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Int J Med Biochem

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- Book with Single Author Cohn PF. Silent myocardial ischemia and infarction. 3rd ed. New York: Marcel Dekker; 1993.
- Editor(s) as author: Norman IJ, Redfern SJ, editors. Mental health care for elderly people. New York: Churchill Livingstone; 1996
- Technical Reports Tschantz BA, Moran B. Modeling of the hydrologic transport of mercury in the Upper East Fork Poplar Creek (UEFPC) watershed. Technical Report for Lockheed Martin Energy Systems: Bethesda, MD, September 2004.
- Supplement Castelli WP. Lipids, risk factors and ischaemic heart disease. Atherosclerosis 1996;124 Suppl:S1-9.
- Epub ahead of print Milbury CA, Li J, Makrigiorgos GM. PCR-based methods for the enrichment of minority alleles and mutations. [Epub ahead of print] Clin Chem February 6, 2009 as doi:10.1373/clinchem.2008.113035.
- Internet Source: American Association for Clinical Chemistry. AACC continuing education. http://www.aacc.org/education-and-career/continuing-education(Accessed June 2016).

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Research Article

Remifentanil induces apoptosis and G1-phase cell cycle arrest in human MCF-7 breast cancer cells

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Abstract

Objectives: Remifentanil, a fentanyl-derivative opioid analgesic acting as a μ-opioid receptor agonist, is a crucial drug in anesthesia due to its numerous benefits during surgical procedures. This study aimed to explore whether remifentanil effectively induced apoptosis in MCF-7 breast cancer cells via possible mechanisms.

Methods: Flow cytometry was performed for Annexin V/7-aminoactinomycin (7-AAD) and DAPI staining, cell cycle assays, and to measure reactive oxygen species (ROS) levels. Immunoassays for lactate dehydrogenase (LDH) and interleukin (IL)-6, as well as a chorioallantoic membrane (CAM) test, were also performed.

Results: Remifentanil effectively suppressed cell proliferation and led to the induction of cell cycle arrest at the G1 phase in MCF-7 cells. Compared with the control group, MCF-7 cells treated with remifentanil had a higher apoptotic rate with nuclear fragmentation, increased LDH release, and lower IL-6 concentrations. Overgeneration of ROS and decreased angiogenic activity were also observed in remifentanil-treated MCF-7 cells.

Conclusion: Remifentanil led to G1-phase arrest and apoptosis in MCF-7 cells. The mechanism of action of remifentanil likely involves the suppression of IL-6 production and angiogenesis, along with enhanced ROS levels and LDH generation. This preliminary study highlighted the need for further experimental evidence from future research to clearly support the significant potential of remifentanil as an anticancer agent for breast cancer.

Keywords: Anticancer effect, apoptosis, MCF-7 breast cancer cells, oxidative stress, remifentanil

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Breast cancer represents one of the most prevalent female malignant tumors globally [1]. According to a recently published statistic in 2023, thirteen percent of malignancies in women are caused by breast cancer, and the greatest number of deaths from breast cancer among women has been reported [1]. However, the global mortality of breast cancer is falling as a better indicator of progress due to early diagnosis through the established system of mammography screening, increased awareness, and improvements in comprehensive treatment options such as adjuvant chemotherapies [1]. The available options include surgery, radiation, chemotherapy, immunotherapy, or hormonal therapies, depending on the stage and subtype of breast cancer [2]. Despite significant advancements in diagnostic and therapeutic approaches against breast cancer, the treatment still remains a challenging endeavor due to the high relapse and lethality rate of the disease, significant side effects, and the lower effectiveness of therapeutic strategies [3].

Patients with breast cancer are most commonly diagnosed as hormone receptor-positive [4]. The MCF-7 (Michigan Cancer Foundation-7) cell line is characterized as the Luminal A subtype of breast cancer, which is an estrogen and progesterone receptor-positive tumor [5]. A primary care approach for the

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majority of solid organ malignancies is surgery [6]. Nonetheless, it is typical for cancer to return following surgery intended to be curative; this frequently results in refractory illness and patient mortality [6]. The biological milieu created by surgery and perioperative care encourages the survival and proliferation of cancer cells that are still present in the primary tumor after the procedure, which may raise the risk of metastasis [6]. Analgesic and anesthetic therapies may influence these processes and, in turn, either positively or negatively increase the likelihood of cancer recurrence, according to mounting data [6].

Fentanyl and its derivative remifentanil are potent opioid analgesics [7]. Remifentanil, as a selective μ-opioid receptor agonist, is used extensively in surgeries for the induction of general anesthesia and the preservation of clinical analgesia [8]. Remifentanil is a valuable agent in anesthetic practice due to its several advantages in surgeries. It has significant advantages, such as its analgesic effect, which is about 100–200 times higher than that of morphine [9]. It metabolizes quickly by non-specific esterases in the blood and tissues and acts swiftly, leading to an abrupt cessation of effects once the infusion stops [8]. This allows precise control over anesthesia depth and quick recovery after surgery [8]. Remifentanil aids in preserving a steady heart rate and blood pressure during surgery, reducing the risk of hemodynamic fluctuations, which is particularly beneficial for high-risk patients and complex procedures [10]. Its pharmacokinetic profile enables easy and precise titration to the desired effect, allowing anesthesiologists to adjust the dose in real time [10]. Due to its rapid metabolism and high plasma clearance rate, remifentanil does not accumulate in the body, even with prolonged use, making it ideal for long surgeries or extended analgesia [10]. Remifentanil can be used in various surgical settings, including general anesthesia, procedural sedation, as an adjunct in regional anesthesia, and even in the intensive care unit for optimal analgesia and sedation [11, 12]. Due to its advantages, remifentanil is often favored over other short-acting opioids and has become the primary choice of therapy in many intensive care units [11, 12]. These advantages make remifentanil a preferred choice for anesthesiologists, particularly where precise control over anesthesia and rapid recovery are critical.

Reactive oxygen species (ROS) are generated during aerobic metabolism within the biological system [13]. Inflammation triggers higher amounts of ROS, which might result in oxidative stress [14]. In addition, oxidative stress is capable of producing an increased inflammatory response [14]. ROS at low levels act as signaling molecules in the regulation of cellular or physiological activities and homeostasis [13]. The overproduction of ROS due to a disturbed redox balance would result in oxidative stress under pathophysiological conditions, which causes a direct oxidizing effect and thereby damage to fundamental cellular elements, including lipids, proteins, and DNA [14].

It has been reported that remifentanil alleviates inflammation and oxidative stress in many experimental models [15, 16]. Remifentanil ameliorated myocardial ischemia and acute lung injury in rats via the inhibition of several proinflammatory cytokines [15, 17]. Oxidative stress and inflammation are crucial mechanisms in the pathogenesis of cancer [14]. In addition, previous *in vitro* studies have demonstrated controversial results that opioids could inhibit or promote the proliferation of various cancer cells [18, 19]. Morphine promoted apoptosis and caused G0/G1 and G2/M phase arrest in hepatocellular and gastric cancer cell lines, respectively [20, 21]. Moreover, a study by Li et al. [22] demonstrated the anticancer effects of fentanyl on MGC-803 gastric cancer cells via cell cycle arrest in the G0/G1 phase.

Specifically, this study examines how remifentanil influences cytotoxicity, apoptosis, cell cycle, inflammation, oxidative stress, and angiogenesis of MCF-7 cells, considering the broader context of using antioxidant and anti-inflammatory agents as an anticancer strategy. However, future research could be employed on animal models and various kinds of cancer cell lines to elucidate the *in vivo* and *in vitro* anticancer effects of remifentanil in detail. Then the preclinical data can be adopted for clinical studies.

Materials and Methods

Cell culture

MCF-7 cells (ATCC, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C with 5% CO_2. Adherent cells were sub-cultured every two days. At a confluency of 80%, the cells were passaged with 0.05% trypsin (Gibco, CA, USA) for further experiments. Stock solutions of remifentanil were dissolved in dimethyl sulfoxide (DMSO) or saline (0.9% NaCl) and subsequently in culture medium. Throughout treatment, the DMSO levels did not rise above 0.1%.

Cell viability

The MCF-7 cell viability was evaluated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay (Beyotime Biotech, Haimen, China). In summary, 150 μL of cell mixture (1×10^4 cells) was added to each well. The cells were incubated overnight and then treated for 24 and 48 h with remifentanil (0.15625, 0.3125, 0.625, 1.25, 2.5, 5, 10 μg/mL). After the removal of media, MTT solution (Sigma Aldrich, Missouri, USA) was applied to each well for 4 h at 37°C. Following the addition of 150 μL of DMSO, the absorbance of each well was determined at 550 nm using a plate reader.

Apoptosis assay by flow cytometry

The apoptosis rate was assessed using an Annexin V/7-aminoactinomycin (7-AAD) detection kit (BD Biosciences, NJ, USA). The cells were exposed to 0.44 μg/mL of remifentanil for 24 and 48 h in 6-well culture plates. Cells were then collected, rinsed with cold PBS, suspended in binding buffer, and stained with PE-Annexin V for 15 minutes. After the addition of 7-AAD (5 μg/mL) and binding buffer, tubes were incubated for 30 min in the dark. Then, a flow cytometer (BD Accuri C6, USA) was used for the quantification of apoptotic cells.

4′,6-Diamidino-2-phenylindole (DAPI) staining

After exposure to remifentanil for 24 and 48 h in 6-well plates, MCF-7 cells (2×10^4 cells/well) were collected, rinsed with PBS, and then preserved in 4% formaldehyde for 10 minutes. After centrifugation, cells were treated with 20 µL DAPI (Invitrogen, USA) in the dark for 20 min at room temperature. Afterwards, the supernatant was removed, the cell pellet was mixed with sterile PBS, and the cell suspension was placed on coverslips. The nuclei of the stained cells were visualized under confocal microscopy (Zeiss LSM 510, USA).

Cell cycle assay by flow cytometry

After 24 and 48 h of treatment with remifentanil (0.44 µg/mL), the cells were collected by 0.05% trypsin for harvesting, washed twice with assay buffer, and fixed in 70% ethanol at 4°C overnight. Following the elimination of ethanol, cells were resuspended in PBS. After staining cells with propidium iodide (PI) containing RNase (20 µg/mL) for 30 min at 37°C in the dark, a flow cytometer (BD Accuri C6, USA) with CellQuest software was used to determine cell cycle distribution by measurements of side and forward scatter.

Flow cytometric analysis of intracellular ROS generation

Dichloro-dihydrofluorescein diacetate (DCF-DA) probe, a ROS-sensitive fluorescent dye, was used to determine ROS generation according to the manufacturer's instructions. After cells were treated for 24 and 48 h with remifentanil, cells suspended in PBS were incubated with DCF-DA (10 µM) for 30 min in an incubator. Finally, the DCF fluorescence of cells was detected via a BD Accuri C6 flow cytometer.

Lactate dehydrogenase (LDH) and interleukin-6 (IL-6) measurement

After treatment with remifentanil (0.44 µg/mL) in MCF-7 cells for 24 and 48 h in 6-well plates, cell supernatant was collected to measure LDH and IL-6 levels using the Roche Cobas 6000 and e411 analyzer and related kits.

Chorioallantoic membrane (CAM) assay for angiogenesis

Fertilized chicken eggs were cultivated at 37°C and 85–90% relative humidity for the purpose of the ex ovo CAM assay. Atak-S chickens raised at the Ministry of Agriculture and Forestry's Poultry Research Institute in Ankara, Turkey, provided the fertilized chicken eggs. The cleaned eggs were incubated for 72 hours, with a 2-hour rotation, in an incubator (CIMUKA 40080 serial number-incubator). Following incubation, the embryo side of the eggs was labeled and examined under a light source. The eggs were cracked using forceps from the opposite side so that the selected region remained on top. After being placed inside the weighing container, the eggs were returned to the incubator and covered with glass lids sterilized with ethylene oxide. The eggs were incubated for an additional three days to increase the visibility of the CAM. Remifentanil (0.44 µg/mL) was applied ex ovo to the chorioallantoic membrane following incubation. Following the application, the groups were observed at 0, 24, and 48 h using a stereomicroscope (S6D; Leica Microsystems, Heerbrugg, Switzerland). Quantitative analysis of the captured images was conducted using the ImageJ program (National Institutes of Health).

Statistical analysis

GraphPad Prism 8 software was used to compare data using a one-way analysis of variance (ANOVA) test. Each experiment was conducted in two biological replicates and three technical replicates. $P<0.05$ was regarded as statistically significant after comparing the variations between means (mean±SD).

Results

Remifentanil inhibited the viability of MCF-7 breast cancer cells

To evaluate the antiproliferative effects of remifentanil, MCF-7 cells were incubated with remifentanil between 0.15625–10 µg/mL for 24 and 48 h. After exposure to remifentanil, a dose- and time-dependent decline in cell viability was observed. The results showed that remifentanil induced cytotoxicity within the range of dosages tested for 24 and 48 h (Fig. 1). As expected, remifentanil administered for 48 h at the dose of 10 µg/mL offered the highest cytotoxic effect. The IC_{50} values of remifentanil were calculated as 0.86 µg/mL for 24 h and 0.44 µg/mL for 48 h treatment. For the selection of remifentanil dosages for 24 and 48 h of treatment in the further experiments, we have taken 0.44 µg/mL, which was the IC_{50} concentration of remifentanil for 48 h in MCF-7 cells.

Remifentanil induced apoptosis in MCF-7 cells

To assess the apoptotic progression of the remifentanil-exposed cells, flow cytometry was used. The percentages of early and late apoptotic cells were significantly increased after 0.44 µg/mL remifentanil treatment for 24 h in MCF-7 cells ($p<0.05$). For 48 h treatment, the results demonstrated that remifentanil (0.44 µg/mL) promoted early and late apoptosis; however, only the proportion of late apoptosis was significantly higher in comparison to the untreated cells ($p<0.05$; Fig. 2a). The percentage of total apoptosis induced by 24 h of remifentanil treatment in MCF-7 cells (18.9%) was considerably higher than that of 48 h of treatment (14%). However, a lower percentage of MCF-7 cells undergoing necrosis after exposure to 0.44 µg/mL remifentanil for 24 h (13.3%) was observed compared to 48 h of treatment (17.3%).

