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ORIGNAL ARTICLE

CIRCULATING MICRORNA-22 AS A BIOMARKER RELATED TO OXIDATIVE STRESS IN HYPOTHYROID WOMEN PATIENT

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Summary

Background: Recent research has linked the spread of microribonucleic acid (miRNA) to numerous disorders, either as a stimulant or an inhibitor. One of these is miRNA-22, which research has connected to oxidative stress and thyroid issues. However, the underlying mechanisms are unknown. This study investigates the expression of miRNA-22 in hypothyroid women and its relationship to the rise in oxidative stress in the patient population.

Materials and Methods: 40 women patients with Hypothyroid and 40 in this study, healthy volunteers who served as controls were included. The levels of serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH) were measured by sandwich assay, while free triiodothyronine (FT3) and thyroxine (T4) levels were measured competitive binding immunoenzymatic assay. To assess lipid profiles, an automated analyzer was employed. By enzyme-linked immunosorbent assay (ELISA), Interleukin 6 (IL-6) levels were measured. Malondialdehyde (MDA), superoxide dismutase activity (SOD), catalase activity (CAT), and advanced oxidation protein products (AOPPs), and assessed using a colorimetric technique. The quantitative polymerase chain reaction was used to evaluate the expression of serum miRNA-22.

Results: Significantly more SOD and CAT activity was identified in patient groups than in the control group ($P<0.05$), also the patient group's AOPP and MDA concentrations were discovered to significantly outweigh those of the control group. ($P< 0.05$). IL-6 levels were significantly higher in the patient group than in ($P<0.05$) the control group. The level of miRNA-22 was higher in the sick group as compared to the control groups ($P<0.05$).

Conclusions: The pathophysiology of oxidative stress brought on by hypothyroidism involves miRNA-22 expression, there is a reciprocal relationship between the increase in gene expression of the miRNA-22 and the increase in oxidative stress, which results in the disease's development.

Key words: Hypothyroid; Oxidative Stress; miRNA-22

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Introduction

Thyroid dysfunction is the most prevalent endocrine disorder worldwide, which is the second after diabetes. Women are more affected by this disease than men, and the percentage increases with age (1). When there are not enough thyroid hormones present in the tissues, hypothyroidism develops. The majority of hypothyroid instances are primary in character, which means that they result from a problem with the thyroid gland itself, such as diseases or therapies that damage thyroid tissue or obstruct the generation of thyroid hormone. They may, to a much lesser extent, be brought on by pituitary and/or hypothalamic illness, which causes shortages in TSH and/or TRH (2).

A key component of the manufacturing of thyroid hormone is hydrogen peroxide. Dual oxidize 1 (DUOX1) and 2 (DUOX2), two insofar enzymes from the NOX family, that generate it in the thyroid gland, have the most experimental support for DUOX2. At each stage of the production of thyroid hormone, including the oxidation of iodide and the subsequent creation of the hormone as well as the coupling reaction involving iodothyronine, hydrogen peroxide serves as an electron acceptor. It is necessary for the function of thyroperoxidase (TPO), the principal enzyme in the manufacture of thyroid hormones. (3).

When the balance between pro-oxidants and antioxidants is disturbed, oxidative stress occurs; Free radicals also contribute to a rise in disease severity for many diseases, including cases of infertility, neuropathy, and retinopathy. Antioxidants are substances that can donate atoms of hydrogen to free radicals produced either through cellular metabolism or from outside sources, producing damage to DNA, lipids, and amino acids, ultimately leading to death of cells (4). The initial line of defense against ROS is provided by members of the superoxide dismutase (SOD) family, which removes extremely reactive superoxide radicals by converting them to hydrogen peroxide (H_2O_2). It is then broken down by the enzymes peroxiredoxin (PRDX), glutathione peroxidase (GPX), and catalase (CAT) (5). The antioxidant system of the cell is supported by the antioxidant enzyme CAT, which is found in peroxisomes and plasma.

One of the potential markers for oxidative damage has been suggested to be advanced oxidation protein products (AOPPs), which results from oxidative and carbonyl stress and increases overall inflammatory activity (6). Malondialdehyde (MDA) is a good biomarker of oxidative stress and damage caused by free radicals in biological samples. MDA's primary source is polyunsaturated fatty acid peroxidation (7).

