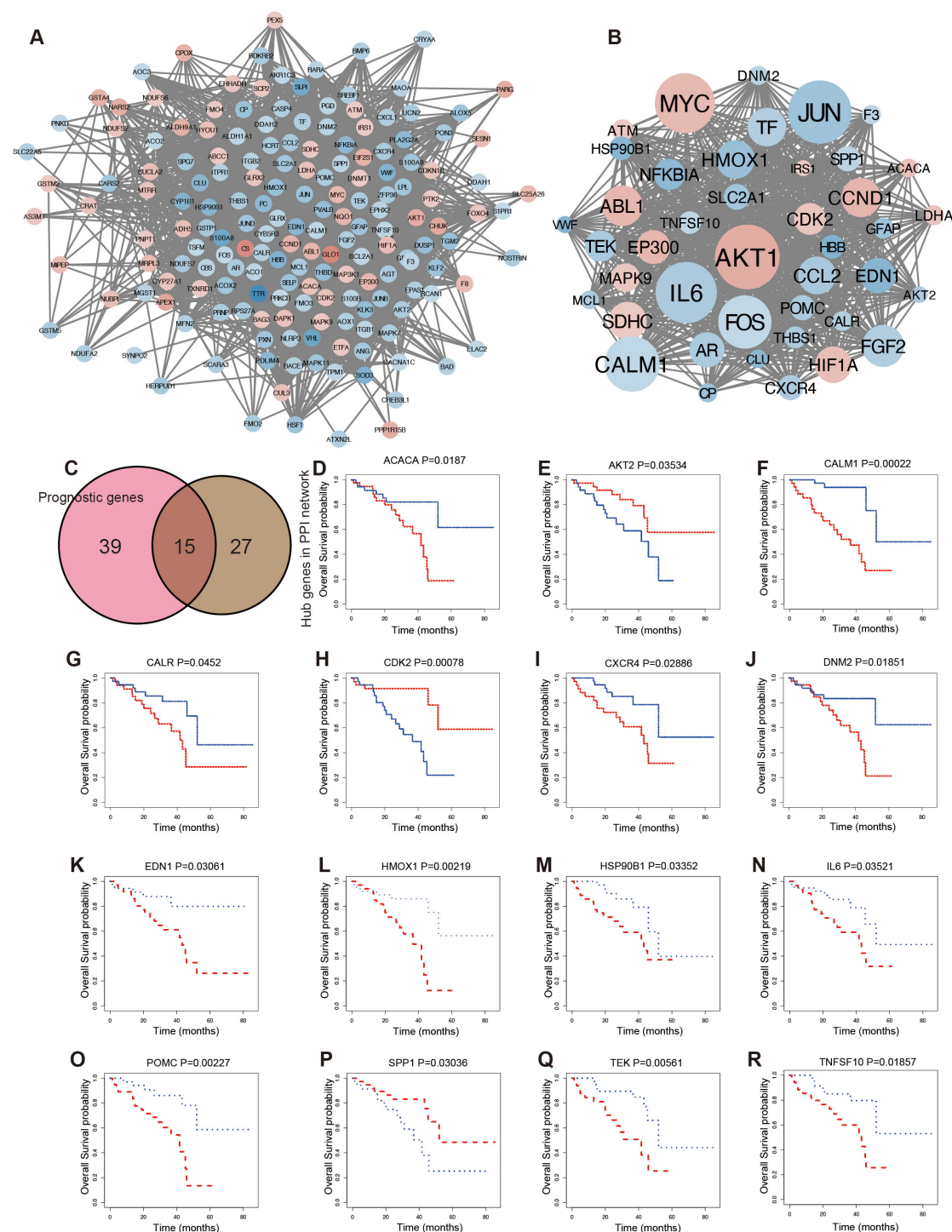


FIGURE 3

Screening prognostic hub OSGs in UVM. **(A)** The protein-protein interaction (PPI) analysis. **(B)** The hub genes network. **(C)** The Venn diagram showing the overlapping of prognosis and hub genes of the PPI network. Kaplan-Meier survival curve for overall survival according to the expression levels of OSGs, including ACACA **(D)**, AKT2 **(E)**, CALM1 **(F)**, CALR **(G)**, CDK2 **(H)**, CXCR4 **(I)**, DNM2 **(J)**, EDN1 **(K)**, HMOX1 **(L)**, HSP90B1 **(M)**, IL6 **(N)**, POMC **(O)**, SPP1 **(P)**, TEK **(Q)**, and TNFSF10 **(R)**.

Further analysis revealed that risk scores were positively correlated with CD4⁺ memory-activated T cells ($\text{cor} = 0.388, P = 0.001078$) and mast cells resting ($\text{cor} = 0.386, P = 0.00108$) while negatively correlated with resting NK cells ($\text{cor} = -0.317, P = 0.007704$) and CD4⁺ memory resting T cells ($\text{cor} = -0.235, P = 0.04937$) (Figure 7C).

Moreover, ESTIMATE scores were compared between the OSG high- and OSG low-risk groups. The OSG high-risk group showed significantly lower immune scores, while stromal scores and total ESTIMATE scores did not differ significantly between the groups (Figure 7D).

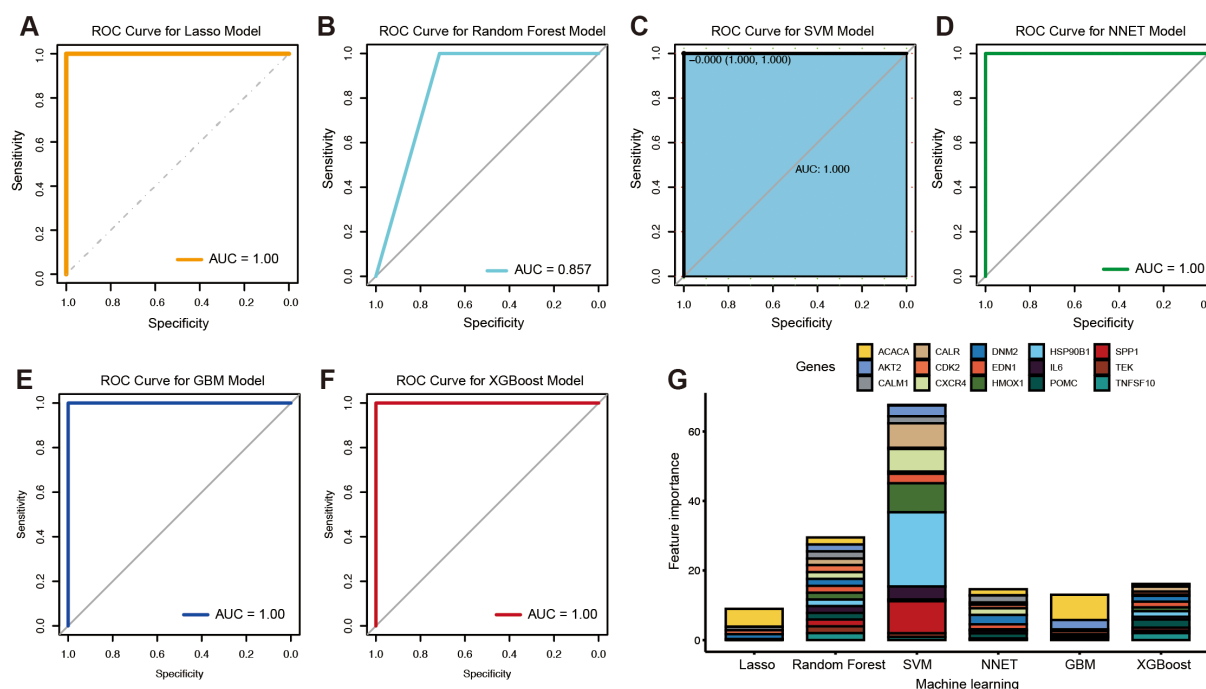


FIGURE 4

Candidate biomarkers identified by the machine learning algorithms. The ROC curves of the LASSO model (A), random forest (B), SVM (C), NNET (D), GBM (E), and XGBoost (F). The feature importance in different machine learning algorithms (G).