Remifentanil led to nuclear fragmentation in MCF-7 cells

Nuclear condensation and apoptotic body formation are considered the core features of apoptosis in cancer cells [23]. DAPI staining was performed to observe whether remifentanil causes nuclear morphological alterations. In the untreated MCF-7 cells, there were rounded and homogeneous nuclei, while cells treated with 0.44 µg/mL remifentanil for 24 and 48 h exhibited apoptotic morphology with nuclear fragmentation and condensation. MCF-7 cells incubated with 48 h of remifentanil showed darker and more intense blue coloration compared to 24 h incubation (Fig. 2b). The results suggest that remifentanil led to cell death, and these data also correlated with the cytotoxicity results and apoptosis data obtained by flow cytometry.

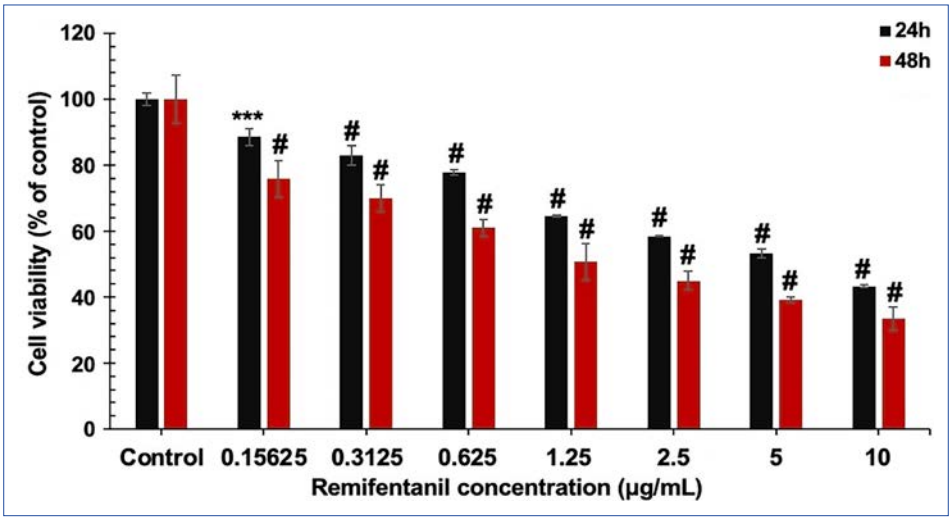

Figure 1. Determination of cell viability by MTT assay after 24 and 48 h remifentanil treatment (0, 0.15625, 0.3125, 0.625, 1.25, 2.5, 5 and 10 µg/mL).

The three separate experiments are shown as the mean±SD. ***: p<0.0005; #: p<0.0001 vs control group. MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; SD: Standard deviation.

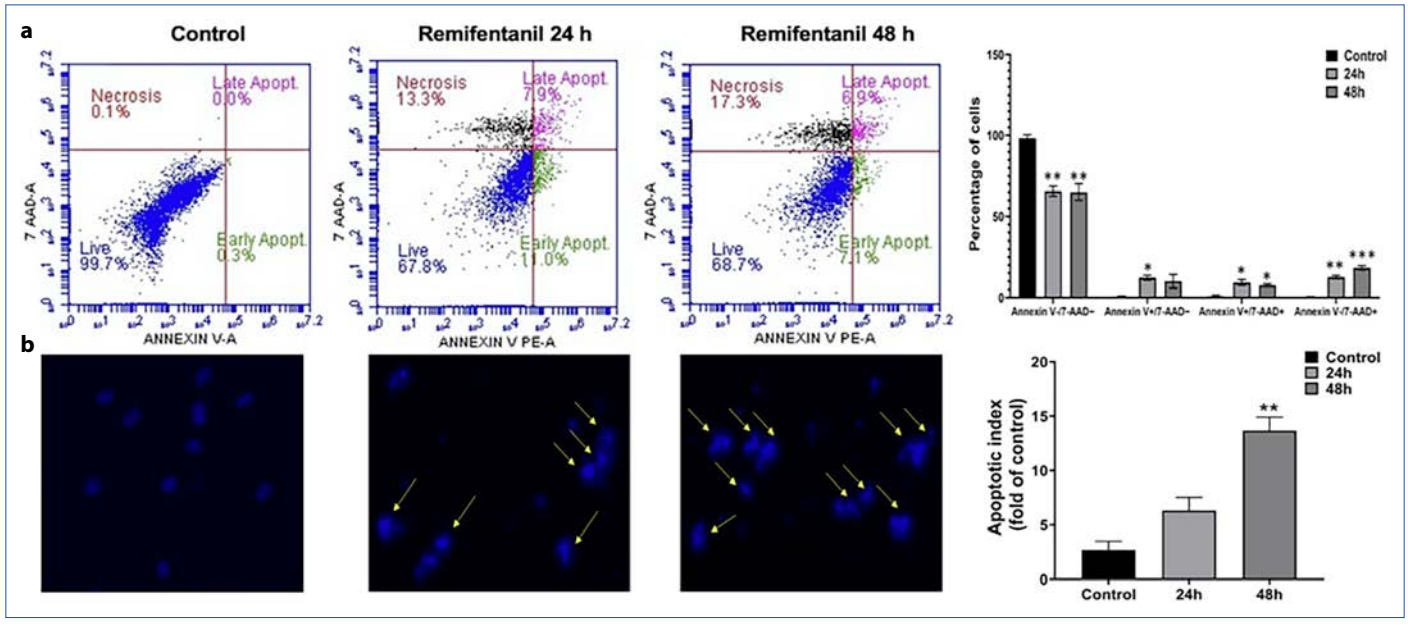

Figure 2. The effect of remifentanil (0.44 µg/mL) on the apoptosis of MCF-7 breast cancer cells after 24 and 48 h treatment. (a) Apoptotic cell death induced by 24 and 48 h remifentanil treatment via Annexin V/7-AAD double staining. The percentage of viable, early, late and necrotic cells was shown in upper right panel. (b) Fluorescence microscopy images at 20x magnification after DAPI staining of MCF-7 cells incubated with remifentanil (0.44 µg/mL) for 24 and 48 h. Apoptotic cells are depicted by the arrows. Quantitative analysis of apoptosis detected by DAPI staining is depicted in right panel.

The data are shown as the mean±SD. *: p<0.05; **: p<0.01; ***: p<0.001 vs control group. MCF-7: Michigan Cancer Foundation-7; V/7-AAD: V/7-aminoactinomycin; DAPI: Diamidino-2-phenylindole.

Remifentanil arrested the MCF-7 cell cycle in the G1 phase

To better clarify the mechanism of remifentanil-induced cytotoxicity, the distribution of cell cycle phases was determined in MCF-7 cells after incubation with 0.44 µg/mL remifentanil for 24 and 48 h. As presented in Figure 3, the cell population after 24 and 48 h of remifentanil treatment was highly accumulated in the G1 phase (28.5% and 37.6%, respectively) compared to the untreated cells (19.5%), which was accompanied by a concomitant decrease in the G2 population. Following 24 and 48 h of treatment with remifentanil, the percentage of MCF-7 cells in the G2 phase was 70.2% and 60.6%, respectively, while 75.9% of the control cells were found to be in the G2 phase (Fig. 3a, b). Moreover, there were no alterations in the S phase among groups. The data suggest that

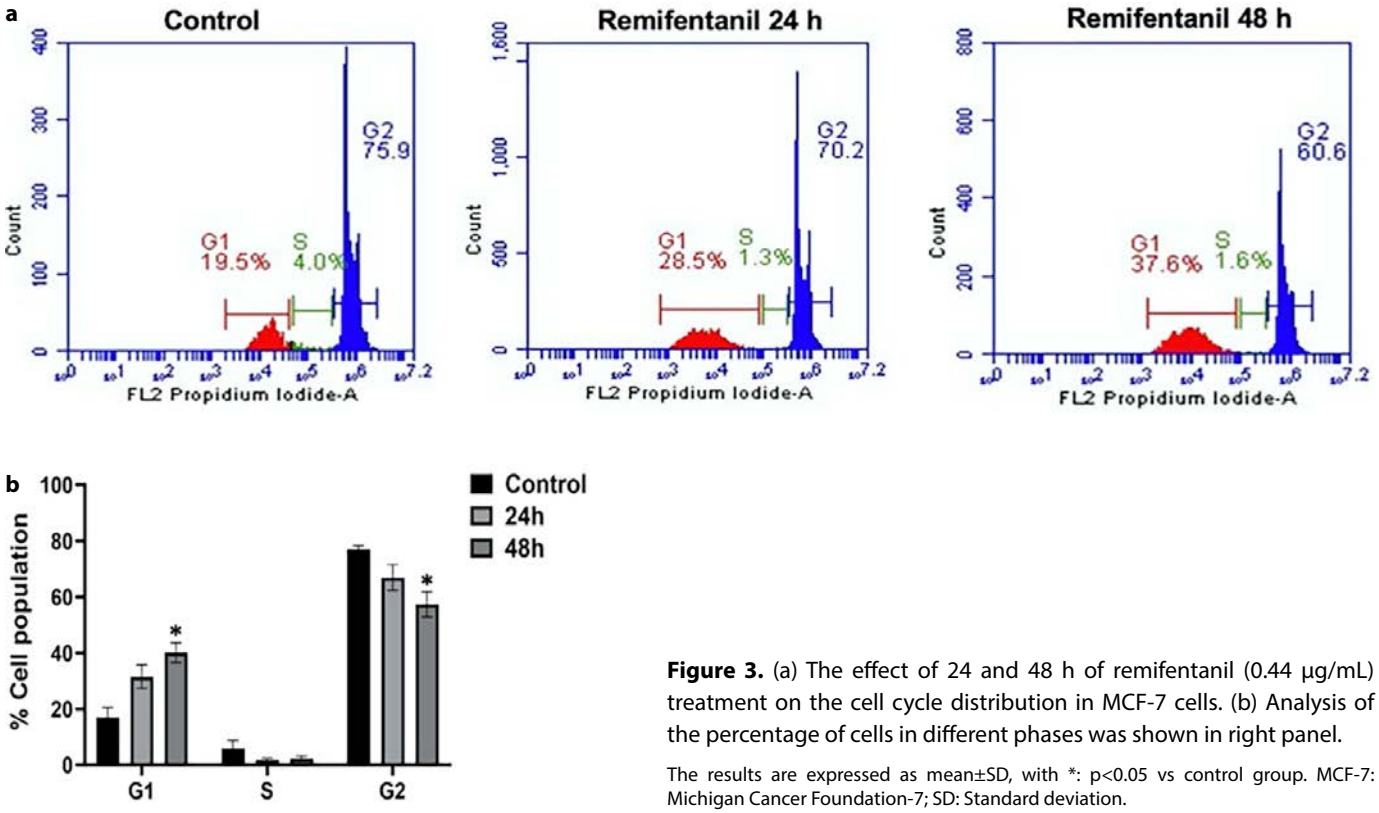

Figure 3. (a) The effect of 24 and 48 h of remifentanil (0.44 µg/mL) treatment on the cell cycle distribution in MCF-7 cells. (b) Analysis of the percentage of cells in different phases was shown in right panel.

The results are expressed as mean±SD, with *: p<0.05 vs control group. MCF-7: Michigan Cancer Foundation-7; SD: Standard deviation.

remifentanil induces apoptosis by causing cell cycle arrest in the G1 phase in MCF-7 cells.

Remifentanil promoted ROS overproduction in MCF-7 cells

In the present study, we evaluated whether remifentanil influences the generation of ROS in MCF-7 cells. Remifentanil exposure caused a rise in ROS production to 42.5% after 24 h and 47.2% after 48 h of 0.44 µg/mL remifentanil treatment compared to the control group (13.7%) ($p<0.01$, Fig. 4a, b).

Remifentanil increased lactate dehydrogenase (LDH) release and repressed interleukin (IL)-6 levels in MCF-7 cell culture media

Remarkable upregulation of LDH release in the supernatant of MCF-7 cells was observed after 48 h of treatment with 0.44 µg/mL remifentanil ($p<0.01$, Fig. 5a). The IL-6 levels for remifentanil-treated MCF-7 cells at a dose of 0.44 µg/mL diminished in a time-dependent manner ($p<0.05$ and $p<0.01$ for 24 and 48 h, respectively; Fig. 5b).

Remifentanil restrained angiogenesis in MCF-7 cells

The CAM model was used to evaluate the effect of remifentanil on angiogenesis *in vivo* [24]. The effect of 24 and 48 h of remifentanil (0.44 µg/mL) on the inhibition of angiogenesis was demonstrated by a decreased percentage of vascular proliferation ($p<0.01$; Fig. 6a, b). The ability of remifentanil to repress angiogenesis might contribute to its antitumor effect in the MCF-7 cell line.

Discussion

Remifentanil, a fentanyl derivative, is an ultra-short-acting, highly effective opioid analgesic agent with its predictable pharmacokinetic profile [8]. It is especially valuable in anesthesia due to its rapid action and metabolism, making it more potent than morphine [9]. In clinical practice, the dosing of remifentanil varies depending on the context of its use [25]. For the induction of anesthesia, a loading remifentanil dose of 1.0 microgram per kilogram (mcg/kg) is administered over 30 to 60 seconds, followed by maintenance of anesthesia typically ranging from 0.1 to 1.0 mcg/kg/minute based on the patient's response and surgical conditions [25]. In the intensive care unit, for sedation, remifentanil is used at a much lower continuous infusion rate, usually between 0.01 and 0.05 mcg/kg/minute [25]. Its pharmacokinetic profile prevents accumulation, even with prolonged use, making it ideal for long surgeries [8]. The drawbacks of using remifentanil in intensive care, such as the potential for hyperalgesia and withdrawal symptoms upon discontinuation, can be effectively controlled [26]. These concerns should not prevent its utilization, as they can be addressed by providing analgesics or opiates after remifentanil infusion and before dose tapering [26]. High doses of remifentanil may pose a risk that necessitates careful monitoring and management to address potential complications [9].