Interleukin (IL-6), a pleiotropic cytokine with different impacts on cells and tissues, is created by numerous distinctive cell sorts, counting safe cells, fibroblasts, endothelial cells, and tumor cells (8). Mediators of inflammation, such as interleukin 6 (IL-6) has been associated with hypothyroidism (9).

Numerous studies have shown the significance of miRNAs for the development of the thyroid gland, differentiation and hormone production. Deregulation of miRNA expression has also been linked to decreased thyroid cell differentiation and thyroid cancer formation (10). MiRNAs are non-coding, single-stranded RNAs with 19–25 nucleotides have a role in the regulation of transcriptional and post-transcriptional gene expression through specific interactions with target genes (11). Several physiologic and pathological processes in which miRNAs are involved function include adipocyte differentiation, metabolism, appetite regulation, and oxidative stress. (12). MiR-22 is a 22 nucleotide protein that is found in a broad range of tissues and sorts of cells. Its expression was first discovered in HeLa cells, however, it was later discovered to be highly conserved across many vertebrate species (13). The purpose of this work was to outline the regulation of miRNA-22-driven molecular gene expression, its connection to oxidative stress and hypothyroid women, development, and to review recent developments in this area.

Methods

Sample Collection

The current study involved 80 individuals who were separated into 2 groups: hypothyroid women and healthy controls. All patient data, including the age, and length of the illness, were recorded. The control group was chosen with care to make sure that none of the participants had thyroid abnormalities or other illnesses.

The average age of the participants in the current study ranged from (18 - 45) years old. For routine check-ups, patients visit, to the Al-Diwaniyah Teaching Hospital. All of the participants in this study were selected by a licensed physician and clinical signs and biochemical tests, such as Triiodothyronine (T3), Thyroxine (T4), and Thyroid Stimulating Hormone (TSH).

Patients' details such as age, gender, BMI, smoking status, family history, and duration of illness are also documented. Done with all laboratory test analyses at the Nabu Scientific Foundation in (Baghdad, Iraq), were used in the current investigation Al-Diwaniyah Teaching Hospital, Baghdad Lab, and Laboratory for Biochemistry at the College of Science at Al-Qadisiyah University are all in Al-Diwaniyah, Iraq.

Exclusion criteria: Women with autoimmune disease, COVID-19, cancer, diabetes, or hyperthyroidism were not included in the study.

Storage

Five milliliters of each subject's blood were taken and separated into serum. To separate the serum from the blood, the gel tube containing the blood was centrifuged at 4000 rpm for 10 to 15 minutes. Four sections of the isolated serum were separated using Eppendorf tubes. For biochemical examination, just one element of the miRNA-22 research was kept at -40°C. The other elements were preserved at -20°C.

Method

The Serum Thyroid-Stimulating Hormone (TSH), Luteinizing Hormone (LH), and Follicle stimulating Hormone (FSH) levels were measured by sandwich assay (Thyroid-Stimulating Hormone Kit, Mindray, China), (Cobas® Elecsys LH Kit, Roche, USA), and (Cobas® Elecsys FSH Kit, Roche, USA) respectively, while Free Triiodothyronine (FT3), and Thyroxine (T4) levels were measured competitive binding immunoenzymatic assay (Free Triiodothyronine Kit, Mindray, China) and (Total Thyroxine Kit, Mindray, China) respectively. Lipid profiles included measurements of total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C), by routine techniques using an automated analyzer (Abbott, USA) from Al-Diwaniyah Teaching Hospital. Human Interleukin 6 (IL-6) levels were measured by Enzyme-Linked Immunosorbent Assay (ELISA) (Human IL-6(Interleukin 6) ELISA Kit, Elabscience®, and USA). Spectrophotometer was used to measure the serum's activity (SOD) (14). By using ultraviolet (UV) spectrometry, (CAT) was calculated (15). The concentrations of (MDA) were measured using a spectrophotometer (16). UV spectroscopy was used to assess the concentration of (AOPP) (6). Quantitative polymerase chain reaction (qPCR) was used to assess the expression of miRNA-22 in serum. In order to extract RNA, A small amount of serum (0.3 mL) was used (TRIzol™ Reagent, Invitrogen, USA). MiRNA with miR-22-RT-primer was used to make the cDNA using (ProtoScript® First Strand cDNA Synthesis Kit, NEB, UK). PCR was conducted using Luna Universal qPCR Master Mix (NEB, UK). The resulting cDNA was mixed with miR-22-specific forward; reverse universal primers (Table 1), and cDNA Bright Green master mix. Gene U6 was employed as an internal control. A comparative threshold cycle (Ct) was used to calculate the relative levels of miR-22 and ($2^{-\Delta\Delta Ct}$), and the results indicated the fold change of expression.