Association between risk score and clinical features

To further accurately assess the prognostic value of OSGs in clinical information, the tumor stage, patient age, gender, and race were used to analyze the correlation with overall survival in this study. Our results demonstrate that the high-risk group consistently exhibited a significantly higher risk score (Supplementary Figure 1A), regardless of whether patients were younger or older than 60 years. Although the overall survival between patients aged >60 and ≤60 did not show a statistically significant difference (Supplementary Figure 1B), further stratified analysis revealed that the high-risk group showed significantly poorer survival outcomes in patients ≤60 years (Supplementary Figure 1C). Similarly, in patients >60 years, the high-risk group also displayed a comparable and statistically significant association with adverse prognosis (Supplementary Figure 1D).

Similarly, in our sex-based subgroup analysis, we observed comparable findings. Although no statistically significant difference in prognosis was detected between male and female patients overall, further stratification revealed that high-risk status remained strongly associated with adverse outcomes in both groups (Supplementary Figures 1E, F). Among female patients, high-risk individuals similarly demonstrated higher risk scores and worse prognosis (Supplementary Figure 1G). Likewise, among male patients, the high-risk group exhibited significantly elevated risk scores and poorer survival outcomes (Supplementary Figure 1H).

Due to limitations in the clinical annotation of TCGA datasets, our race-stratified analysis was restricted to comparing “White”

versus “Other” populations. Despite this broad categorization, consistent patterns emerged regarding high-risk patients who demonstrated significantly elevated risk scores and worse overall survival in the White subgroup ($P = 0.000989$, Supplementary Figures 1I–K).

Similarly, high-risk individuals of other racial subgroups showed poorer survival outcomes ($P = 0.03089$, Supplementary Figure 1L). Notably, our risk stratification model revealed consistent prognostic value in both subgroups. In early-stage (I–II) patients, high-risk individuals exhibited significantly elevated risk scores (mean score: 2.326 vs. 0.453; $P = 1.1317E-06$, Supplementary Figure 1M). When categorizing patients into early-stage (I–II) and late-stage (III–IV) disease, we observed that while the overall survival difference between these groups did not reach statistical significance, early-stage cases demonstrated a trend toward better prognosis (Supplementary Figure 1N, $P = 0.2286$). Additionally, early-stage patients within high-risk groups showed worse clinical outcomes (log-rank $P = 0.000774$, Supplementary Figure 1O). In advanced-stage (III–IV) patients, similar results showed comparable risk score differentiation (mean score: 3.123 vs. 0.543; $P = 0.000105$, Supplementary Figure 1M), with poorer survival in the high-risk group (log-rank $P = 0.01491$, Supplementary Figure 1P).

Additionally, we could calculate each patient’s total points and the corresponding overall survival probability using the constructed nomogram (Supplementary Figure 1Q). Calibration curves demonstrated high consistencies between the predicted overall survival and the actual overall survival rates at 1 year (Supplementary Figure 1R), 3 years (Supplementary Figure 1S), and 5 years (Supplementary Figure 1T). These results reinforced

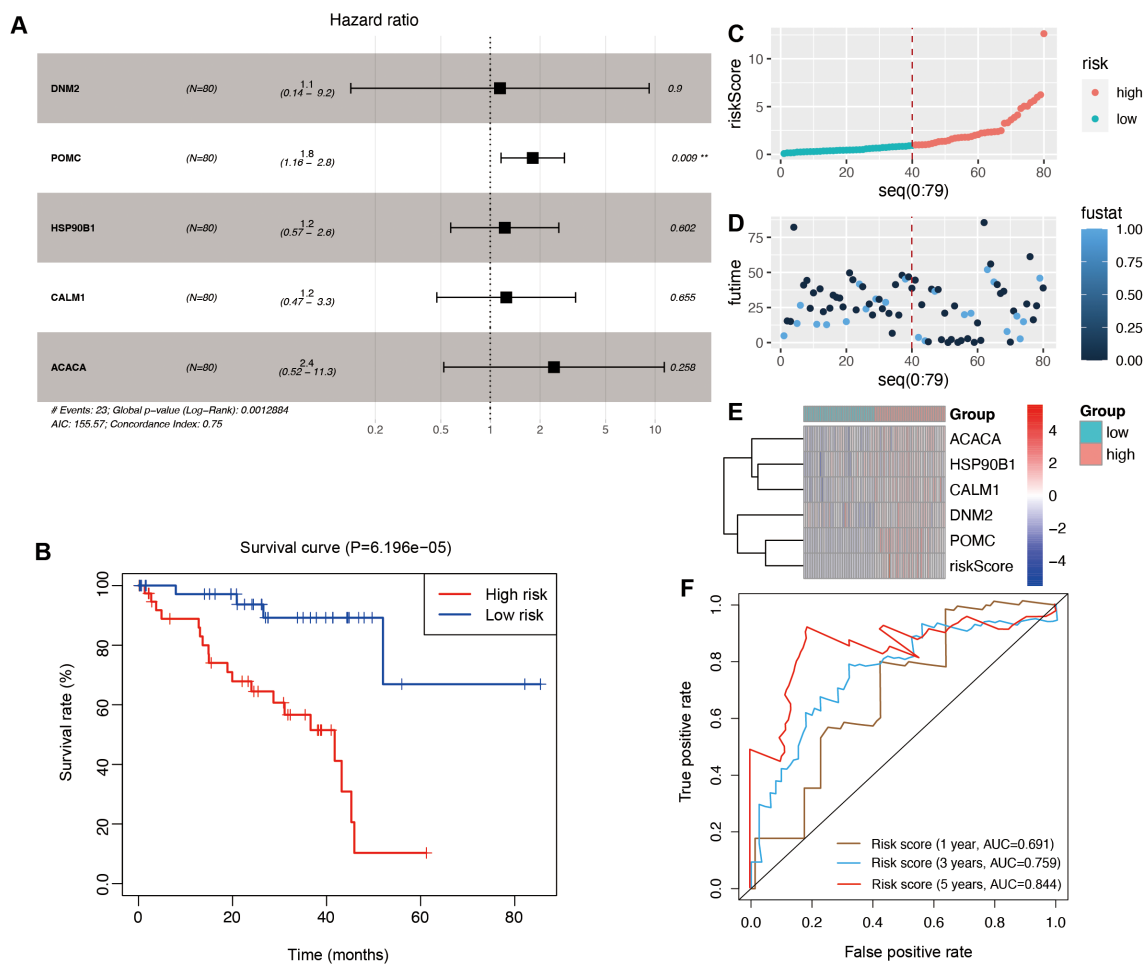


FIGURE 5

The construction of the risk signature model in the TCGA cohort. (A) The multivariate regression analysis. (B) Survival curves to evaluate the risk stratification ability of OSGs. (C) Risk plots to illustrate the risk scores of different risk groups. (D) Risk plots to illustrate the survival status of different risk groups. (E) Heatmap showing the expression levels and risk scores in the risk model. (F) ROC curves to evaluate the sensitivity and specificity of the risk signature to predict the 1-, 3-, and 5-year overall survival.

that our risk stratification model maintained robust predictive value across age, sex, race subgroups, and disease stages, potentially identifying high-risk patients who might benefit from more aggressive therapeutic interventions regardless of initial staging.