Research in the literature has increasingly supported the anticancer effects of fentanyl and its derivatives [22, 27, 28]. However, studies focusing on the apoptotic effects of

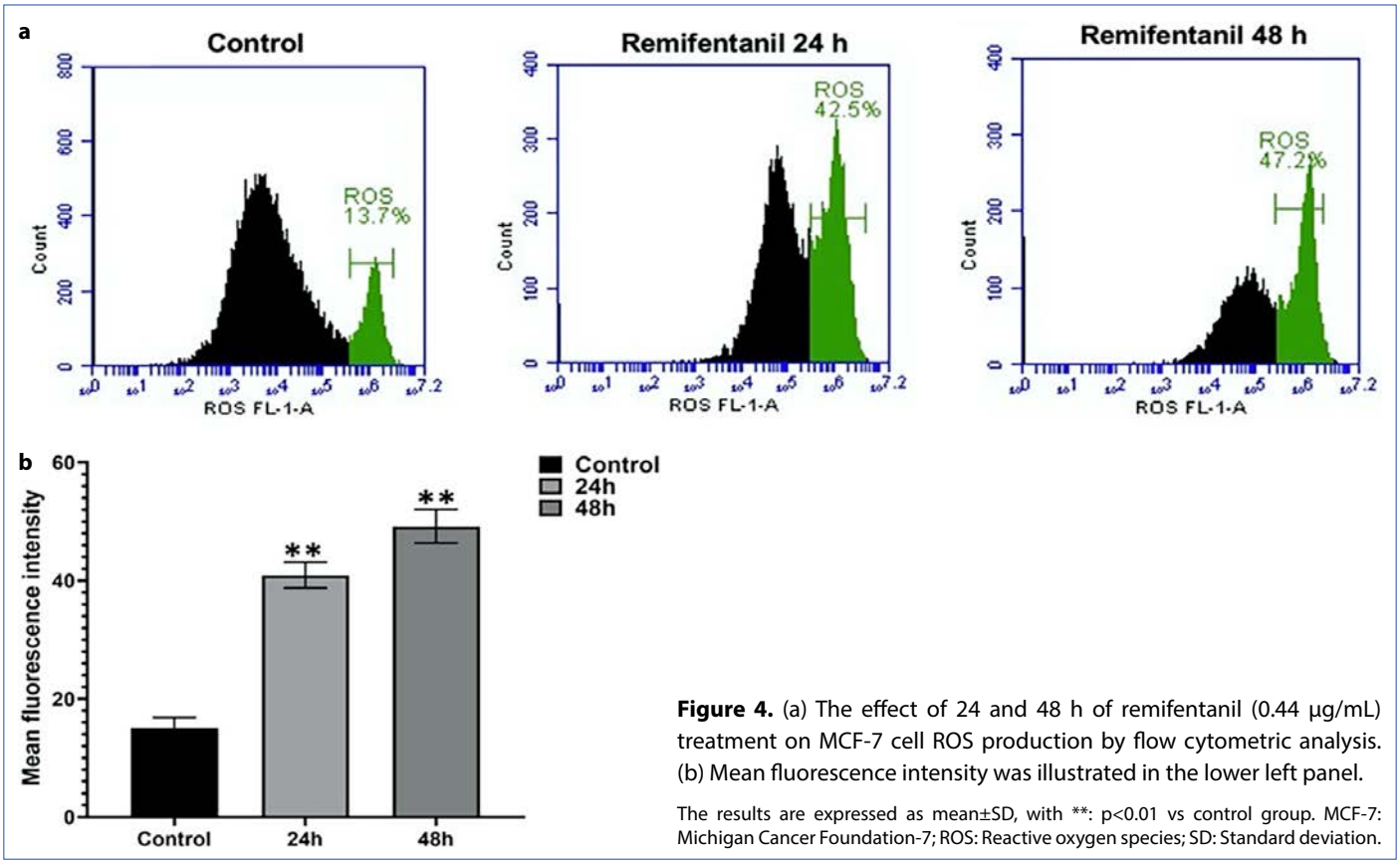

Figure 4. (a) The effect of 24 and 48 h of remifentanil (0.44 µg/mL) treatment on MCF-7 cell ROS production by flow cytometric analysis. (b) Mean fluorescence intensity was illustrated in the lower left panel.

The results are expressed as mean±SD, with **: p<0.01 vs control group. MCF-7: Michigan Cancer Foundation-7; ROS: Reactive oxygen species; SD: Standard deviation.

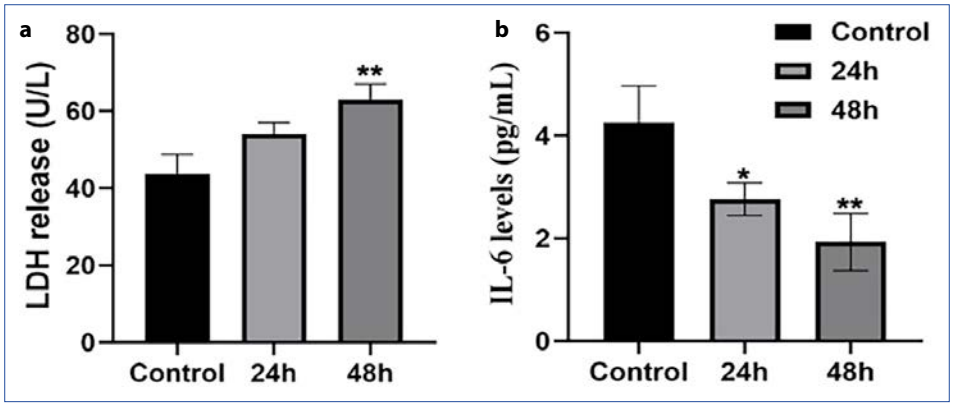

Figure 5. Levels of (a) LDH and (b) IL-6 in MCF-7 cell culture media after 24 and 48 h exposure of remifentanil (0.44 µg/mL).

The results are expressed as mean±SD, with *: p<0.05; **: p<0.01 vs control group. LDH: Lactate dehydrogenase; IL-6: Interleukin-6.

remifentanil in different cancer cells are limited in number [29–31]. The beneficial effects of remifentanil in normal cell lines at nanomolar levels have been previously reported [32, 33]. These data may demonstrate the high safety and selectivity profile of remifentanil due to its decreased cytotoxicity to normal cells and antitumor effects on various cancer cell lines [29–31], even though the applied concentrations of remifentanil vary. Another opioid agent, morphine, also showed higher cytotoxicity against the human MCF-7 breast cancer cell line than normal human cells [34] and significantly increased the proliferation of MDA-MB-231 breast cancer cells at low concentrations but demonstrated cytotoxic effects at higher concentrations [35].

In addition, a growing body of evidence has indicated good anti-inflammatory and antioxidant activities of remifentanil in various animal disease models [15–17]. Based on the confirmed relationship between inflammation, oxidative stress, and the carcinogenesis process, we have therefore proposed that remifentanil can exert anticancer properties via anti-inflammatory and antioxidant mechanisms of action.

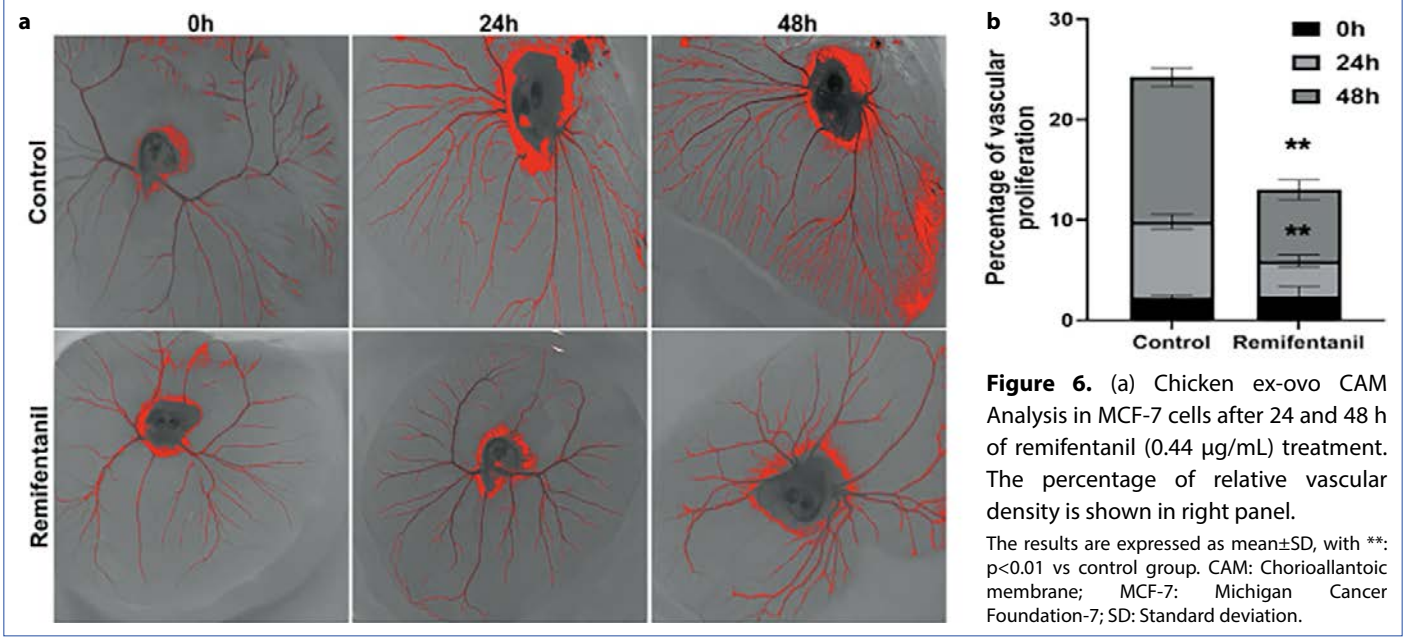

Figure 6. (a) Chicken ex-ovo CAM Analysis in MCF-7 cells after 24 and 48 h of remifentanil (0.44 µg/mL) treatment. The percentage of relative vascular density is shown in right panel.

The results are expressed as mean±SD, with **: $p<0.01$ vs control group. CAM: Chorioallantoic membrane; MCF-7: Michigan Cancer Foundation-7; SD: Standard deviation.

In this study, remifentanil significantly diminished cell proliferation in a time-dependent manner and also, as expected, promoted apoptosis. In addition, apoptotic nuclear morphology, as evidenced by fragmented nuclei, was observed after remifentanil treatment according to DAPI staining. Supporting this, a previous study indicated that remifentanil inhibited cell viability and triggered apoptotic cell death in C6 glioma cells [29]. Another fentanyl derivative, sufentanil, has been shown to lead to apoptosis and a decrease in the growth of MDA-MB-231 breast cancer cells [28]. Moreover, the arrest of cell cycle progression is thought to be an efficient approach for the elimination of cancer cells via apoptosis [36].

Our findings indicate that remifentanil treatment leads to an arrest of MCF-7 cells in the G1 phase, accompanied by a diminished cell population in the G2 phase. This suggests that remifentanil may promote apoptosis by halting cell cycle progression at the G1 phase. Cancer cells usually develop a defective G1/S checkpoint, whereas the G2/M checkpoint is often intact and crucial for the survival of cancer cells [37]. Thus, remifentanil, as a potential anticancer agent, could lead to G1 arrest rather than arrest in the S or G2/M phases. In addition, it is known that ROS-induced DNA damage activates the DNA damage response (DDR) [38]. Based on this, the oxidative stress induced by elevated ROS generation after remifentanil treatment could activate DDR and subsequently lead to cell cycle arrest. Thus, the remifentanil-induced mechanisms involved in DDR require further investigation.

Moreover, as supporting evidence, Yang et al. [39] demonstrated that the upregulation of ROS levels plays a key role in apoptosis through inhibiting cell proliferation, activating pro-apoptotic protein expression, and triggering cell cycle arrest in breast cancer cells. It can be suggested that the inhibition of cell proliferation and cell cycle arrest induced by remifentanil treatment might be mediated by ROS.

ROS accumulation has been found to promote cancer cell death via irreversible oxidative damage to cellular structures, which is the mechanism of many chemotherapeutic drugs [40]. We observed that exposure to remifentanil for 24 and 48 h caused elevated levels of ROS in MCF-7 cells. In a study by Yao et al. [41], fentanyl was shown to induce ROS generation in A549 lung cancer cells. The apoptosis-inducing effects of remifentanil might be attributed to increased production of ROS.

The main enzyme, lactate dehydrogenase (LDH), catalyzes the aerobic glycolysis of pyruvate in cancer cells to produce ATP [42]. It has been shown that increased LDH release into the culture supernatants of cancer cells is related to irreversible cell death due to cell membrane damage and disruption of membrane integrity [42]. The higher amount of LDH in the culture supernatant is a supportive marker for the cytotoxic potential of remifentanil in MCF-7 cells, as supported by similar *in vitro* results with other cytotoxic agents [43].

IL-6, as one of the inflammatory cytokines, exhibits abnormal expression in many cancer cells, with a critical role in tumor progression [44]. In the current study, remifentanil reduced the production of IL-6 in MCF-7 cells. Similarly, remifentanil restricts the release of IL-6 in LPS-induced BV2 microglia cells and an acute lung injury rat model [17, 45]. ROS levels are also critical to inducing IL-6 gene expression [46], and IL-6 can also promote the production of ROS [47]. Depending on the tissue microenvironment and concentration, IL-6 may exhibit either pro- or anti-inflammatory actions [48]. Interestingly, in the present study, the downregulation of IL-6 levels coincided with increased ROS levels in MCF-7 cells exposed to remifentanil, which could be related to the induction of ROS-mediated signaling pathways associated with IL-6 or the alterations in transcriptional activation of the IL-6 gene through ROS production.

Furthermore, tumor progression is profoundly affected by angiogenesis, and the inhibition of angiogenesis might block

the growth of cancer cells [49]. The inhibition of angiogenesis, along with decreased vascular proliferation, was observed after remifentanil treatment according to the CAM assay. The inhibitory effect of remifentanil on IL-6 cytokine production and angiogenesis in MCF-7 cancer cells is possibly related to its ability to induce apoptotic cell death.