Table 1. Primers used for qPCR experiments.

Primers	Sequence	Size	Product Size (bp)
miR-22_RT	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACTAAAGC	47	------
miR-22For	GAGCTGCACTGACCAGTAGG	20	99
miR-22Rev	GTGCTGGCAGATGGATCACT	20	
U6 For	GAGAAGATTAGCATGGCCCCT	21	60
U6 Rev	ATATGGAACGCTTCACGAATTTGC	24	

Statistical Analysis

The Statistical Package for Social Sciences (SPSS) version 28 is used to conduct the statistical analysis. The data are presented as a mean and a standard deviation. Independent-Samples T-Test or a nonparametric ranking

(Mann–Whitney U test) were used to evaluate various groups for normal and non-normal distribution data. A ($P > 0.05$) was regarded as significant throughout the entire investigation. The connection between two continuous variables was found using the correlation coefficient (r).

Results

In this study, 40 patients and 40 healthy controls were present. Table 2 displays the characteristics of the patients as well as the outcomes of the biochemical tests. The body mass index (BMI) values in hypothyroid patients were significantly higher than those in the control group. While T3, T4, LH, and FSH levels decreased in the hypothyroid group, TSH levels were noticeably greater than in the control group. While HDL-C was substantially greater in the control group, TC, TG, and LDL levels in the hypothyroid group were significantly higher than those in the other groups ($P < 0.05$).

Table 2. The study groups' clinical and laboratory features.

Parameters	Groups (Mean ± SD)		p-value
	Control	Hypothyroid	
Number	40	40	
Age (years)	34.56 ± 7.90	32.004 ± 9.365	0.616
BMI (Kg/m²)	25.2 ± 0.74	28.3 ± 1.08 *	0.001
TSH (µIU/mL)	3.30675 ± 1.139	7.7646 ± 1.5209 *	0.001
T3 (ng/ml)	1.105 ± 0.2984	0.3744 ± 0.146535 *	0.001
T4 (µg/dL)	9.4135 ± 2.5489	3.88165 ± 1.37065 *	0.001
LH (mIU/mL)	5.616 ± 1.3541	3.775 ± 1.16591*	0.001
FSH (mIU/mL)	7.6275 ± 2.167	4.335 ± 1.401	0.001
TC (mg/dl)	144.35 ± 21.68	205.07 ± 28.7 *	0.001
TG (mg/dl)	106.075 ± 30.08	192.575 ± 31.726 *	0.001
HDL-C (mg/dl)	51.175 ± 10.27	41.75 ± 6.865 *	0.001
LDL-C (mg/dl)	85.98 ± 26.336	118.81 ± 39.1358 *	0.001

*Mean statistically significant differences between patient groups as compared to the control group ($P < 0.05$) Body mass index (BMI) .TSH stands for thyroid stimulating hormone, T3 for triiodothyronine, and T4 for thyroxine. LH stands for luteinizing hormone, FSH for follicle-stimulating hormone. TC for total cholesterol, and TG for triglyceride. LDL stands for low density lipoprotein and HDL-C stands for high density lipoprotein.

SOD and CAT activity contrasted with the control group was increased significantly ($P < 0.05$) in the hypothyroid group (Figure 1), and (Figure 2). The AOPP and MDA levels in the hypothyroid group was all significantly higher than in the control group (Figure 3), and (Figure 4). IL-6 level was increased in the hypothyroid group (Figure 5). When compared to a control group, the hypothyroid group had significantly greater serum levels of miRNA-22 expression, according to qPCR miRNA analysis (Figure 6).