CALM1 as a prognostic and diagnostic biomarker

A total of five key genes were screened using machine learning and prognosis analysis in the TCGA training dataset. Further prognostic analysis of individual genes in the GSE84976 (Supplementary Figures 2A–E) database demonstrated that there were four key genes with consistent correlation of overall survival, with genes having high expression showing poor overall survival, including ACACA (Supplementary Figure 2A, log-rank $P = 0.000538$), CALM1 (Supplementary Figure 2B, log-rank $P = 0.019632$), DNM2 (Supplementary Figure 2C, log-rank $P = 0.03332$), and POMC (Supplementary Figure 2E, log-rank $P =$

0.002665). HSP90B showed no significant correlation with either expression or overall survival in the GSE84976 validation data (Supplementary Figure 2D, log-rank $P = 0.407925$).

Moreover, the ICGC validation set also revealed consistent correlations between overall survival and three key genes: ACACA, CALM1, and DNM2, all showing high expression associated with poor prognosis (Supplementary Figures 3A–C), aligning with our initial results. HSP90B1 exhibited a discordant prognostic trend compared to our training data and GSE84976 validation cohort, with higher expression correlating with better survival in the ICGC data ($P = 0.044791$, Supplementary Figure 3D). POMC showed no significant correlation with either expression or overall survival in the ICGC validation cohort ($P = 0.762312$, Supplementary Figure 3E). Based on their reproducible prognostic associations, ACACA, CALM1, and DNM2 were prioritized for subsequent mechanistic studies.

The expression levels of key genes were determined in the TCGA training and ICGC testing data (Supplementary Figures 4A–J). The results revealed consistent expression between the high- and low-risk

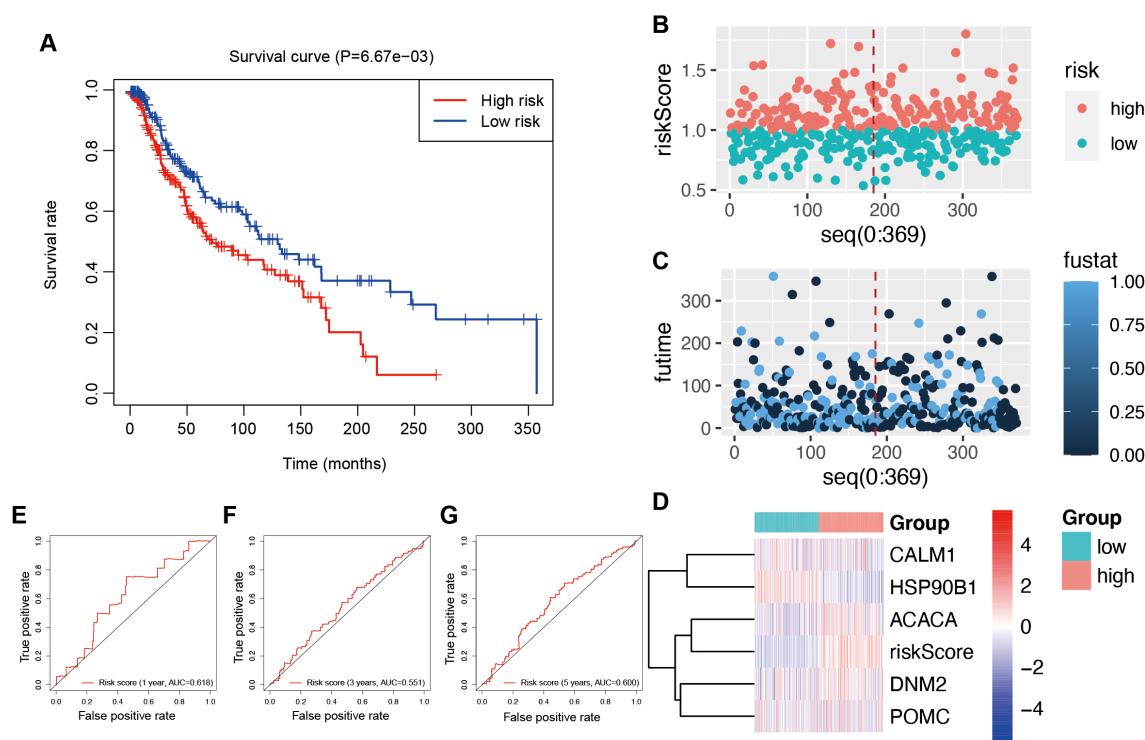


FIGURE 6

Validation of the risk signature model in the ICGC database. (A) Survival curves to investigate the risk stratification ability of OSGs. (B) The risk scores of the high- and low-risk groups. (C) The survival status of the high- and low-risk groups. (D) The expression levels and risk scores in the risk model. ROC curves to evaluate the sensitivity and specificity of the risk signature to predict the 1-year (E), 3-year (F), and 5-year (G) overall survival.

groups, including ACACA (Supplementary Figures 4A,F), CALM1 (Supplementary Figures 4B, G), DNM2 (Supplementary Figures 4C, H), and POMC (Supplementary Figures 4E, J). In our ICGC validation analyses, we observed a discordant expression pattern of HSP90B1 between the TCGA training set and the ICGC validation set. Specifically, HSP90B1 was significantly upregulated in the high-risk group within the TCGA cohort ($P = 0.000159$, Supplementary Figure 4D), whereas it showed higher expression in the low-risk group in the ICGC dataset ($P = 3.611\text{E}-31$, Supplementary Figure 4I). We opted not to include HSP90B1 in further analyses to ensure the robustness of our model.

In addition, ROC analysis was performed to evaluate diagnostic efficacy. In the TCGA training cohort, CALM1 demonstrated the highest AUC (0.9225), followed by POMC (AUC = 0.8556) and ACACA (AUC = 0.8356) (Supplementary Figure 5A). Similarly, in the ICGC validation data, CALM1 maintained high diagnostic performance (AUC = 0.8378) (Supplementary Figure 5B), exceeding the predefined threshold of AUC >0.8. We further evaluated the diagnosis of these genes using clinical samples, including three UVM tumor samples and matched adjacent normal tissues. The results demonstrated that CALM1 and HSP90B1 exhibited superior discriminatory power, with significantly higher AUC values compared to other candidate genes (Supplementary Figure 5C). Based on these findings, CALM1 was selected for further functional analysis to elucidate its biological role.