Evidence suggests that anesthetic and pain management techniques can significantly impact underlying biological processes, potentially altering the risk of cancer recurrence for better or worse [50]. In a recent study, the combination of remifentanil and dexmedetomidine showed improvement in analgesia following surgery while lowering immunosuppression by regulating T lymphocyte subsets [51]. Despite concerns about remifentanil-induced hyperalgesia [26], it remains a preferred choice due to its various benefits [52]. While our data point to potential benefits for cancer patients, further research is essential. Comprehensive *in vitro* and *in vivo* experiments, along with clinical trials, are needed to fully understand and confirm the anticancer effects of remifentanil. These studies should involve combinations with other agents and various cancer cell lines to fully explore the potential of remifentanil in cancer therapy. Since most current evidence on the effects of anesthetic and analgesic agents on cancer cells comes from experimental models, there is a need for more retrospective and prospective studies to establish a relationship between anesthesia and improved cancer outcomes.

The current study contains a number of limitations. Firstly, only the MCF-7 cell line was used in this *in vitro* study. Animal models and various kinds of cancer cell lines could be used to determine and validate the *in vivo* and comprehensive *in vitro* anticancer impacts of remifentanil in further studies. In our study, we used 0.44 µg/mL remifentanil for experiments conducted for 24 and 48 h. The beneficial effect of remifentanil on healthy cell lines at the nanomolar level [32,33] and its antitumor effects on different cancer cell lines at the micromolar level have been reported in studies [29,31]. Due to the application of higher remifentanil concentrations in cancer cell lines compared to normal cells, remifentanil treatment at the micromolar level could lead to potential side effects. More experimental data are needed to investigate the effects of remifentanil at two different concentrations ($2\times IC_{50}$, IC_{50}) to explore dose-dependent toxicity in non-cancerous cells in future studies.

It should also be considered that cancer patients frequently need to take other analgesics and antitumor medications simultaneously [41]. Further studies should also be conducted to determine the molecular mechanisms by which remifentanil impacts cancer cell sensitivity to common chemotherapy. Moreover, by presenting more reliable data regarding the toxicity, stability, and negative effects of remifentanil, future research applying various animal models and clinical trials will generate stronger results to clarify the potential of remifentanil for clinical application.

Conclusion

This study, for the first time, delineated that remifentanil showed significant cytotoxic and apoptotic effects in MCF-7 cells, which could be associated with G1 cell cycle arrest, elevated ROS production, and inhibition of IL-6 release and angiogenesis *in vitro*. Any off-target effects or interactions with standard cancer therapies require thorough investigation. This preliminary study warrants more experimental *in vitro* and *in vivo* evidence from future research to clearly support the great potential of remifentanil as an anticancer agent against breast cancer.

Authorship Contributions: Concept – E.K.S., A.K.Y., O.F.K.; Design – E.K.S., A.K.Y., O.F.K.; Supervision – A.K.Y., O.F.K.; Data collection &/or processing – E.K.S., A.K.Y., A.C., O.F.K.; Analysis and/or interpretation – E.K.S., A.K.Y., A.C., O.F.K.; Literature search – E.K.S., A.K.Y., A.C., O.F.K.; Writing – E.K.S., A.K.Y., A.C., O.F.K.; Critical review – E.K.S., A.K.Y., A.C., O.F.K.

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References

1. Siegel RL, Miller KD, Wagle NS, Jemal A. Cancer statistics, 2023. CA Cancer J Clin 2023;73(1):17–48. [CrossRef]
2. Wang Y, Minden A. Current molecular combination therapies used for the treatment of breast cancer. Int J Mol Sci 2022;23(19):11046. [CrossRef]
3. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2021;71(3):209–49. [CrossRef]
4. Pfeiffer RM, Webb-Vargas Y, Wheeler W, Gail MH. Proportion of U.S. trends in breast cancer incidence attributable to long-term changes in risk factor distributions. Cancer Epidemiol Biomarkers Prev 2018;27(10):1214–22. [CrossRef]
5. Subik K, Lee JF, Baxter L, Strzepek T, Costello D, Crowley P, et al. The expression patterns of ER, PR, HER2, CK5/6, EGFR, Ki-67 and AR by immunohistochemical analysis in breast cancer cell lines. Breast Cancer Auckl 2010;4:35–41. [CrossRef]
6. Duff S, Connolly C, Buggy DJ. Adrenergic, inflammatory, and immune function in the setting of oncological surgery: their effects on cancer progression and the role of the anesthetic technique in their modulation. Int Anesthesiol Clin 2016;54(4):48–57. [CrossRef]
7. Ziesenitz VC, Vaughns JD, Koch G, Mikus G, van den Anker JN. Pharmacokinetics of fentanyl and its derivatives in children: A comprehensive review. Clin Pharmacokinet 2018;57(2):125–49. [CrossRef]

8. Feldman PL. Insights into the chemical discovery of remifentanil. Anesthesiology 2020;132(5):1229–34. [CrossRef]
9. Santonocito C, Noto A, Crimi C, Sanfilippo F. Remifentanil-induced postoperative hyperalgesia: Current perspectives on mechanisms and therapeutic strategies. Local Reg Anesth 2018;11:15–23. [CrossRef]
10. Komatsu R, Turan AM, Orhan-Sungur M, McGuire J, Radke OC, Apfel CC. Remifentanil for general anaesthesia: A systematic review. Anaesthesia 2007;62(12):1266–80. [CrossRef]
11. Reade MC, Finfer S. Sedation and delirium in the intensive care unit. N Engl J Med 2014;370(5):444–54. [CrossRef]
12. Barr J, Fraser GL, Puntillo K, Ely EW, Gélinas C, Dasta JF, et al. Clinical practice guidelines for the management of pain, agitation, and delirium in adult patients in the intensive care unit. Crit Care Med 2013;41(1):263–306. [CrossRef]
13. Droge W. Free radicals in the physiological control of cell function. Physiol Rev 2002;82(1):47–95. [CrossRef]
14. Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB. Oxidative stress, inflammation, and cancer: How are they linked? Free Radic Biol Med 2010;49(11):1603–16. [CrossRef]
15. Zhou Q, Song J, Wang Y, Lin T. Remifentanil attenuates cardiac dysfunction, lipid peroxidation and immune disorder in rats with isoproterenol-induced myocardial injury via JNK/NF-KB p65 inhibition. Ann Transl Med 2020;8(8):551. [CrossRef]
16. Shen J, Zhan Y, He Q, Deng Q, Li K, Wen S, et al. Remifentanil Promotes PDIA3 expression by activating p38MAPK to Inhibit Intestinal ischemia/reperfusion-induced oxidative and endoplasmic reticulum stress. Front Cell Dev Biol 2022;10:818513. [CrossRef]
17. Zhang Y, Du Z, Zhou Q, Wang Y, Li J. Remifentanil attenuates lipopolysaccharide-induced acute lung injury by downregulating the NF-kappaB signaling pathway. Inflammation 2014;37(5):1654–60. [CrossRef]
18. Ma Y, Ren Z, Ma S, Yan W, He M, Wang D, et al. Morphine enhances renal cell carcinoma aggressiveness through promotes survivin level. Ren Fail 2017;39(1):258–64. [CrossRef]
19. Harimaya Y, Koizumi K, Andoh T, Nojima H, Kuraishi Y, Saiki I. Potential ability of morphine to inhibit the adhesion, invasion and metastasis of metastatic colon 26-L5 carcinoma cells. Cancer Lett 2002;187(1–2):121–7. [CrossRef]
20. Dai H, Feng J, Nan Z, Wei L, Lin F, Jin R, et al. Morphine may act via DDX49 to inhibit hepatocellular carcinoma cell growth. Aging Albany NY 2021;13(9):12766–79. [CrossRef]
21. Qin Y, Chen J, Li L, Liao CJ, Liang YB, Guan EJ, et al. Exogenous morphine inhibits human gastric cancer MGC- 803 cell growth by cell cycle arrest and apoptosis induction. Asian Pac J Cancer Prev 2012;13(4):1377–82. [CrossRef]
22. Li C, Qin Y, Zhong Y, Qin Y, Wei Y, Li L, et al. Fentanyl inhibits the progression of gastric cancer through the suppression of MMP-9 via the PI3K/Akt signaling pathway. Ann Transl Med 2020;8(4):118. [CrossRef]
23. Hacker G. The morphology of apoptosis. Cell Tissue Res 2000;301(1):5–17. [CrossRef]
24. Mitrevska K, Merlos Rodrigo MA, Cernei N, Michalkova H, Splichal Z, Hynek D, et al. Chick chorioallantoic membrane (CAM) assay for the evaluation of the antitumor and antimetastatic activity of platinum-based drugs in association with the impact on the amino acid metabolism. Mater Today Bio 2023;19:100570. [CrossRef]
25. Battershill AJ, Keating GM. Remifentanil : A review of its analgesic and sedative use in the intensive care unit. Drugs 2006;66(3):365–85. [CrossRef]
26. Joly V, Richebe P, Guignard B, Fletcher D, Maurette P, Sessler DI, et al. Remifentanil-induced postoperative hyperalgesia and its prevention with small-dose ketamine. Anesthesiology 2005;103(1):147–55. [CrossRef]
27. Wu W, Wei N, Jiang CN, Cui S, Yuan J. Effects of sufentanil on human gastric cancer cell line SGC-7901 *in vitro*. Cent Eur J Immunol 2014;39(3):299–305. [CrossRef]
28. Li M, Gu K, Kong Q, Wang G, Gu J. Sufentanil inhibits the metastasis and immune response of breast cancer via mediating the NF-kappaB pathway. Immunopharmacol Immunotoxicol 2023;45(6):663–71. [CrossRef]
29. Xu J, Xu P, Li Z, Xiao L, Yang Z. The role of glycogen synthase kinase-3beta in glioma cell apoptosis induced by remifentanil. Cell Mol Biol Lett 2013;18(4):494–506. [CrossRef]
30. Chang J, Zhang W. Remifentanil modulates the TLR4-mediated MMP-9/TIMP1 balance and NF-kappaB/STAT3 signaling in LPS-induced A549 cells. Exp Ther Med 2023;25(2):79. [CrossRef]
31. Liang W, Ke J. Remifentanil reduces the proliferation, migration and invasion of HCC cells via lncRNA NBR2/miR-650/TIMP3 axis. Int J Exp Pathol 2022;103(2):44–53. [CrossRef]
32. Kwon JY, Park BS, Kim YH, Kim YD, Kim CH, Yoon JY, et al. Remifentanil protects human keratinocytes against hypoxia-reoxygenation injury through activation of autophagy. PLoS One 2015;10(1):e0116982. [CrossRef]
33. Yoon JY, Park CG, Park BS, Kim EJ, Byeon GJ, Yoon JU. Effects of remifentanil preconditioning attenuating oxidative stress in human dermal fibroblast. Tissue Eng Regen Med 2017;14(2):133–41. [CrossRef]
34. Hatsukari I, Hitosugi N, Ohno R, Hashimoto K, Nakamura S, Satoh K, et al. Induction of apoptosis by morphine in human tumor cell lines *in vitro*. Anticancer Res 2007;27(2):857–64.
35. Sezer G, Caner A, Onal MG, Cumaoglu A. Morphine counteracts the effects of paclitaxel in triple-negative breast cancer cells. Indian J Med Res 2022;156(1):70–6. [CrossRef]
36. Otto T, Sicinski P. Cell cycle proteins as promising targets in cancer therapy. Nat Rev Cancer 2017;17(2):93–115. [CrossRef]
37. Yan Y, Hein AL, Greer PM, Wang Z, Kolb RH, Batra SK, et al. A novel function of HER2/Neu in the activation of G2/M checkpoint in response to gamma-irradiation. Oncogene 2015;34(17):2215–26. [CrossRef]
38. Kuczler MD, Olseen AM, Pienta KJ, Amend SR. ROS-induced cell cycle arrest as a mechanism of resistance in polyaneuploid cancer cells (PACCs). Prog Biophys Mol Biol 2021;165:3–7. [CrossRef]
39. Yang B, Zhu W, Zheng Z, Chai R, Ji S, Ren G, et al. Fluctuation of ROS regulates proliferation and mediates inhibition of migration by reducing the interaction between DLC1 and CAV-1 in breast cancer cells. *In Vitro* Cell Dev Biol Anim 2017;53(4):354–62. [CrossRef]

40. Arfin S, Jha NK, Jha SK, Kesari KK, Ruokolainen J, Roychoudhury S, et al. Oxidative stress in cancer cell metabolism. Antioxidants Basel 2021;10(5):642. [CrossRef]
41. Yao J, Ma C, Gao W, Liang J, Liu C, Yang H, et al. Fentanyl induces autophagy via activation of the ROS/MAPK pathway and reduces the sensitivity of cisplatin in lung cancer cells. Oncol Rep 2016;36(6):3363–70. [CrossRef]
42. Yao H, Yang F, Li Y. Natural products targeting human lactate dehydrogenases for cancer therapy: A mini review. Front Chem 2022;10:1013670. [CrossRef]
43. Gabr SA, Elsaed WM, Eladl MA, El-Sherbiny M, Ebrahim HA, Asseri SM, et al. Curcumin modulates oxidative stress, fibrosis, and apoptosis in drug-resistant cancer cell lines. Life Basel 2022;12(9):1427. [CrossRef]
44. Hodge DR, Hurt EM, Farrar WL. The role of IL-6 and STAT3 in inflammation and cancer. Eur J Cancer 2005;41(16):2502–12. [CrossRef]
45. Huang Y, Cai Q, Liu H, Wang Y, Ma W. Remifentanil inhibits the inflammatory response of BV2 microglia and protects PC12 cells from damage caused by microglia activation. Bioengineered 2022;13(5):13944–55. [CrossRef]
46. Henriquez-Olguin C, Altamirano F, Valladares D, Lopez JR, Allen PD, Jaimovich E. Altered ROS production, NF-kappaB activation and interleukin-6 gene expression induced by electrical stimulation in dystrophic mdx skeletal muscle cells. Biochim Biophys Acta 2015;1852(7):1410–9. [CrossRef]
47. Wu Y, Antony S, Meitzler JL, Doroshow JH. Molecular mechanisms underlying chronic inflammation-associated cancers. Cancer Lett 2014;345(2):164–73. [CrossRef]
48. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochim Biophys Acta 2011;1813(5):878–88. [CrossRef]
49. Rajabi M, Mousa SA. The role of angiogenesis in cancer treatment. Biomedicines 2017;5(2):34. [CrossRef]
50. Wall T, Sherwin A, Ma D, Buggy DJ. Influence of perioperative anaesthetic and analgesic interventions on oncological outcomes: A narrative review. Br J Anaesth 2019;123(2):135–50. [CrossRef]
51. Zhang Y, Jiang W, Luo X. Remifentanil combined with dexmedetomidine on the analgesic effect of breast cancer patients undergoing modified radical mastectomy and the influence of perioperative T lymphocyte subsets. Front Surg 2022;9:1016690. [CrossRef]
52. Yi S, Cao H, Zheng W, Wang Y, Li P, Wang S, et al. Targeting the opioid remifentanil: Protective effects and molecular mechanisms against organ ischemia-reperfusion injury. Biomed Pharmacother 2023;167:115472. [CrossRef]