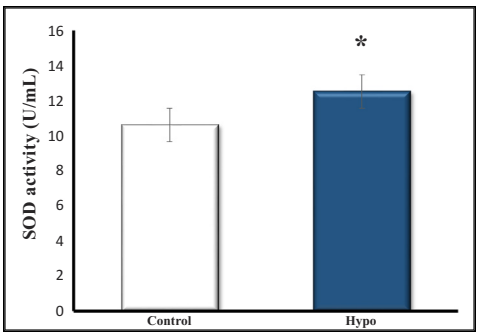

Figure 1. SOD activity U/ml was compared between the hypothyroid and control groups with a P-value >0.03. * Mean statistically significant differences between patient groups as compared to the control group ($P < 0.05$).

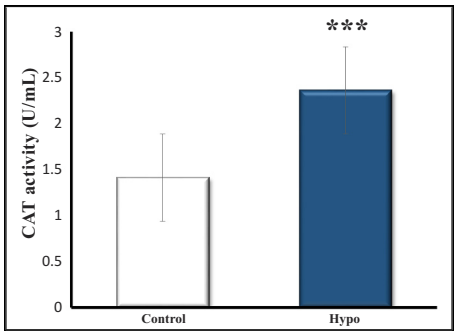

Figure 2. Comparison of CAT activity U/ml in the hypothyroid and control groups, as well as other study groups (P-value < 0.001). * Mean statistically significant differences between patient groups as compared to the control group (P < 0.05).

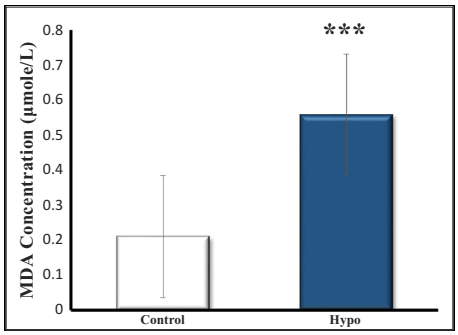

Figure 3. MDA level comparison between control and hypothyroid groups, as well as other study groups, in mol/L (P-value < 0.001). * Mean statistically significant differences between patient groups as compared to the control group (P < 0.05).

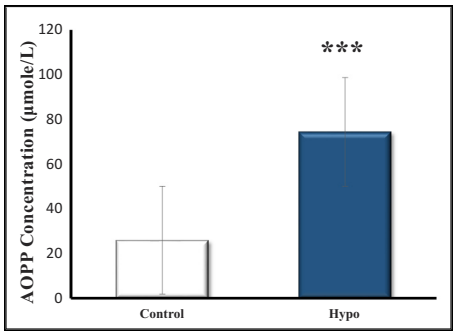

Figure 4. AOPPs level mol/L in the control and hypothyroid groups, among other examined groups (P-value < 0.001). * Mean statistically significant differences between patient groups as compared to the control group (P < 0.05).

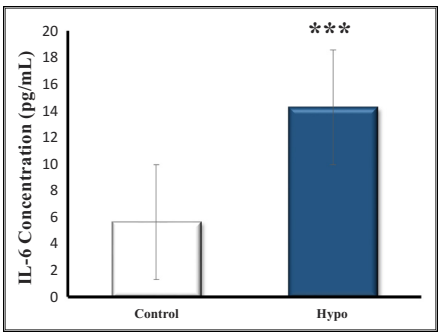

Figure 5. Comparison of IL-6 level pg/mL control and hypothyroid group in various study groups (P-value < 0.001). * Mean statistically significant differences between patient groups as compared to the control group (P < 0.05).

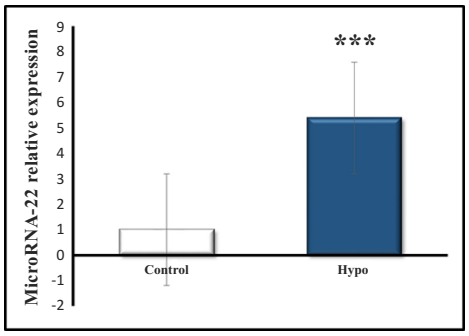

Figure 6. The levels of microRNA-22 expression in the various study groups—control and hypothyroid group—were compared. (P-value = 0.001). * Mean statistically significant differences between patient groups as compared to the control group ($P < 0.05$).

There were extremely weak positive connections with CAT but significant positive strong associations with TSH, MDA, and IL-6 in the hypothyroid group (Figure 7).