The effect of H_2O_2 on the viability of UVM cells

To evaluate the impact of oxidative stress on cell viability, UVM cells (MP65, MM28) were treated with various concentrations of H_2O_2 (0, 100, 200, 300, and 400 $\mu\text{mol/L}$) for 24 h. Cell viability was assessed using the CCK-8 assay. The results indicated that cell viability decreased significantly in a dose-dependent manner in MP65 (Figure 8A) and MM28 (Supplementary Figure 6A). A concentration of 200 $\mu\text{mol/L}$ (cell viability: 57.2% in MP65 and 47.3% in MM28) was selected for subsequent experiments.

CALM1 overexpression attenuates H_2O_2 -induced oxidative stress in UVM cells

To further investigate the role of CALM1 in H_2O_2 -induced oxidative stress, UVM cells were transfected with the CALM1 overexpression vector (oeCALM1 group) or a control vector and then treated with H_2O_2 . Western blot analysis confirmed low expression of CALM1 in H_2O_2 -treated cells compared to the control (CN) group in MP65 cells (Figure 8B). In the H_2O_2 -challenged environment (200 $\mu\text{mol/L}$, 24 h duration), the CALM1 overexpression system achieved a robust 1.44 ± 0.012 -fold increase in CALM1 expression in MM28 (Western blot quantification, normalized to GAPDH, $P = 5.36\text{E}-05$, Supplementary Figure 6B), meeting the predetermined threshold for

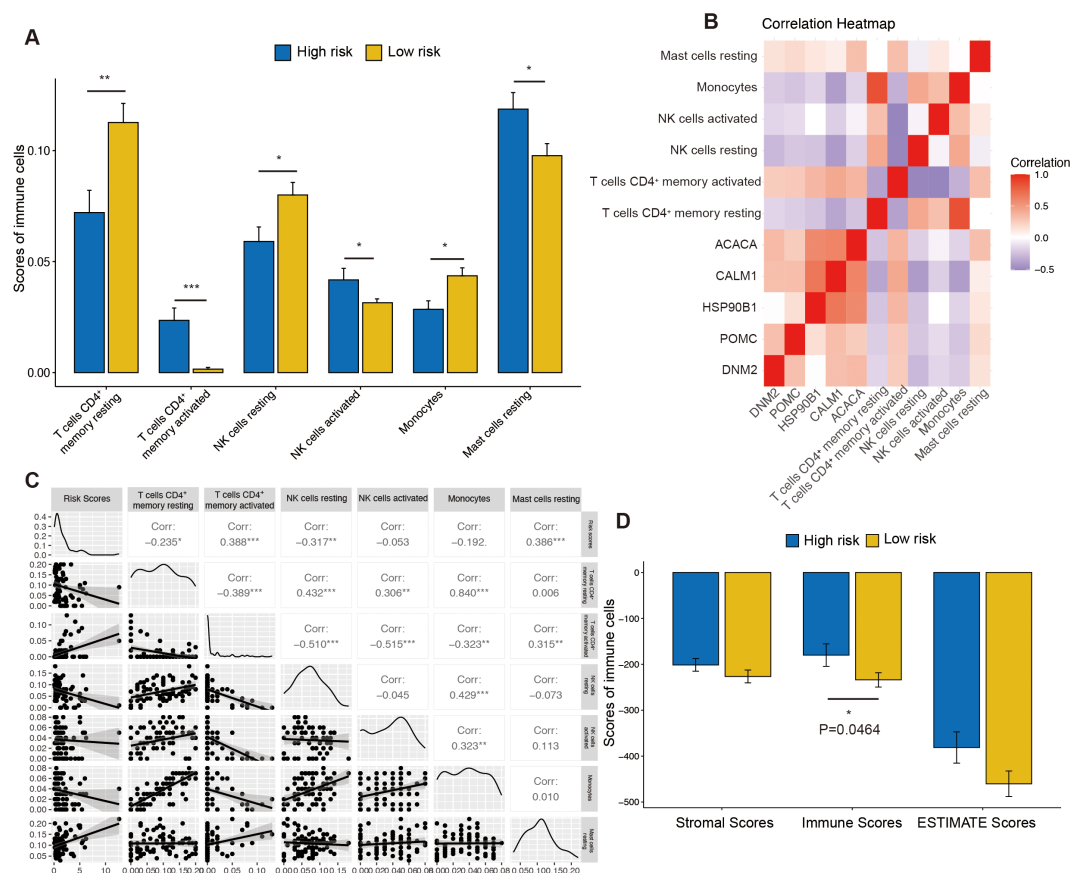


FIGURE 7

The immune function between the high- and low-risk groups. (A) The difference of immune cell infiltration scores between the high- and low-risk groups. (B) The heatmap was generated to show the relationship between OSGs and immune cells. (C) The correlation analysis between risk scores and immune cells. (D) The difference of stromal scores, immune scores, and estimate scores between the high- and low-risk groups. "ns" represents no statistical significance; * represents $P < 0.05$, ** represents $P < 0.01$, and *** represents $P < 0.001$.

successful genetic manipulation. This overexpression efficiency remained stable throughout the oxidative stress exposure period (24 h post-treatment), establishing an appropriate experimental platform for probing CALM1's functional role in redox regulation.

To evaluate the effect of CALM1 on oxidative stress, the levels of SOD, MDA, and LDH were measured using ELISA assays. H_2O_2 treatment significantly decreased SOD levels ($P = 0.03698$; Figure 8C) and increased MDA and LDH levels ($P = 0.008023$ and $P = 0.007522$, respectively; Figures 8D, E), compared to untreated controls (CN). Notably, oeCALM1 effectively reversed the reduction in SOD and suppressed the accumulation of MDA and LDH (Figures 8C–E) in H_2O_2 -treated MP65 cells. Compared with the H_2O_2 negative control group (vector + H_2O_2), oeCALM1 also restored the expression of SOD2 ($P = 0.02858$) and CAT ($P = 0.00918$), both of which were suppressed by H_2O_2 treatment (Figure 8F). This protective pattern was consistently replicated in MM28 cells, where oeCALM1 similarly mitigated H_2O_2 -mediated SOD suppression (50.39% recovery, $P = 0.00046$, Supplementary Figure 6C) and reduced oxidative damage markers (MDA: 23.53% decrease; LDH: 29.81% decrease; both $P < 0.05$ vs. H_2O_2 -treated vector controls, Supplementary Figures 6D, E). Western blot results also revealed that oeCALM1 restored the expression of SOD2 ($P = 0.02858$) and CAT ($P = 0.00918$) in H_2O_2

treatment (Supplementary Figure 6F). The concordant results across both cell lines demonstrated the robust capacity of CALM1 to counteract oxidative stress regardless of cellular context.