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Research Article

Correlation study between collagen type 1 C telopeptide and thyroid stimulating hormone at the sedentary lifestyle individuals

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Abstract

Objectives: A sedentary lifestyle can be defined as spending most of your time without physical activity, such as sitting or lying down for long periods during the day. This lifestyle is considered the least energy-consuming state in the body, which means accumulating a lot of calories in the body. Collagen type 1 C-telopeptide (CTX-1) is the C-terminal telopeptide of fibrillar collagens, such as collagen type I, and has an important role in stabilizing protein tertiary and quaternary structures for bone tissues. It is usually used as a biomarker in serum samples to assess the bone turnover rate. Thyroid-stimulating hormone (TSH) is a peptide hormone secreted by the pituitary gland in the brain. TSH is responsible for sending a signal to the thyroid gland to increase or decrease the amount of hormones it secretes. This study aims to show the correlation between CTX-1 and TSH in individuals who have a sedentary lifestyle, which in turn causes osteoporosis in those individuals. This shows the harms of sedentary lifestyle conditions on individuals.

Methods: The study was designed based on selecting two groups of male individuals (the first group: 25 individuals living a sedentary lifestyle, and the second group: 25 individuals who have physical activity regularly). Blood samples were collected from all individuals in the study after obtaining their consent. The blood samples of the individuals were used to measure several parameters, including CTX-1, TSH, T4, and T3. The t-test statistical method was used to determine the clinically important values.

Results: The results of the study, after comparing the individuals of the sedentary lifestyle group and the active lifestyle group by the t-test statistical method, showed the following: A significant increase in the level of CTX-1 in the individuals of the Sedentary Lifestyle Group. A significant decrease in the levels of T3, T4, and TSH in the individuals of the Sedentary Lifestyle Group. The presence of an inverse or negative correlation between CTX-1 and TSH in the individuals of theSedentary Lifestyle Group.

Conclusion: The results of this study concluded the importance of physical activity for the body, as the study showed that a sedentary lifestyle causes several disorders, including secondary hypothyroidism, which in turn can cause osteoporosis in individuals.

Keywords: Collagen type 1C telopeptide, sedentary lifestyle, thyroid stimulating hormone

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A sedentary lifestyle can be defined as spending most of your time without physical activity, such as sitting or lying down for long periods during the day. This lifestyle is considered the least energy-consuming state in the body, which means accumulating a lot of calories in the body [1]. A sedentary lifestyle has many characteristics, including spending most of your time playing electronic games while sitting, lack of physical activity, and others. Recently, the percentage of a sedentary lifestyle has increased due to the technological development that the world is witnessing. A sedentary lifestyle (inactive lifestyle) has many health problems and complications, including an increased incidence of diabetes, obesity,

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osteoporosis, psychological stress, and other complications (Fig.1). Laziness is considered a contributing cause of bone diseases, the most important of which is osteoporosis, for many reasons, including that laziness and a sedentary lifestyle cause hypothyroidism, and thus a decrease in the function of thyroid hormones can cause weakness in the development and growth of bone tissue [2].

Osteoblasts are defined as cells responsible for manufacturing and forming bones by producing the membrane containing many organic compounds, for example, collagen, which makes the bone more flexible, and calcium ions with phosphate ions, which make the bone more solid [3]. This membrane is known as the bone matrix. Osteoblasts play an important role during the formation and growth of skeletal bones. In adults, the body can activate osteoblasts in the event of a need for cell renewal, a defect, or depletion of the cell matrix. Osteoblasts regulate osteoclast cells to maintain bone mass [4].

Collagen type 1 C-telopeptide (CTX-1) is the C-terminal telopeptide of fibrillar collagens like collagen type I and has an important role in stabilizing protein tertiary and quaternary structures for bone tissues. It is usually used as a serum biomarker to assess the bone turnover rate [5]. It is considered a more specific marker of bone resorption than any other test currently available because it is intracellular in osteoblast cells. When these cells begin resorption and degradation under osteoporotic conditions, CTX-1 is released into the bloodstream [6].

Thyroid-stimulating hormone (TSH) is defined as a peptide hormone secreted via the pituitary gland in the brain. TSH is responsible for sending a signal to the thyroid gland to increase or decrease the amount of hormones it secretes, and its amount depends on the amount of thyroxine (T4) and the decrease in triiodothyronine (T3) present in the blood in the normal state. The level of TSH can be affected by many factors, including thyroid hormones, psychological state, some medications, sedentary lifestyle, and others [7]. Low levels of TSH can cause a slow heart rate, joint swelling and stiffness, dry skin, constipation, growth disturbances, and more. The level of TSH decreases and causes secondary hypothyroidism because it leads to inhibition of T4 and T3 production from the thyroid gland. The TSH decrease has several factors and conditions because it is under the control of the hypothalamus and the brain, as any psychological conditions can lead to this [8].

The current study aims to show the health complications resulting from a sedentary lifestyle on individuals. These complications are shown by measuring many biomarkers for all study individuals, then comparing and finding correlations between these biomarkers. The biomarkers included T3, T4, TSH, and CTX-1. In addition, the most important health complications resulting from a sedentary lifestyle are secondary hypothyroidism and osteoporosis.

Materials and Methods

Study design

This study included in its design the selection of two groups of individuals chosen according to specific lifestyles, where individuals were divided into two groups:

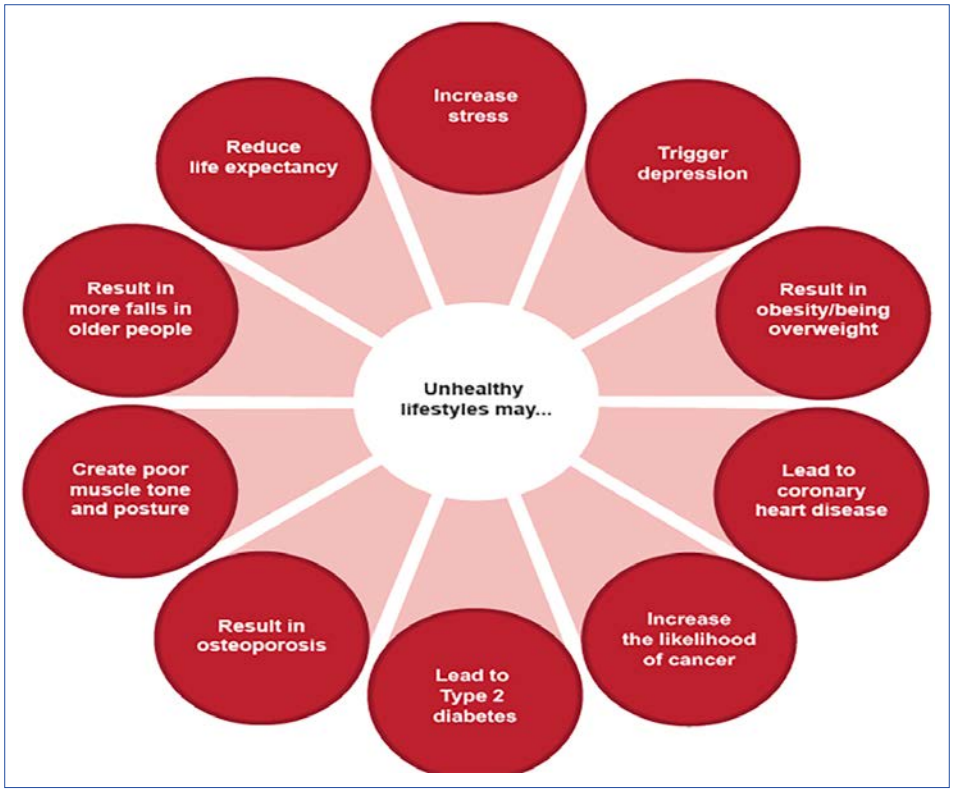

Figure 1. The sedentary lifestyle complications.

Table 1. The biochemical parameters kits details

Biochemical marker	Principle test	Company name for the kit	Lot number
CTX1	Sandwich ELISA	BioVendor	Ac.02
TSH	Kinetic Fluorescence Immunoassay	TOSOH	1007471001-017F - 01/17
T4	Kinetic Fluorescence Immunoassay	TOSOH	1007371001-017E - 01/17
T3	Kinetic Fluorescence Immunoassay	TOSOH	1007271001-110G - 11/20

CTX 1: Collagen type 1 C-telopeptide; TSH: Thyroid-stimulating hormone.

Table 2. Comparison between Sedentary Lifestyle Group and Active Lifestyle Group according to some biochemical parameters

Biochemical parameters	Sedentary Lifestyle Group Mean±SD (n=25)	Active Lifestyle Group Mean±SD (n=15)	Comparison of two groups (p)
CTX 1 (ng/ml)	2.28±1.13	0.91±0.20	0.001*
TSH (µIU/ml)	0.64±0.31	4.15±2.24	0.001*
T4 (nmol/L)	51.5±28.6	107.4±3.7	0.001*
T3 (nmol/L)	0.87±1.5	1.89±0.41	0.01*

*: Significant value is (P-value less than 0.05). SD: Standard deviation; CTX 1: Collagen type 1 C-telopeptide; TSH: Thyroid-stimulating hormone.

- Sedentary Lifestyle Group: Included 25 individuals who had an inactive and sedentary lifestyle and did not practice any sports activity, aged 30–40 years of male gender. These cases were collected from electronic game halls and cafes.
- Active Lifestyle Group: Included 25 individuals who had an active lifestyle and practiced sports activity regularly, aged 30–40 years of male gender. These cases were collected from gyms.

All study individuals were selected according to a special questionnaire directed to them and collected and analyzed statistically (see appendix section), also based on the specifications stipulated in the American College of Rheumatology (ACR) guideline (2022) [9]. The current study was conducted in Baghdad for the period from May to June 2024. Oral and written consent was taken from the individuals to include them in the study in order to achieve research ethics, according to research ethics in my college (No. 312, May 2024). (There is a research ethics committee in my college that approves all research for researchers after being informed of all details through a seminar.) The study was done after the (2013) Helsinki Declaration on the Ethics Approval for Scientific Research.

Measurements and methods

After identifying the study individuals from both groups, their blood samples were taken. These samples were centrifuged to obtain pure serum for use in measuring the specified biochemical parameters, which are CTX-1, TSH, T4, and T3. In this study, we used special kits to measure these biochemical parameters from reputable manufacturers, where all measurement details from the company name, chemical principle of measurement, and other details are shown in Table 1.

Statistical analysis

In the current study, the statistical method known as the t-test was used, which is based on the mean, standard deviation, and p-value to clarify the clinical significance of valuable biochemical parameters. The choice of this statistical method was based on the research characteristics present in the research, as we wanted to compare only two groups. This is consistent with the characteristics of the t-test statistical method. The statistical program, version 18 (2022), was used to analyze the data [10].

Results

Results of the current study showed that there are clinical differences with significant values for biochemical markers when we compared the two groups (first group: sedentary lifestyle and second group: active lifestyle), as the results showed the following:

- Significant increase in the level of CTX-1 in the Sedentary Lifestyle Group compared to the Active Lifestyle Group.
- Significant decrease in the levels of TSH, T4, and T3 in the Sedentary Lifestyle Group compared to the Active Lifestyle Group.

In addition, the correlation curve was made between CTX-1 and TSH to determine whether there is a correlation between them. It was found that there is an inverse or negative relationship between CTX-1 and TSH within the Sedentary Lifestyle Group (Table 2 and Figs. 2-6).

Discussion

A sedentary lifestyle is defined as the lack of physical activity for a person, which means that the individual lives a sedentary lifestyle. These conditions are a source and cause of many disorders, the most important of which are metabolic disorders, which in turn affect several organs in the body, the most important of

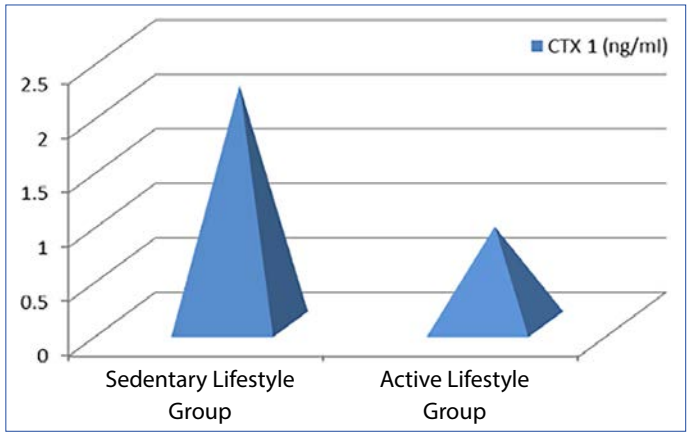

Figure 2. Comparison between Sedentary Lifestyle Group and Active Lifestyle Group according to CTX 1 biochemical parameters.
CTX 1: Collagen type 1 C-telopeptide.