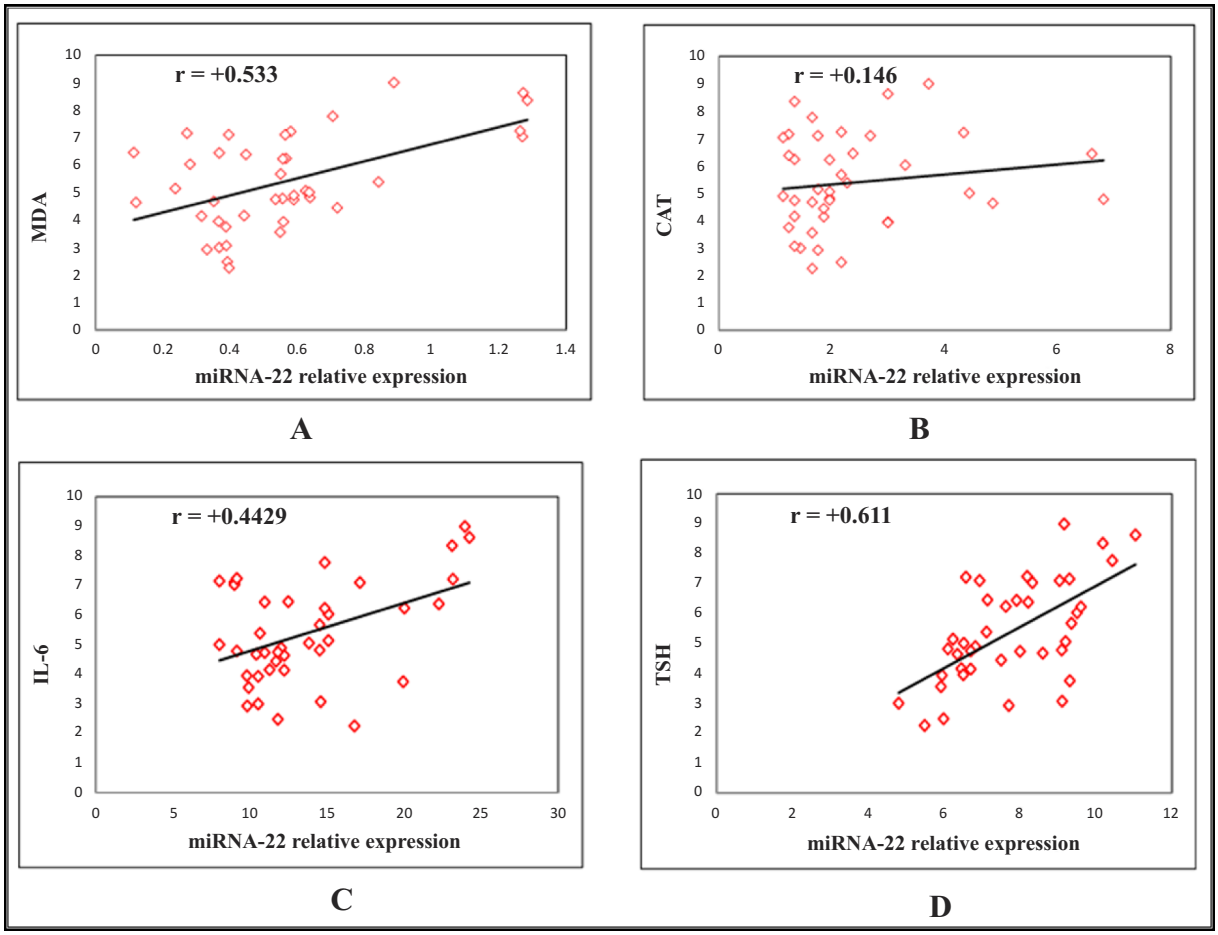

Figure 7. Correlation between microRNA-22 and other biomarkers in hypothyroid group (A) with MDA, (B) with CAT, (C) with IL-6, (D) with TSH. r: means value of correlation coefficient.

Additionally, in the hypothyroid group, there were highly significant positive correlations between TSH and MDA, TSH and AOPP, and MDA and AOPP (Figure 8).