CALM1 overexpression attenuates H_2O_2 -induced apoptosis in UVM cells

Next, we examined the effect of CALM1 on apoptosis induced by oxidative stress. Flow cytometry analysis showed that H_2O_2 significantly increased the rate of apoptosis in MP65 cells, compared to untreated controls (Figure 9A). Importantly, this effect was attenuated by oeCALM1 transfection (Figures 9A, B), while necrosis remained unaffected. Compared with the CN group, the expression levels of CASP3 (caspase-3; $P = 0.000797$) and BAX ($P = 0.00021$) were significantly elevated following H_2O_2 treatment (Figure 9C). Overexpression of CALM1 mitigated the H_2O_2 -induced increases in CASP3 ($P = 0.00355$) and BAX ($P = 0.01071$) expression levels (Figure 9C). These protective effects were consistently observed in MM28 cells, where oeCALM1 similarly attenuated both the percentage of apoptotic cells (49.14% reduction, $P = 0.000464$, Supplementary Figures 7A, B) and the

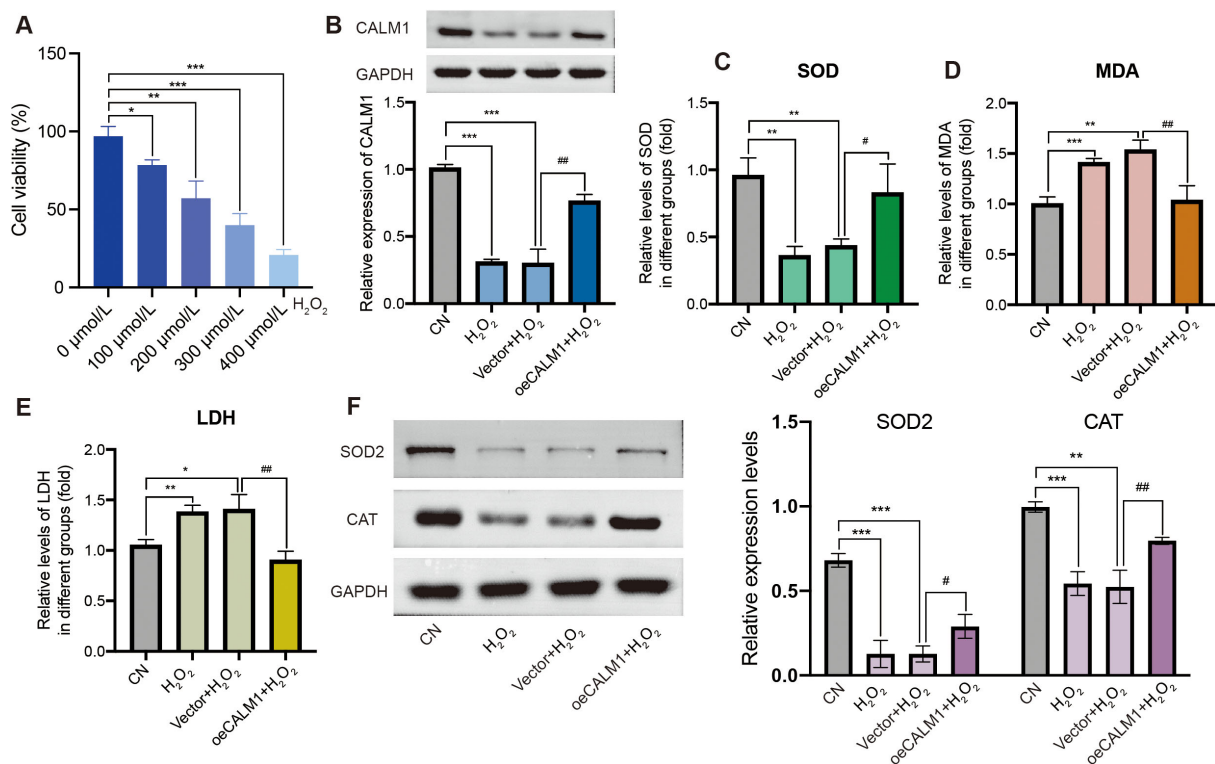


FIGURE 8

CALM1 overexpression attenuated H_2O_2 -induced oxidative stress in MP65 cells. (A) Cell viability was inhibited by H_2O_2 . (B) The expression level of CALM1 was detected by Western blot upon transfection of H_2O_2 -induced MP65 cells with negative control (vector) or CALM1 overexpression. The activity of SOD (C), MDA (D), and LDH (E) in H_2O_2 -induced MP65 cells was determined by ELISA. (F) Western blot evaluating the expression levels of SOD2 and CAT. * or # represents $P < 0.05$, ** or ## represents $P < 0.01$, and *** or ### represents $P < 0.001$. "0 $\mu\text{mol/L}$ " denotes the untreated control.

expression levels of CASP3 (50.60% decrease) and BAX (26.62% decrease) following H_2O_2 exposure (Supplementary Figure 7C).

CALM1 overexpression attenuates H_2O_2 -induced proliferation, migration, and invasion in UVM cells

Our investigation extended to examine the impact of CALM1 overexpression on proliferation, migration, and invasion under H_2O_2 -induced oxidative stress (200 $\mu\text{mol/L}$, 24 h). CCK-8 assays demonstrated that oeCALM1 significantly enhanced cell proliferation by 37.58% ($P = 0.00383$, Supplementary Figure 8A) in MP65 cells and 43.35% ($P = 0.00734$, Supplementary Figure 8B) in MM28 cells at 72 h, effectively reversing the H_2O_2 -mediated growth inhibition. This pro-proliferative effect was consistently observed across both cell lines. Furthermore, transwell assays revealed that oeCALM1 restored migratory capacity by 1.51-fold ($P = 0.00288$) in MP65 cells and 1.62-fold ($P = 0.00436$) in MM28 cells, compared to H_2O_2 -treated controls (Supplementary Figures 8C, D). The oeCALM1 increased invasive potential by 1.84-fold ($P = 0.00069$) in MP65 cells and 2.22-fold ($P = 6.013 \times 10^{-6}$) in MM28 cells, relative to oxidative stress conditions (Supplementary Figures 8E, F). The concordant results in both

MP65 and MM28 cell lines established CALM1 as a multifunctional regulator capable of counteracting oxidative stress-induced impairment of critical oncogenic processes. These findings suggest CALM1 may serve as a key mediator in maintaining cellular functionality during redox imbalance.

Discussion

UVM is a highly aggressive and potentially devastating form of ocular cancer, characterized by a poor prognosis and high metastatic potential (2). Despite advancements in early diagnosis and local treatment, the survival rate for patients with UVM remains dismally low, underscoring the urgent need for reliable biomarkers to enhance diagnostic accuracy and predict clinical outcomes (24–26). Recent research has increasingly focused on the role of oxidative stress in UVM pathogenesis, revealing that oxidative stress not only contributes to tumor progression but also impacts therapeutic resistance (19, 27, 28). Studies have identified key molecular pathways—such as the HIF-1 signaling axis—involved in regulating oxidative stress responses across various diseases (29, 30). Furthermore, elevated levels of ROS and disrupted antioxidant mechanisms have been observed in UVM, suggesting the presence of potential therapeutic targets (31, 32).