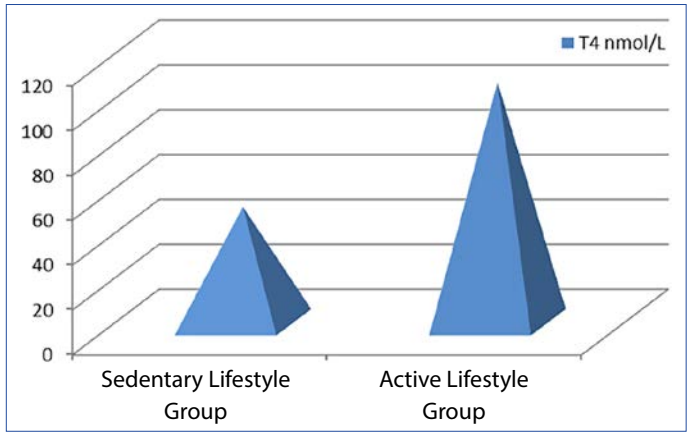

Figure 4. Comparison between Sedentary Lifestyle Group and Active Lifestyle Group according to T4 biochemical parameters.
T4: Thyroxine.

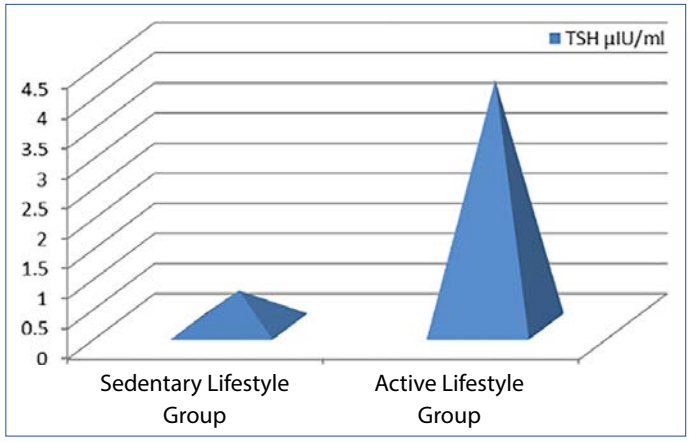

Figure 3. Comparison between Sedentary Lifestyle Group and Active Lifestyle Group according to TSH biochemical parameters.
TSH: Thyroid-stimulating hormone.

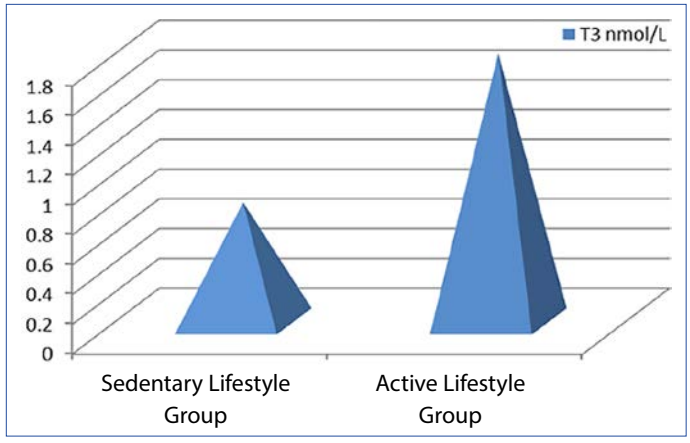

Figure 5. Comparison between Sedentary Lifestyle Group and Active Lifestyle Group according to T3 biochemical parameters.
T3: Triiodothyronine.

which are the bones [2]. Living a sedentary lifestyle suggests to the body that it does not need energy, and this is interpreted by the body as nerve signals sent to the brain to take what is necessary. In such conditions, the brain begins to control the change in metabolic pathways, as we see weight gain through the accumulation of fat in individuals. This is one of the paths that the body takes to get rid of unburned calories to produce energy [11]. On the other hand, the body begins to inhibit the secretion of the TSH hormone, which is the hormone that controls the production of thyroid hormones that play an important role in the process of energy production in biological metabolism. The brain begins sending signals to the pituitary gland via the hypothalamus to inhibit the production of the TSH hormone, and thus the thyroid gland is inhibited and the production of thyroid hormones (T3 and T4), which play an important role in regulating and producing energy in the body, is reduced. This is why we see that individuals who live a sedentary lifestyle sometimes suffer from secondary hypothyroidism [12].

Secondary hypothyroidism causes many disorders and diseases, the most important of which is osteoporosis. Osteo-

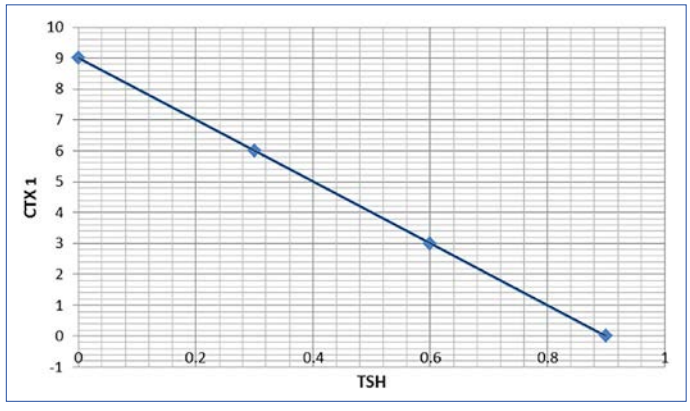

Figure 6. Correlation between CTX 1 and TSH within Active Lifestyle Group.

porosis is a disease that affects the human skeletal system, where there is an imbalance between the activity of osteoblasts and osteoclasts [13]. The decrease in the T3 hormone inhibits the process of bone repair, and therefore bone resorption begins to accelerate due to the destruction of os-

teoblasts. One of the most important compounds that result from this destruction process is CTX-1, which is released into the bloodstream in large quantities. CTX-1 is known as the collagen compound or collagen responsible for binding the bone matrix inside and between cells. The release of CTX-1 into the bloodstream means that it has left the osteoblasts and has no role in the process of building bone tissue, and thus the bone becomes fragile, which is what we call osteoporosis [14].

The results of this study showed that there is an increase in the level of CTX-1 in individuals who live a sedentary lifestyle compared to individuals who practice sports activity regularly. Also, the study found a decrease in the level of TSH in individuals who live a sedentary lifestyle compared to individuals who practice sports activity regularly. These results mean that individuals who have a sedentary lifestyle suffer from secondary hypothyroidism, and this disorder has led to inhibition of the function of osteoblasts and thus increased bone resorption and the release of CTX-1 into the bloodstream. These conditions occur due to the effective role of thyroid hormones in the development and growth of cells in general, and the loss of these hormones means the inactivity of these processes. This was also demonstrated by our current study by proving the negative relationship between TSH and CTX-1 in individuals who live a sedentary lifestyle.

Our current study is consistent with the results of Roberts HM, Law RJ (2019), who also demonstrated high CTX-1 levels in individuals who live a sedentary lifestyle [15]. Also, our study is consistent with Di Blasio, A., and Di Dalmazi, G. (2022), who also demonstrated low TSH levels in individuals who live a sedentary lifestyle and thus suffer from secondary hypothyroidism [16]. This is explained by our study results by the existence of a negative or inverse relationship between CTX-1 and TSH in sedentary lifestyle individuals.

The current research opens up future research horizons on the effects of a sedentary lifestyle on human health, as we hope to conduct research on how to treat secondary hypothyroidism by modifying lifestyle to avoid medications. On the other hand, we hope to conduct in-depth research on osteoporosis, hypothyroidism, and their relationship to lifestyle.

Conclusion

Our current study demonstrated the important function of physical activity for the human body after comparing individuals who engage in physical activity with individuals who have a sedentary lifestyle. The current study concluded that a sedentary lifestyle causes secondary hypothyroidism, which in turn can cause osteoporosis. This conclusion was shown through the results and examinations conducted on all individuals in the study, which included CTX-1, TSH, T4, and T3. In addition, we found through the study the existence of an inverse relationship between CTX-1 and TSH in individuals who have a sedentary lifestyle.

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Ethics Committee Approval: The study was approved by The Ministry of Higher Education and Scientific Research, Health and Research Institutions Ethics Committee (No: 312, Date: 03/05/2024).

Authorship Contributions: Concept – M.S.K.; Design – M.S.K.; Supervision – M.S.K.; Funding – N.B.R.; Materials – M.S.K.; Data collection &/or processing – N.B.R.; Analysis and/or interpretation – N.B.R.; Literature search – M.S.K.; Writing – M.S.K.; Critical review – N.B.R.

Conflict of Interest: The authors declare that there is no conflict of interest.

Use of AI for Writing Assistance: No AI technologies utilized.

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References

1. Park JH, Moon JH, Kim HJ, Kong MH, Oh YH. Sedentary lifestyle: Overview of updated evidence of potential health risks. Korean J Fam Med 2020;41(6):365–73. [CrossRef]
2. Raffin J, de Souto Barreto P, Le Traon AP, Vellas B, Aubertin-Leheudre M, Rolland Y. Sedentary behavior and the biological hallmarks of aging. Ageing Res Rev 2023;83:101807. [CrossRef]
3. Mizoguchi T, Ono N. The diverse origin of bone-forming osteoblasts. J Bone Miner Res 2021;36(8):1432–47. [CrossRef]
4. Guo J, Wang F, Hu Y, Luo Y, Wei Y, Xu K, et al. Exosome-based bone-targeting drug delivery alleviates impaired osteoblastic bone formation and bone loss in inflammatory bowel diseases. Cell Rep Med 2023;4(1):100881. [CrossRef]
5. Martlı HF, Saylam B, Er S, Yücel Ç, Tez M. Evaluation of preoperative procollagen type 1 N-terminal peptide and collagen type 1 C-telopeptide levels in the prediction of postoperative hypocalcemia in patients undergoing parathyroidectomy due to primary hyperparathyroidism. Langenbecks Arch Surg 2023;408(1):71. [CrossRef]
6. Zhu X, Chen L, Pan L, Zeng Y, Fu Q, Liu Y, et al. Correlation between bone mineral density and bone metabolic markers in postmenopausal women with osteoporotic fractures at different C-terminal telopeptide of type 1 collagen levels: A retrospective analysis study. Menopause 2023;30(11):1139–46. [CrossRef]
7. Van Uytfanghe K, Ehrenkranz J, Halsall D, Hoff K, Loh TP, Spencer CA, et al. Thyroid stimulating hormone and thyroid hormones (triiodothyronine and thyroxine): An American Thyroid Association-commissioned review of current clinical and laboratory status. Thyroid 2023;33(9):1013–28. [CrossRef]
8. Babić Leko M, Gunjača I, Pleić N, Zemunik T. Environmental factors affecting thyroid-stimulating hormone and thyroid hormone levels. Int J Mol Sci 2021;22(12):6521. [CrossRef]
9. Grayson PC, Ponte C, Suppiah R, Robson JC, Craven A, Judge A, et al; DCVAS Study Group. 2022 American College of Rheumatology/European Alliance of Associations for rheumatology classification criteria for eosinophilic granulomatosis with polyangiitis. Ann Rheum Dis 2022;81(3):309–14. [CrossRef]

10. Khalaf MS, Alsaady ZA. The role of neuregulin 4 status in female's primary infertility patients. J Med Chem Sci 2023;6(7):1577–81.
11. Verdú E, Homs J, Boadas-Vaello P. Physiological changes and pathological pain associated with sedentary lifestyle-induced body systems fat accumulation and their modulation by physical exercise. Int J Environ Res Public Health 2021;18(24):13333. [CrossRef]
12. Wu K, Zhou Y, Ke S, Huang J, Gao X, Li B, et al. Lifestyle is associated with thyroid function in subclinical hypothyroidism: A cross-sectional study. BMC Endocr Disord 2021;21(1):112. [CrossRef]
13. Du J, Ji H, Jin J, Gao S, Yan X, Hu S. Pituitary adenoma secondary to primary hypothyroidism: Two case reports. Medicine Baltimore 2020;99(8):e19222. [CrossRef]
14. Brown JP, Don-Wauchope A, Douville P, Albert C, Vasikaran SD. Current use of bone turnover markers in the management of osteoporosis. Clin Biochem 2022;109–110:1–10. [CrossRef]
15. Roberts HM, Law RJ, Thom JM. The time course and mechanisms of change in biomarkers of joint metabolism in response to acute exercise and chronic training in physiologic and pathological conditions. Eur J Appl Physiol 2019;119(11–12):2401–20. [CrossRef]
16. Di Blasio A, Di Dalmazi G, Izzicupo P, Bucci I, Giuliani C, Di Baldassarre A, et al. Serum TSH and daily physical activity in a cohort of nonagenarians: Results from the Mugello Study. J Funct Morphol Kinesiol 2022;7(3):56. [CrossRef]

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Research Article

Hematological effect of *Mucuna pruriens, Justicia carnea* and their combination on phenylhydrazine-induced anemia in rat

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Abstract

Objectives: Anemia, a widespread public health concern, affects millions globally, particularly in developing countries. Traditional medicinal plants, including *Mucuna pruriens* and *Justicia carnea*, have been used to manage anemia due to their potential hematopoietic and antioxidant properties. This study investigated the hematological effects of *Mucuna pruriens*, *Justicia carnea*, and their combination on phenylhydrazine-induced anemia in rats, aiming to provide insight into their therapeutic potential.

Methods: The acute oral toxicity test (LD50) was conducted using the Up-and-Down procedure. Anemia was induced in all rats, excluding the normal control group, via a single-dose intraperitoneal injection of 80 mg/kg phenylhydrazine. Thirty (30) adult male albino rats were assigned into six (6) groups of five (5) rats, consisting of the normal control, anemic control, and treated groups (standard drug—Astyfer 1.5 mg/kg, 200 mg/kg of ethanol leaf extracts of *Mucuna pruriens, Justicia carnea*, and their combined leaf extracts). Treatment was given orally once per day and lasted for 21 days. Blood samples were collected two weeks into treatment and three weeks after treatment. Hematological parameters were determined using standard biochemical methods. The parameters analyzed were hemoglobin (HB), packed cell volume (PCV), and red blood cell count (RBC).