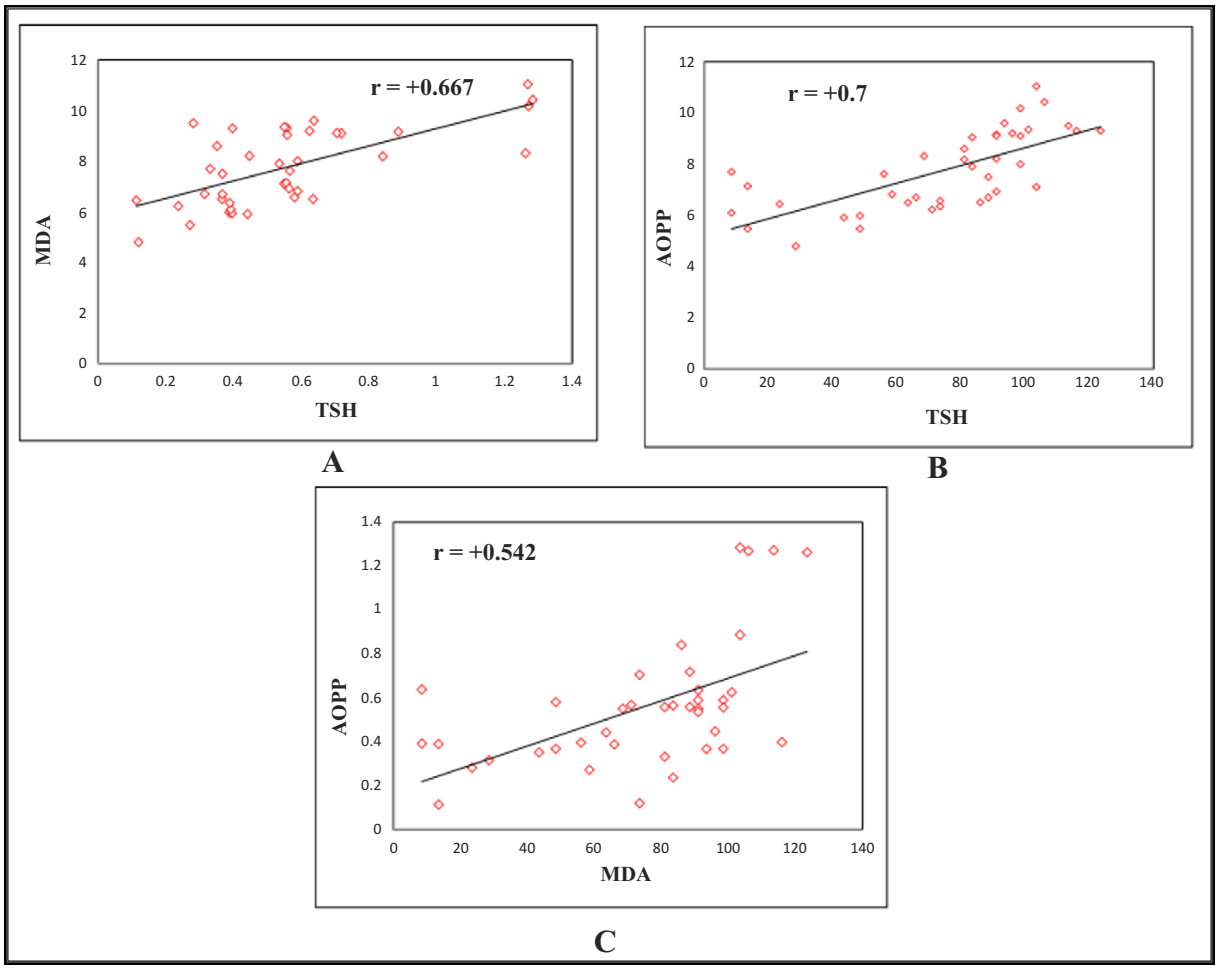

Figure 8. Correlation between (A) TSH with MDA, (B) TSH with AOPP, and (C) MDA with AOPP in hypothyroid group. r: means value of correlation coefficient.

Discussion

The metabolism of lipids is significantly influenced by thyroid hormones. Any thyroid hormone shortage has a propensity to lead to hyperlipidemia, which is a recognized risk factor for the emergence of atherosclerotic disease. According to this study, overt hypothyroidism is associated with increased TC, TG, and LDL-C concentrations. This discovery is consistent with other research, such as those conducted by (Murgod and Soans 2012) (17).