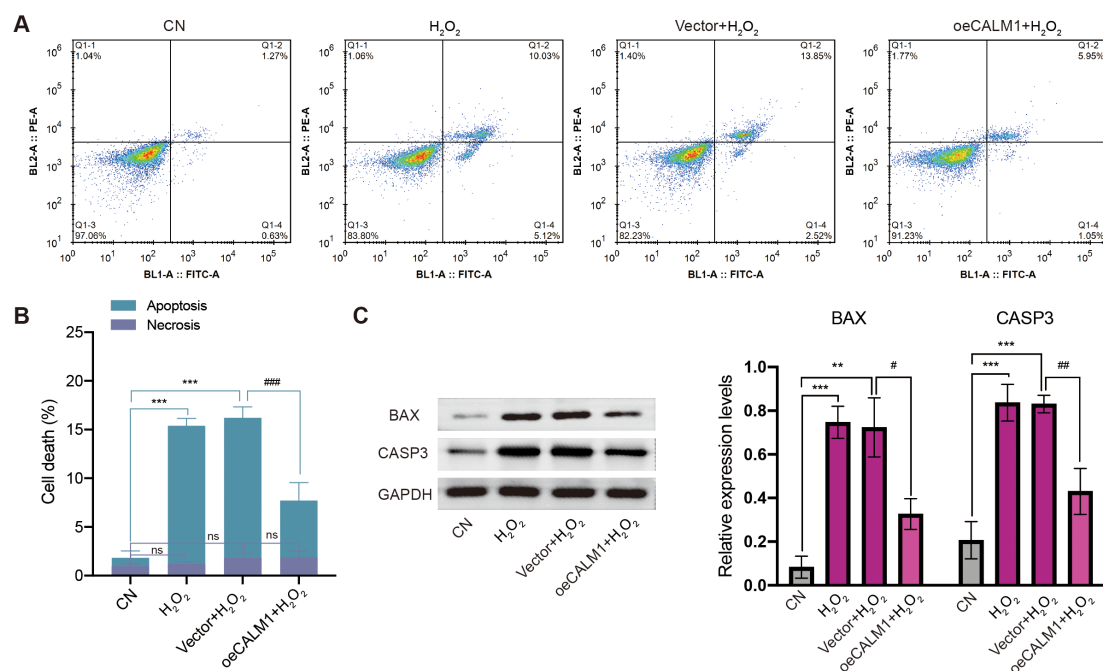


FIGURE 9

CALM1 overexpression attenuated H₂O₂-induced apoptosis in MP65 cells. (A) After transfection with negative control (vector) or CALM1 overexpression, MP65 cells were determined by Annexin V-FITC/propidium iodide (PI) staining. (B) Percentage of apoptotic cell death and necrosis. (C) The expression level of BAX and CASP3 (caspase 3) was detected by Western blot. Three independent experiments were carried out. "ns" represents no statistical significance; * or # represents $P < 0.05$, ** or ## represents $P < 0.01$, and *** or ### represents $P < 0.001$.

However, the specific genes that respond to oxidative stress and influence UVM progression remain poorly characterized. The molecular mechanisms and key regulatory genes involved in this process require further elucidation. Therefore, deeper investigation into the interplay between oxidative stress and UVM biology could pave the way for novel diagnostic tools and targeted therapies to improve patient outcomes.

In this study, we identified 185 oxidative stress-related DEGs between control and UVM samples. Among them, 15 intersecting genes were identified between hub genes from the PPI networks and prognostic genes. Multiple machine learning algorithms (LASSO regression, random forest, SVM, NNET, GMB, and XGBoost) were employed to identify novel diagnostic genes, including ACACA, CALM1, HSP90B1, DNM2, and POMC. These five genes were subsequently used to construct a prognostic risk signature model. Patients classified as high risk exhibited poorer prognoses and demonstrated elevated immune infiltration scores for CD4⁺ memory-activated T cells, activated NK cells, and resting mast cells. Additionally, functional experiments showed that CALM1 overexpression attenuated H₂O₂-induced oxidative stress and apoptosis in MP65 cells.

ACACA (acetyl-CoA carboxylase alpha) is a biotin-containing enzyme that catalyzes the carboxylation of acetyl-CoA to malonyl-CoA (33), participating in lipid metabolism and acetyl-CoA metabolic processes (34). Downregulation of ACACA suppresses the malignant progression of prostate cancer (35). In hepatocellular carcinoma, ACACA has been identified as a central gene associated with poor prognosis (36). Consistent with these findings, our results

indicated that high ACACA expression was associated with worse prognosis in UVM patients. Interestingly, its downregulation in lung fibroblasts was found to trigger an inflammatory phenotypic shift (37). CALM1 (calmodulin 1) is a member of the EF-hand calcium-binding protein family, involved in G2/M cell cycle transition and calcium signal transduction by modulating a wide range of enzymes (38–40). CALM1 has shown high diagnostic and prognostic value in several cancers, including UVM, bladder cancer, and breast cancer (41), which aligns with our findings linking high CALM1 expression to poor overall survival in UVM patients. Prior studies reported a positive correlation between CALM1 expression and macrophage/neutrophil infiltration in skin cutaneous melanoma (41). Our study supports this, suggesting CALM1 may modulate the tumor microenvironment. Furthermore, in esophageal squamous cell carcinoma, CALM1 was shown to promote tumor progression and reduce sensitivity to EGFR inhibitors (42). In ovarian cancer, it was associated with tumor classification and immune status (43). These findings are consistent with our observation that CALM1 overexpression alleviated H₂O₂-induced oxidative stress, reduced MDA and LDH accumulation, and inhibited apoptosis in UVM cells. CALM1 may regulate tumor apoptosis through multiple signaling pathways. It activates the Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) pathway, inhibits mitochondrial apoptosis by reducing the Bax/Bcl-2 ratio, and promotes glioma cell survival (44). CALM1 can also activate the NF- κ B pathway by binding to I κ B kinase (IKK) (45), upregulating anti-apoptotic proteins to inhibit prostate cancer apoptosis (46). Further studies have shown that activated Ca²⁺/CaMKII can

enhance CaMKII/NF- κ B interaction and NF- κ B activation (47). In addition, CALM1 has been implicated in AKT regulation, especially in PIK3CA-mutated breast cancer (48). The activation of CaMKII also promotes PI3K/Akt signaling, facilitating anti-apoptotic mechanisms in prostate cancer (49). This pathway enhances glucose uptake, promotes glycolysis, and inhibits apoptosis in hepatocellular carcinoma (50). Although CALM1 is known to inhibit apoptosis in many cancers, its role in UVM apoptosis remains underexplored. Notably, CALM1 may activate Ca^{2+} /CaMKII signaling, while excessive Ca^{2+} can induce apoptosis in some cancer types (51). In GNAQ/11-mutant UVM cells, mutations increase cytosolic calcium and trigger p53-dependent apoptosis (52). Moreover, reduced Ca^{2+} flux in BAP1^{+/-} cells hinders apoptosis despite DNA damage accumulation in UVM with germline BAP1-inactivating mutations (53).