Results: The LD50 results revealed no mortality or signs of toxicity at doses up to 5000 mg/kg body weight. The findings of this study revealed that, two weeks into treatment, all treatment groups showed a significant increase ($p<0.05$) in their HB, PCV, and RBC levels compared with the anemic control. At the end of the treatment (three weeks), the HB of groups treated with 200 mg/kg *Justicia carnea* and the combined extract were significantly higher ($p<0.05$) than in the other groups.

Conclusion: The combination of *Mucuna pruriens* and *Justicia carnea* offered a modest additional benefit, although the improvement was not substantially greater than the individual effects of each extract.

Keywords: Anemia, combination, *justicia carnea, mucuna pruriens*, phenylhyrazine-induced, rat

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Anemia, a prevalent blood disorder affecting individuals across all age groups, disproportionately impacts the elderly, women of childbearing age, and infants. Although not a disease itself, anemia often arises from underlying conditions [1]. With over 400 types, mostly rare, anemia is characterized by a reduced number of circulating red blood cells [2]. The most common form, iron-deficiency anemia, results from insufficient iron, a crucial component of hemoglobin. Heavy or persistent bleeding, particularly menstruation in women of childbearing age, is the primary cause of iron deficiency. Iron-deficiency anemia can also result from gastrointestinal bleeding caused by disorders like erosive gastritis, peptic ulcers, and inflammatory bowel disease [3].

The use of plants in treating anemia has garnered significant attention in recent research, highlighting various species with potential therapeutic effects. Anemia is reportedly managed

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with plant-derived treatments in traditional medicine practices. Several studies have demonstrated the efficacy of specific plant extracts in ameliorating anemia symptoms. For instance, *Mangifera indica* and *Telfairia occidentalis* showed significant anti-anemic effects in rabbits, with increased hemoglobin levels observed after treatment with their extracts [2]. A study on *Falcaria vulgaris* indicated that its aqueous extract significantly improved hematological parameters in rats, suggesting its potential as an anti-anemic agent [4]. Ethnobotanical research in Nigeria identified ten plants, including *Sorghum bicolor* and *Terminalia catappa*, traditionally used for anemia treatment, confirming their nutritional and phytochemical value [5]. Over the last decade, Nigeria has seen a surge in the cultivation and utilization of *Mucuna pruriens* and *Justicia carnea* for various medicinal applications, notably the treatment of anemia. These plants are increasingly becoming integral to traditional medicine practices in the region.

Mucuna pruriens (velvet bean/cowage), native to Africa and Asia, has been used in traditional medicine for its therapeutic properties, including nervous system regulation, reproductive health, and disease management [6,7]. Its seeds and extracts have been studied for their potential to treat infertility, nervous disorders, and male sexual dysfunction, as well as their aphrodisiac, neuroprotective, and anti-parkinsonian effects [8]. Research has also demonstrated its ability to increase hemoglobin levels and red blood cell count in anemic animal models [9].

Justicia carnea, native to Brazil, has been used in traditional South American medicine for its anti-inflammatory, analgesic, and antioxidant properties. It has also been employed to manage inflammation and gastrointestinal disorders [10–12]. Research has shown that *Justicia carnea* leaf extract may have hematinic effects, increasing hemoglobin levels and red blood cell count in experimental animals [13].

For centuries, traditional cultures have employed combined plant extract therapy, or polyherbal therapy, to enhance healing, balance bodily systems, and minimize side effects [14,15]. This approach leverages synergies between active compounds in multiple plants to achieve greater benefits than individual extracts. Rooted in ancient practices like Ayurveda, Traditional Chinese Medicine, and indigenous healing, combined therapy harnesses the power of plant interactions [16]. When applied correctly by knowledgeable practitioners, combined plant extract therapy offers numerous benefits. However, improper use can pose risks. Success relies on informed formulation, blending traditional wisdom with modern scientific insight [17]. In treating anemia, particularly iron-deficiency anemia, traditional medicine systems have explored combined plant extract therapies. Plant-based treatments incorporating iron-rich herbs, absorption enhancers, and vitality boosters can effectively manage anemia [18].

This study explored the potential therapeutic benefits of ethanol leaf extracts of *Mucuna pruriens* and *Justicia carnea* and their combined extract on hematological parameters in phenylhydrazine-induced anemia in rats. Anemia's significant impact on human health necessitates innovative treatments. *Mucuna pruriens* and *Justicia carnea* have been traditionally used to treat various health conditions, including anemia [13,19]. Investigating their combined effects may reveal synergistic or additive actions in alleviating anemia-related symptoms. Hematological parameters serve as crucial indicators of overall health [20]. By examining the changes in hematological parameters post-treatment with combined plant extracts, researchers can uncover valuable insights into the biological mechanisms that contribute to their therapeutic efficacy in combating anemia. While individual studies have investigated the effects of *Mucuna pruriens* and *Justicia carnea* extracts on hematological parameters [13,19], there is a notable gap in the literature regarding their combined effects. Few studies have explored the synergistic or additive effects of combining plant extracts, particularly in the context of treating anemia or related conditions [21]. This study aims to contribute to the existing body of knowledge by exploring the combined effects of *Mucuna pruriens* and *Justicia carnea* ethanol leaf extracts on hematological parameters in phenylhydrazine-induced anemia in rats. It also aims to contribute to the development of novel therapeutic strategies for managing anemia and related disorders.

Materials and Methods

Helsinki declaration – ethical approval

This study adhered to international guidelines for laboratory animal care and use, with approval from the Institutional Research and Ethical Clearance Committee, Faculty of Basic Medical Sciences, with the approval number: ESUCOM/FBMS/ETR/2024/04.

Sample collection and identification

Fresh leaves of *Mucuna pruriens* and *Justicia carnea* were harvested from a garden located at Ebeano Tunnel, Fidelity Estate, Enugu. They were identified by a botanist in the Department of Botany and deposited in the herbarium with voucher numbers NAUH-15 and NAUH-203B for *Mucuna pruriens* and *Justicia carnea*, respectively.

Sample preparation/extraction

The leaves of *Mucuna pruriens* and *Justicia carnea* were properly rinsed separately with distilled water and air-dried at room temperature (25 ^{o}C). The dried leaves were ground into a powdery form using a grinding mill. One hundred grams (100 g) of each of the powdered leaves was subjected to Soxhlet extraction as follows: the sample was wrapped in filter paper and placed in the thimble of the Soxhlet apparatus. The condenser and heating mantle were connected securely. An initial 500 ml volume of ethanol was added to the round-bottom flask via a funnel, passing through the sample-containing thimble. The condenser's inlet and outlet were connected to a hose for continuous cold water circulation during extraction. Finally, the heat source was activated, positioned 5 cm from the flask. The resulting filtrate was concentrated in a water

bath at 50 ºC. For the combined dose, the extracts of *Mucuna pruriens* and *Justicia carnea* were combined in equal proportions (1:1 ratio), reconstituted in distilled water to achieve the designated treatment group doses, and then administered orally once daily via gavage.

Experimental animal

A total of 30 male albino rats, weighing 150–200 g, were acquired from the animal house of Nnamdi Azikiwe University, Awka, Anambra State, and acclimatized for one week in the animal facility. The animals were housed in well-ventilated cages under controlled environmental conditions (27°C±3°C, 12-hour light/dark cycle). They were provided with commercial rodent chow and ad libitum access to water.

Acute toxicity study (LD50)

The acute oral toxicity was assessed using the Up-and-Down Procedure (UDP) as described by Bruce [22]. Six rats were randomly selected and subjected to a limit test with a dose of 5000 mg/kg of the extract. Each animal was then closely monitored for 48 hours for death or signs of toxicity.

Induction of anemia

Anemia was induced in the rats through a single-dose intraperitoneal administration of phenylhydrazine (80 mg/kg b.w.). Anemia was confirmed after 48 hours. Blood samples were collected via retro-orbital sinus puncture for hematological analysis. Blood samples were also collected before anemia induction to establish baseline hematological parameters.

Experimental design and treatment

Thirty (30) rats were assigned into six groups of five rats each.

- **Group 1:** Normal control with no induction or treatment.
- **Group 2:** Negative control with anemia and no treatment.
- **Group 3:** Anemic rats treated with the standard drug (Astyfer 1.5 mg/kg).
- **Group 4:** Anemic rats treated with 200 mg/kg of *Mucuna pruriens*.
- **Group 5:** Anemic rats treated with 200 mg/kg of *Justicia carnea*.
- **Group 6:** Anemic rats treated with 200 mg/kg of the combined *Mucuna pruriens* and *Justicia carnea*.

Treatments were administered orally once daily for a period of three weeks.

Blood sample collection and hematological analysis

Blood samples were collected at two weeks into treatment and again at the end of the three-week treatment period via orbital sinus puncture using a sterile syringe. Samples were transferred to heparinized tubes for hematological analysis. The following hematological parameters were analyzed: packed cell volume (PCV), hemoglobin (HB), and red blood cell (RBC) count.

Packed cell volume (PCV) estimation

Packed cell volume (PCV) was estimated using the microhematocrit technique described by Ochei and Kolhatkar [23]. Blood samples (2 ml) were collected in heparinized capillary tubes, sealed with plasticine to prevent leakage, and subjected to centrifugation at 12,000 g for five minutes. Following centrifugation, the tubes were placed on a microhematocrit reader scale, and the PCV was measured as a percentage.

Red blood cell (RBC) count

Total red blood cell counts were determined according to Ochei and Kolhatkar's [23] hematological protocol. A 0.02 ml aliquot of EDTA-anticoagulated whole blood was diluted (1:20) with 10% sodium carbonate (Na_2CO_3), loaded into a Neubauer hemocytometer, and examined under a light microscope. RBCs were counted in designated squares and summed to obtain the total RBC count.

Determination of hemoglobin (HB) concentration

Hemoglobin (HB) concentration was determined using the cyanmethemoglobin method, as described by Ochei and Kolhatkar [23].

Principle

Hemoglobin reacts with Drabkin's solution, containing potassium ferricyanide, potassium cyanide, and potassium dihydrogen phosphate, to form cyanmethemoglobin. This reaction produces a colored compound that is measured colorimetrically.

Procedure

A 0.02 ml whole blood sample was diluted 1:250 with 5 ml of Drabkin's solution in a test tube. After mixing and a 10-minute incubation, the absorbance was measured at 540 nm using a colorimeter, with Drabkin's solution as the blank. The absorbance reading was then multiplied by a standard hemoglobin factor (36.8 g/dl) to calculate the actual hemoglobin concentration.

Statistical analysis

Data analysis was performed using IBM SPSS version 29 statistical package (SPSS Inc., Chicago, Illinois, USA). Values were presented as mean ± standard deviation (SD). The statistical significance of the results between groups was determined using one-way analysis of variance (ANOVA), and multiple comparisons were carried out using Tukey's post hoc test. Differences between means were considered statistically significant at $p<0.05$.

Results

Acute toxicity test

The extract demonstrated a high margin of safety in rats, with no behavioral changes or mortality observed at doses as high as 5000 mg/kg body weight during the 48-hour toxicity study.

Effect of the extracts on packed cell volume (PCV)

As presented in Table 1, a statistically significant reduction in packed cell volume (PCV) was observed ($p<0.05$) in all experimental groups (2–6) relative to the normal control group (1) following induction. After two weeks of treatment with the standard drug and extracts, all treatment groups (3–6) exhibited recovery, with no statistically significant difference ($p>0.05$) compared to the normal control group (1) but significantly increased ($p<0.05$) compared to group 2. At three weeks, a significant elevation in PCV was observed in groups 3–6 relative to group 2, surpassing initial baseline levels. Notably, groups 5 and 6 displayed the highest PCV values.

Effect of the extracts on hemoglobin (HB)

Table 2 indicates that HB levels decreased significantly ($p<0.05$) in all the experimental groups following induction. However, the HB levels of all treatment groups (2–6) demonstrated a significant increase ($p<0.05$) in weeks 2 and 3, with group 5 showing the most significant increase ($p<0.05$), followed closely by group 6 compared to the other groups.

Effect of the extracts on red blood cell (RBC) count

Table 3 shows that RBC counts decreased significantly ($p<0.05$) in all groups following induction. However, the RBC counts in all treatment groups (3–6) showed a significant increase ($p<0.05$) by the third week of treatment compared to group 2, with groups 4 and 5 showing the highest RBC counts at the final measurement, approaching normal control levels.

Discussion

The primary objective of this study was to assess the effect of *Mucuna pruriens* and *Justicia carnea* leaf extracts, alone and in combination, on hematological parameters in phenylhydrazine-induced anemia in albino rats, with a focus on potential synergistic effects. The results of the acute oral toxicity study demonstrated that the extracts did not induce any behavioral alterations or mortality in rats at doses up to 5000 mg/kg over a 48-hour observation period. This suggests a high safety profile for the extract, indicating potential safety for short-term use.

The outcomes of hematological investigations are crucial for the diagnosis, management, and monitoring of various diseases, as well as for evaluating the severity of blood-related damage. This study demonstrated a statistically significant ($p<0.05$) decline in packed cell volume (PCV), hemoglobin (HB) concentration, and red blood cell (RBC) count following phenylhydrazine administration in all experimental groups (2–6). Phenylhydrazine damages RBCs by generating reactive oxygen species, causing lipid peroxidation, hemoglobin oxidation, and premature destruction [24].

PCV measurement provides valuable diagnostic information on red blood cell proportion, aiding in the detection and monitoring of various conditions, including anemia, dehydration, and polycythemia. This parameter is essential for evaluating oxygen-carrying capacity and hematological well-being. Notably, from the results of this study, a marked increase in packed cell volume (PCV) levels was observed in all treatment groups following the induction-induced decrease. It was also observed that the untreated group exhibited a modest spontaneous recovery by the final week, suggesting the presence of intrinsic compensatory mechanisms that may mitigate the initial damage over time. Groups treated with *Mucuna pruriens* and *Justicia carnea*, alone or combined, showed notable PCV improvements. Combination treatment yielded the highest PCV levels, closely followed by the group treated with *Justicia carnea* alone, indicating effectiveness in restoring PCV levels and blood health. The results suggest that *Mucuna pruriens* and *Justicia carnea* possess therapeutic potential in managing hypochromic conditions, such as anemia, characterized by reduced PCV. The observed PCV increase implies stimulation of erythropoiesis or enhanced erythrocyte survival. Notably, synergism was observed with combined administration.