The results showed an increase in both MDA and AOPP in women with hypothyroidism when compared with the control group. The reason for increasing in oxidative stress in hypothyroidism is probably complex in nature. In spite of being described as in a hypo metabolic state, a slower removal rate in hypothyroidism might lead to higher lipid peroxide levels (18). The additional hypothesized processes linked to increased oxidative stress in hypothyroidism include hyperlipidemia, reduced cholesterol clearance, a weak antioxidant system, effects of thyroid hormones on control of antioxidant enzymes, and excess TSH (19). Increased in the present study oxidative stress was demonstrated by elevated levels of MDA and AOPP among the women hypothyroid group when compared to controls, showing that oxidative damage to lipids and proteins has increased, respectively. Our discovery of elevated lipid peroxidation is consistent with (Zai, Gill *et al.* 2021) (Haribabu, Reddy *et al*. 2013) (18, 20). Earlier research shown that elevated TSH levels may directly influence protein carbonylation and lipid peroxidation, causing levels of MDA and AOPP to rise (21). TSH overproduction has been linked to a higher oxidative stress as well as small amounts of inflammation (21). Increased End products of lipid peroxidation include

an excessive amount of reactive aldehydes. Found increase in MDA level as one such lipid peroxidation byproduct that promotes changes to protein structure and function (18). According to reports, MDA can establish covalent connections with proteins that allow for an irreversible link and promote the development of AOPP (22). As a result, the higher AOPP seen in the patient group could be attributed to enhanced lipid peroxidation. And our discovery of an important correlation between TSH and AOPP noticed in the patient group suggests that excess TSH might also be responsible for the increased AOPP levels, observed. We discovered that the patient group's AOPP levels were affected by both TSH and MDA as showed in (Fig.8).

Two enzymes engaged in the oxidation of hydrogen peroxide and its generation, SOD and CAT, are significantly influenced by the body's thyroid function. In the hypothyroid state, a rise in SOD activity will speed up hydrogen peroxide synthesis while a fall in catalase activity will slow down its elimination. Our findings showed an increase activity of CAT and SOD in women with hypothyroid which is a reflex mechanism against increased oxidative stress induced by hypothyroidism, the results agree with (Chattopadhyay, Zaidi *et al*. 2003) (23).

The results that were found showed a statistically significant increase in the concentration of IL-6 in the women with hypothyroidism when compared with the control group. Similar to our results, a correlation between hypothyroidism and low-grade inflammation was indicated previously (24, 25). The results observed suggest that elevated levels of pro-inflammatory cytokines may be associated with hypothyroidism. Cytokine production, including IL-6, has been linked to hypothyroidism (26). Our results are consistent with (Gómez-Zamudio, Mendoza-Zubieta *et al*. 2016) (27). The IL-6 elevation may be caused by the elevation of TSH in the hypo group. Researchers found that patients with greater TSH levels had higher serum levels of the cytokine IL-6 in a study by Sieminska *et al*, which was linked to the emergence of the metabolic syndrome (28).

Our findings demonstrated for the first time a statistically significant increase in miRNA-22 expression in women with hypothyroidism. This could be explained by the relationships that were reached, as a solid rapport existed. Found between miRNA-22 and MDA. It is possible that the reason for the increase in oxidative stress in this group led to an increase in the expression of miRNA-22, and another reason could also be that the rise of TSH It has a relationship with the rise of miRNA-22, where this relationship was confirmed by correlations that worked between miRNA-22 and TSH, where a strong positive relationship was observed between them as showed in (Figure 7).

Conclusion

Serum circulating microRNAs play important role in common pathogenic mechanisms and may be particularly useful markers and targets for treating hypothyroidism, where the results showed the highest statistical evidence for the level of microRNA-22 was in the hypothyroid group. A change in microRNA-22 expression may suggest that this biomarker has a function in the development of hypothyroidism. This research discovered a strong correlation between miRNA-22's gene expression and its stimulus of oxidative stress. However, more studies and scientific research are needed to determine the microRNA expression and show the pathways of its effect in stimulating or suppressing diseases and thus can benefit from this in determining treatment pathways.

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Conflict of Interest Statement

The authors have no conflicts of interest regarding the publication of this article.

Adherence to Ethical Standards

The study was approved by the ethical committee at the University of Al-Qadisiyah (registration code CMUQ 3187 on 28.8.2022).

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