CALM1 is involved in the process of oxidative stress through the Ca^{2+} signaling pathway. Oxidative stress could activate the TRPM8 channel to induce Ca^{2+} and pro-apoptotic signals in prostate cancer (54). Oxidative stress activated the Ca^{2+} -CaMKII cascade to inhibit early autophagy induction, which led to mitochondria fragmentation and loss of mitochondrial membrane potential (55). Mitochondrial calcium uniporter (MCU)-mediated oxidative stress could increase mitochondrial calcium and decrease mitochondrial membrane potential in osteoblasts (56). The activation of the iNOS pathway led to higher reactive oxygen species and nitric oxide production, which accelerated gastric cancer cell apoptosis (57). The activation of oxidative stress induced the proliferation of leukemia cancer cells through cytosolic Ca^{2+} influx (58). The dysregulation of cytosolic Ca^{2+} also decreased mitochondrial function and increased oxidative stress (59). The mitochondrial oxidative stress was induced by *Clostridium botulinum* neurotoxin A via activation of the Ca^{2+} signaling pathway in neuroblastoma and glioblastoma tumor (60). The activated Ca^{2+} /CaMKII axis increased NOX4 expression, creating a feedforward loop of oxidative damage (60). Additionally, Ca^{2+} signaling mediated airway inflammation in response to oxidative stress through activation of the ERK pathway (61). These findings revealed that Ca^{2+} signaling served as a central regulator of oxidative stress responses. HSP90B1 (heat shock protein 90 beta family member 1) encodes an ATP-dependent molecular chaperone involved in protein stabilization and folding (62). Previous studies have suggested that HSP90B1 is a promising candidate for cancer diagnosis and prognosis (63) and plays a role in regulating cisplatin sensitivity in bladder cancer (64). DNM2 (dynamin 2) is a member of the GTPase protein family (65). It has been associated with poor prognosis in various cancers (66). Interestingly, downregulation of DNM2 was linked to worse outcomes and older age in neuroblastoma patients (67). Our findings are consistent, showing that higher DNM2 expression correlates with worse survival in UVM. POMC (proopiomelanocortin) is involved in physiological processes including pigmentation and inflammation (68). It has been identified as a survival-related gene in colorectal cancer (69). Moreover, POMC-negative expression was associated with better response to paclitaxel and carboplatin chemotherapy in lung cancer (70).

Nowadays, the diversification of treatment strategies has contributed to improved clinical outcomes for patients. Clinical

trial results have demonstrated that anti-PD-1 antibodies can achieve durable clinical benefits in patients with UVM (71). Oxidative stress has been reported to regulate programmed death-ligand 1 (PD-L1) expression on tumors, thereby influencing tumor responses to immune checkpoint inhibitors (72). In our analysis, significant differences in immune cell infiltration levels were observed between the OSG high-risk and OSG low-risk groups. Elevated infiltration scores of CD4⁺ memory-activated T cells and activated NK cells were found in the high-risk group, suggesting that CD4⁺ T lymphocytes and NK cells may contribute to antitumor activity in UVM (73, 74). Dysregulated T-cell infiltration may alter antigen presentation to CD4⁺ T cells and impair epitope recognition, potentially contributing to autoimmune or inflammatory diseases (75). T-cell homeostasis has been associated with Fas-mediated apoptosis (76). Our results showed a negative correlation between CD4⁺ memory resting T cells and ACACA expression ($r = -0.25$, $P = 0.042$). Inhibition of ACACA function has been reported to enhance memory CD4⁺ T-cell formation via fatty acid metabolism (77), and ACACA has also been shown to suppress Th9 differentiation in naive CD4⁺ T cells through fatty acid synthesis (78). CALM1 was positively correlated with CD4⁺ T cells ($r = 0.405$, $P = 0.001$), which aligns with previous findings (79). Although CALM1 has not been directly studied in T lymphocytes, its known role in Ca^{2+} signaling suggests possible involvement in T-cell functions such as activation and differentiation (80), including CD8⁺ and CD4⁺ T cells (81). Memory and recall responses by CD8⁺ T cells require Ca^{2+} channel expression in CD4⁺ T cells (82). Furthermore, exosomal regulation of Ca^{2+} signaling has been shown to reduce CD40L expression and suppress CD4⁺ T-cell activation and proinflammatory cytokine secretion (83). Differentiation of naive CD4⁺ T cells into Th17 and Treg subsets is also dependent on Ca^{2+} signaling (84).

Other studies have indicated that CD8⁺ T cells, macrophages, and NK cell infiltration are associated with poor prognosis in UVM (74, 85). Hepatic NK cells have been shown to occupy the same niche as uveal melanoma micrometastases in the liver sinusoids (86). Low-risk primary UVM tumors are characterized by reduced HLA class I expression and increased NK cell infiltration, which is associated with a decreased risk of disease recurrence (87). Consistent with these findings, our results also revealed high levels of resting NK cell infiltration in low-risk UVM patients (Figure 7A). Calcium channels involved in Ca^{2+} signaling have been found to regulate the homeostasis of secretory lysosomes and their interaction with mitochondria in human NK cells (88). Deletion of calcium channels in NK cells has been shown to impede autophagic flux and lead to the accumulation of dysfunctional mitochondria, contributing to increased oxidative stress (88). Moreover, our results showed that immune scores were lower in the high-risk group, indicating poorer prognosis—contrary to findings in gastric cancer (89). This oxidative stress-related prognostic model may therefore have value in guiding clinical immunotherapy for UVM.

In our analysis, the dysregulated genes were significantly enriched in several critical signaling pathways, including apoptosis, FoxO, and HIF-1 pathways. These pathways are closely associated with oxidative stress in tumors and play crucial roles in tumorigenesis and progression. Prior studies have shown that FoxO transcription

factor knockdown reduces UVM cell proliferation (90), while inhibition of FoxO3a promotes proliferation and invasion in UVM cells (91). Similarly, knockdown of HIF-1 significantly impairs UVM tumor progression (92), and silencing HIF-1 α reduces UVM cell migration, invasion, and adhesion (93). Increased HIF-1 α expression has also been associated with worse prognosis in UVM patients (94), suggesting that the FoxO and HIF-1 signaling pathways contribute to UVM progression. Notably, our findings highlight a particularly strong association between these pathways and UVM. The enrichment of dysregulated genes in the apoptosis, FoxO, and HIF-1 pathways underscores the critical role of oxidative stress regulation in UVM biology. Consistent with our results, other studies have shown that ROS production, oxidative stress signaling, and FoxO activity are pivotal in cancer development and progression (95). The uncontrolled activation of antioxidant signaling has been implicated in breast cancer progression via HIF-1 and FoxO pathways (96). Kim et al. demonstrated that FoxO3a acts as an anti-melanogenic factor mediating antioxidant-induced depigmentation, thereby influencing melanogenesis (97). Although no reports have directly linked the FoxO pathway to antioxidant activity in UVM, loss of FoxO function has been shown to confer growth and survival advantages to melanoma cells (98). Additionally, apoptosis under physiological conditions is tightly regulated by oxidative stress (99), and impaired signaling may disrupt the balance of apoptosis, contributing to tumor development (100). Our results revealed that the dysregulated genes were enriched in key pathways—FoxO, HIF-1, and PI3K-Akt—all of which are closely linked to oxidative stress regulation. Previous studies have reported that the PI3K signaling pathway is enriched in high-risk UVM groups and associated with worse prognosis (101). PI3K-Akt pathway-related risk scores have been shown to reflect distinct immune statuses and mutation landscapes in UVM patients (102). Furthermore, activation of the PI3K-Akt pathway and enhancement of DNA damage response mechanisms may help mitigate treatment side effects in UVM (103). Further investigation of these signaling pathways could provide deeper insights into the molecular mechanisms driving UVM progression and help identify novel therapeutic targets.

However, several limitations must be acknowledged in this study. First, although numerous novel oxidative stress-related biomarkers were identified as prognostic genes for predicting overall survival in UVM—and an external validation cohort was employed—these findings were derived entirely from bioinformatics analysis. Larger sample sizes are needed to further validate the robustness of these results. Second, potential biases may have been introduced by the machine learning algorithms, including overfitting and limitations inherent to database selection. Future research should aim to confirm the effectiveness of this five-biomarker-based diagnostic model using larger, independent cohorts. Third, the current database does not contain treatment-related information, making it impossible to incorporate this factor in the present study. We acknowledge this limitation and will ensure that treatment history is systematically recorded in future clinical sample collections to allow for more comprehensive analyses in subsequent research. Fourth, while we have successfully collected and analyzed RNA expression data from

three UVM tissue samples and three paired adjacent normal tissues for preliminary validation, we acknowledge that the current sample size is insufficient to perform statistically meaningful survival prognosis analysis. The limited number of UVM cases ($n = 3$) lacks the statistical power required to correlate gene expression patterns with clinical outcomes such as overall survival. This preliminary analysis was primarily designed as a proof-of-concept validation of RNA expression trends observed in our larger genomic datasets.

Conclusion

In conclusion, this study comprehensively investigated the role of OSGs in UVM, revealing their significant involvement in key signaling pathways—including HIF-1, FoxO, PI3K-Akt, and apoptosis—which are closely associated with tumor progression and oxidative stress regulation. Through differential gene expression analysis, protein-protein interaction networks, and multiple machine learning algorithms, we identified and validated several prognostic hub OSGs, including ACACA, CALM1, DNM2, POMC, and HSP90B1, which were all associated with poor survival outcomes in UVM patients. A robust risk signature model was constructed, demonstrating strong predictive accuracy for overall survival, particularly for 5-year prognosis. Furthermore, our findings emphasized the impact of risk scores on immune infiltration, with high-risk groups exhibiting distinct immune cell profiles. Experimental validation further confirmed the protective role of CALM1 in alleviating H₂O₂-induced oxidative stress and apoptosis in UVM cells. CALM1 upregulation also mitigated the inhibitory effects of H₂O₂ on key cellular processes, including proliferation, migration, and invasion, highlighting its potential as a therapeutic target. Collectively, these findings provide valuable insights into the molecular mechanisms underlying UVM and offer promising avenues for the development of targeted therapies and prognostic biomarkers.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The requirement of ethical approval was waived by The First Affiliated Hospital of Anhui Medical University for the studies involving humans because the research poses no more than minimal risk to participants, including anonymized analysis with no impact on care or privacy. The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board also waived the requirement of written informed consent for participation from the participants or the participants' legal

guardians/next of kin. Written informed consent was not obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

YWu: Conceptualization, Writing – original draft. XC: Writing – original draft, Formal Analysis, Data curation. MH: Writing – original draft, Formal Analysis, Methodology. RC: Software, Writing – original draft, Data curation. YWa: Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

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Conflict of interest

The authors declare that they have no known competing interests or personal relationships that could have appeared to influence the work reported in this paper.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fonc.2025.1618601/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

Combining the OSGs and clinical variables. (A) The difference of risk scores in patients with age > 60 and age ≤ 60. (B) The survival curve showing no significant difference between age > 60 and age ≤ 60. (C) The survival curve in age ≤ 60 patients. (D) The survival curve in age > 60 patients. (E) The difference of risk scores in female and male patients. (F) The survival curve showing no significant difference between female and male. (G) The survival curve in female patients. (H) The survival curve in male patients. (I) The difference of risk scores in different race. (J) The survival curve showing no significant difference between white patients and others. (K) The survival curve in white patients. (L) The survival curve in non-white patients. (M) The difference of risk scores in different stages. (N) The survival curve showing no significant difference between early stage and advance stage patients. (O) The survival curve in early stage patients. (P) The survival curve in advance stage patients. (Q) Nomogram for predicting the 1-year, 3-years and 5-years overall survival. Calibration plot of the nomogram for predicting 1-year (R), 3-years (S) and 5-years (T).

SUPPLEMENTARY FIGURE 2

The correlation of five genes and overall survival in GSE84976 validation dataset. Survival curve showing the prognostic values of five genes of risk model, including ACACA (A), CALM1 (B), DNM2 (C), HSP90B1 (D), POMC (E).

SUPPLEMENTARY FIGURE 3

The correlation of five genes and overall survival in ICGC validation dataset. Survival curve showing the prognostic values of five genes of risk model, including ACACA (A), CALM1 (B), DNM2 (C), HSP90B1 (D), POMC (E).

SUPPLEMENTARY FIGURE 4

The relative expression levels of five OSGs in training and validation dataset. The genes expression in TCGA training dataset: ACACA (A), CALM1 (B), DNM2 (C), HSP90B1 (D), POMC (E). The genes expression in ICGC testing dataset: ACACA (F), CALM1 (G), DNM2 (H), HSP90B1 (I), POMC (J).

SUPPLEMENTARY FIGURE 5

ROC curves showing the comparisons. (A) ROC curves of ACACA, POMC, HSP90B1, CALM1, DNM2 in TCGA cohort as training cohort. (B) ROC curves of ACACA, POMC, HSP90B1, CALM1, DNM2 in ICGC cohort as testing dataset. (C) ROC curves of ACACA, CALM1, POMC, HSP90B, DNM2 in clinical samples as testing dataset.

SUPPLEMENTARY FIGURE 6

CALM1 overexpression attenuated H₂O₂-induced oxidative stress in MM28 cells. (A) Cell viability was inhibited by H₂O₂. (B) The expression level of CALM1 was detected by western blot upon transfection of H₂O₂-induced MM28 cells with negative control (vector) or CALM1 overexpression. The activity of SOD (C), MDA (D) and LDH (E) in H₂O₂-induced MM28 cells was determined by ELISA. (F) Western blot evaluating the expression levels of SOD2 and CAT using western blot. * or # represents P<0.05, ** or ## represents P<0.01, *** or ### represents P<0.001. "0 μmol/L" denotes the untreated control.

SUPPLEMENTARY FIGURE 7

CALM1 overexpression attenuated H₂O₂-induced apoptosis in MM28 cells. (A) After transfection with negative control (vector) or CALM1 overexpression, MM28 cells were determined by Annexin V-FITC/propidium iodide (PI) staining. (B) Percentage of apoptotic cell death and necrosis. (C) The expression level of BAX and CASP3 (caspase 3) were detected by western blot. Three independent experiments were carried out. "ns" represents no statistical significance; * or # represents P<0.05, ** or ## represents P<0.01, *** or ### represents P<0.001.

SUPPLEMENTARY FIGURE 8

Effects of CALM1 on the proliferation, migration and invasion in UVM cells. (A) The proliferation curve of MP65 cells in different group. (B) The proliferation curve of MM28 cells in different group. (C) The micrographs of migration assay in MP65 and MM28 cells. (D) box plot representation of the migration assay analysis in MP65 and MM28 cells. (E) Images of invasion assay in MP65 and MM28 cells. (F) box plot of the invasion assay in MP65 and MM28 cells.

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