Hemoglobin (HB), a vital protein within erythrocytes, facilitates the transport of oxygen from the pulmonary system to peripheral tissues and concurrently returns carbon dioxide to the lungs [25]. Hemoglobin concentrations serve as critical biomarkers for assessing the blood's oxygen transport capacity, facilitating the diagnosis of anemia and monitoring the efficacy of therapeutic interventions aimed at reestablishing optimal hematological function. This study examined how different treatments affected hemoglobin levels in induced anemia across four treatment groups. As noted earlier, post-induction, a pronounced decline in hemoglobin concentrations was observed across all experimental groups, demonstrating that the induced condition effectively diminished the blood's capacity to transport oxygen—a typical consequence of anemia.

Over the course of treatment, a significant increase ($p<0.05$) in hemoglobin concentrations was observed across all treatment groups, with the exception of the untreated control group. The most pronounced improvement in hemoglobin levels was observed in the *Justicia carnea*-treated group, followed closely by the group treated with the combined extract. The *Mucuna pruriens*-treated group demonstrated a notable, albeit lesser, increase. Standard drug treatment resulted in moderate recovery, slightly lower than the extract treatments. The results indicate that *Mucuna pruriens* and *Justicia carnea* exhibited beneficial effects on hemoglobin concentrations, with *Justicia carnea* demonstrating the most significant effect, which was statistically similar ($p>0.05$) to that of the combined extract. The combination of *Mucuna pruriens* and *Justicia carnea* appears to offer a synergistic effect, leading to higher hemoglobin recovery than *Mucuna pruriens* treatment alone.

Red blood cell count serves as a crucial diagnostic tool, measuring the number of red blood cells and aiding in the detection of various blood-related disorders [26]. This study examined how various treatments affected red blood

Table 1. Effect of the leaf extracts *Mucuna Pruriens, Justicia carnea* and their combination on the packed cell volume of anemic rats

	Normal control	Anemic control	Standard drug Astyfer -1.5mg/kg	200mg/kg *Mucuna pruriens*	200mg/kg *Justicia carnea*	200mg/kg *Mucuna pruriens* +*Justicia carnea*	p
Initial (%)	44.00±2.12	40.60±4.34	41.30±2.33	48.00±5.83	46.70±5.61	46.00±4.69	0.074
After induction (%)	48.00±2.10	26.00±3.37*	24.13±0.85*	24.75±3.77*	23.13±2.10*	23.13±0.85*	0.00*
2 Weeks into treatment (%)	51.80±1.20**	36.00±1.41	53.00±4.08**	53.25±3.10**	52.75±2.06**	52.50±2.08**	0.00**
3 weeks into treatment (final) %	55.14±2.17**	46.34±2.84	50.25±4.90**	55.67±2.24**	57.78±2.63**	57.10±0.56**	0.00**

Values are mean±SD; *p: Significantly different from the control (p<0.05); **p: Significantly different from anemic control (p<0.05). SD: Standard deviation.

Table 2. Effect of the leaf extracts *Mucuna pruriens, Justicia carnea* and their combination on the hemoglobin of anemic rats

	Normal control	Anemic control	Standard drug Astyfer-1.5mg/kg	200 mg/kg *Mucuna pruriens*	200 mg/kg *Justicia carnea*	200 mg/kg *Mucuna pruriens* +*Justicia carnea*	p
Initial (g/dl)	20.88±1.05	20.80±3.74	18.56±1.15	21.14±2.71	20.90±2.23	19.46±1.23	0.40
After induction (g/dl)	22.18±2.06	8.90±1.12*	8.00±0.14*	8.90±0.29*	8.32±0.87*	9.20±0.64*	0.00*
2 Weeks into treatment (g/dl)	25.10±3.20**	9.05±0.34	11.90±1.61**	12.60±1.10**	12.48±0.96**	11.10±0.26**	0.00**
3 weeks into treatment (g/dl)	27.58±4.31	26.10±1.55	25.08±2.96	22.4±3.84	30.10±1.19***	28.85±1.51***	0.016***

Values are mean±SD; *p: Significantly different from the control (p<0.05); **p: Significantly different from anemic control (p<0.05); ***p: Significantly different from normal control, anemic control, standard drug and 200 mg/kg *Mucuna pruriens* groups (p<0.05).

Table 3. Effect of the leaf extracts *Mucuna Pruriens, Justicia carnea* and their combination on the red blood cell count of anemic rats

	Normal control	Anemic control	Standard drug Astyfer-1.5mg/kg	200 mg/kg *Mucuna pruriens*	200 mg/kg *Justicia carnea*	200 mg/kg *Mucuna pruriens* +*Justicia carnea*	p
Initial ($\times10^{12}$/L)	4.90±0.40	5.02±0.31	4.80±0.21	4.82±0.34	4.92±0.41	4.38±1.06	0.51
After induction ($\times10^{12}$/L)	6.89±0.80	3.58±0.38*	3.35±0.34*	3.35±0.51*	3.33±0.39*	3.30±0.18*	0.00*
2 Weeks into treatment ($\times10^{12}$/L)	8.90±0.60**	3.53±0.21	3.45±0.20**	4.80±0.16**	4.40±0.16**	4.95±0.26**	0.00**
3 weeks into treatment ($\times10^{12}$/L)	10.78±0.59***	7.75±0.37	8.35±1.32	10.13±0.54***	9.98±1.38***	9.15±0.95***	0.002***

Values are mean±SD; *p: Significantly different from the normal control (p<0.05); **p: Significantly different from anemic control and standard drug (p<0.05); ***p: Significantly different from anemic control and standard drug (p<0.05).

cell (RBC) counts after inducing anemia in five groups. Initially, RBC levels were similar and not significantly different ($p>0.05$) across all groups. Following induction, RBC counts significantly ($p<0.05$) dropped in all treatment groups, demonstrating the successful induction of anemia. Notably, the untreated group also showed a substantial decrease in RBC count, which remained consistently low throughout the study, emphasizing the severity of the condition when left untreated.

During the treatment phase, groups administered *Mucuna pruriens* and *Justicia carnea*, both as individual treatments and in combination, exhibited significant recovery in RBC counts. Notably, *Mucuna pruriens* and *Justicia carnea* individually yielded comparable results, indicating equivalent efficacy. The combined treatment of both herbs resulted in a modestly enhanced recovery, although the expected synergistic effect was not prominently observed. These findings suggest that *Mucuna pruriens* and *Justicia carnea* may have additive, rather than synergistic, effects on RBC count recovery. The standard drug treatment group also exhibited an increase in RBC counts, although the recovery was less pronounced compared to the leaf extract treatment groups. In contrast, the normal control group demonstrated a significant surge in RBC count by the final week, likely attributable to the natural recovery process or enhanced erythropoietic activity in the absence of any stressors or interventions. This finding suggests that the leaf extract treatments may have promoted a more robust recovery in RBC counts compared to the standard drug treatment. The results indicate that *Mucuna pruriens* and *Justicia carnea* are effective in restoring RBC levels following the induced reduction, indicating their potential as therapeutic agents in treating conditions like anemia. The combination of *Mucuna pruriens* and *Justicia carnea* offered a modest additional benefit, although the improvement was not substantially greater than the individual effects of each extract.

These results align with previous research, which has established that *Mucuna pruriens* and *Justicia carnea* possess erythropoietic properties. Previous research on *Mucuna pruriens*' hematological effects revealed its erythropoietic potential, attributed to its high L-DOPA content, which promotes red blood cell production [27]. Research conducted on *Justicia carnea* has substantiated its ethnopharmacological application in anemia treatment, attributed to its considerable iron content and potential erythropoietic effects [13]. Our study confirms these findings, demonstrating improved hematological parameters. The synergistic effect observed is consistent with other studies showing improved therapeutic outcomes from combining herbal remedies [21].

Based on these results, the combination of *Mucuna pruriens* and *Justicia carnea* offered a modest additional benefit in improving hematological parameters, indicating potential synergistic effects. Therefore, *Mucuna pruriens* and *Justicia carnea* may stimulate erythroid progenitor cells, promoting erythropoiesis and increasing red blood cell production. The extracts may enhance hemoglobin synthesis, leading to improved oxygen delivery to tissues and organs. The study provides and adds to preliminary evidence for the use of these traditional medicinal plants in the management of anemia. It also contributes to the development of novel treatment options for anemia, providing an alternative to conventional therapies. However, further studies are needed to elucidate optimal dosages and potential interactions with conventional medications.

Limitations of the study

The treatment duration of 21 days may not be sufficient to fully assess the long-term effects of the extracts and therefore, further study on long term effect is recommended. The study only tested a single dose of 200mg/kg, which may not be the optimal dose for therapeutic effects. There is need for graded dosage which may potentially boost the synergistic effect of the extracts.

Conclusion

The efficacy of *Mucuna pruriens* and *Justicia carnea* treatments was assessed in rats with phenylhydrazine-induced anemia through various hematological parameters. Notably, hemoglobin levels exhibited significant enhancements in treatment groups, with the combination of *Mucuna pruriens* and *Justicia carnea* yielding the most substantial improvements. Furthermore, red blood cell counts demonstrated marked recovery in treated groups, particularly with *Mucuna pruriens* and *Justicia carnea*, suggesting potential erythropoietic benefits. These results indicate that these treatments effectively replenish red blood cells and promote overall blood health. This study provides evidence supporting the traditional use of *Mucuna pruriens* and *Justicia carnea* for enhancing erythropoiesis, suggesting their potential as natural remedies for improving red blood cell production and potentially treating related health conditions.

Ethics Committee Approval: The study was approved by The ESUT Institutional Research Ethics Committee (No: ESUCOM/FBMS/ETR/2024/04, Date: 12/02/2024).

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References

1. World Health Organization, UNICEF. Iron deficiency anaemia: Assessment, prevention and control. Geneva: World Health Organization; 2001.
2. Ogbe RJ, Adoga GI, Abu AH. Antianaemic potentials of some plant extracts on phenylhydrazine-induced anaemia in rabbits. J Med Plants Res 2010;4(8):680–4.
3. Illustrated Medical Dictionary. 1st ed. London: Dorling Kindersley Limited; 2002.
4. Goorani S, Koohi MK, Zangeneh A, Zangeneh MM, Moradi R. Pharmacological evaluation of anti-anemic property of aqueous extracts of *Falcaria vulgaris* leaf in rats. Comp Clin Pathol 2019;28(5):1221–7. [CrossRef]
5. Gbadamosi IT, Moody JO, Yekini AO. Nutritional composition of ten ethnobotanicals used for the treatment of anaemia in Southwest Nigeria. Eur J Med Plants 2012;2(2):140–50. [CrossRef]
6. Zahra W, Birla H, Singh SS, Rathore AS, Dilnashin H, Singh R, et al. Neuroprotection by *Mucuna pruriens* in neurodegenerative diseases. Neurochem Res 2022;47(7):1816–29. [CrossRef]
7. Rakesh B, Praveen N. Biotechnological approaches for the production of L-DOPA: A novel and potent anti-Parkinson's drug from *Mucuna pruriens* (L.) DC. In Research trends in medicinal plant sciences, Vol. 6. AkiNik Publications, 2020. p. 179–204.
8. Ahmad MK, Mahdi AA, Shukla KK, Islam N, Jaiswar SP, Ahmad S. Effect of *mucuna pruriens* on semen profile and biochemical parameters in seminal plasma of infertile men. Fertil Steril 2008;90(3):627–35. [CrossRef]
9. Naman K, Oseni H, Enoh E. Anti-anaemic potential of methanolic leaf extract of *Mucuna pruriens* on phenylhydrazine (PHZ)-induced anaemic albino Wistar rats. FUDMA J Sci 2020;4(3):370–4. [CrossRef]
10. Ukpabi-Ugo JC, Ndukwe PAC, Iwuoha AG. Hepatoprotective effect of methanol extract of *Justicia carnea* leaves on carbon tetrachloride-intoxicated albino rats. Biochem Anal Biochem 2019;8:381.
11. Anigboro AA, Avwioroko OJ, Ohwokevwo OA, Pessu B, Tonukari NJ. Phytochemical profile, antioxidant, α-amylase inhibition, binding interaction, and docking studies of *Justicia carnea* bioactive compounds with α-amylase. Biophys Chem 2021;269:106529. [CrossRef]
12. Nduche MU, Offor IC. Anatomical studies of Sida acuta Burm, Spigelia anthelmia Linn, Centrosema pubescens Benth, Pueraria phaseoloides (Roxb) Benth, *Justicia carnea* Lindl and their taxonomic significance. Int J Res Pharm Biosci 2019;6(2):21–32.
13. Onyeabo C, Achi NK, Ekeleme-Egedigwe CA, Ebere CU, Okoro CK. Haematological and biochemical studies on *Justicia carnea* leaves extract in phenylhydrazine-induced anaemia in albino rats. Acta Sci Pol Technol Aliment 2017;16(2):217–30. [CrossRef]
14. Aslam MS, Ahmad MS, Mamat AS, Ahmad MZ, Salam F. An updated review on polyherbal formulation: A global perspective. Syst Rev Pharm 2016;7:35–41. [CrossRef]
15. Che CT, Wang ZJ, Chow MS, Lam CW. Herb-herb combination for therapeutic enhancement and advancement: Theory, practice and future perspectives. Molecules 2013;18(5):5125–41. [CrossRef]
16. Patwardhan B, Warude D, Pushpangadan P, Bhatt N. Ayurveda and traditional Chinese medicine: A comparative overview. Evid Based Complement Alternat Med 2005;2(4):465–73. [CrossRef]
17. Niemeyer K, Bell IR, Koithan M. Traditional knowledge of western herbal medicine and complex systems science. J Herb Med 2013;3(3):112–9. [CrossRef]
18. Bhatnagar RS, Padilla-Zakour OI. Plant-based dietary practices and socioeconomic factors that influence anaemia in India. Nutrients 2021;13(10):3538. [CrossRef]
19. Okorie N, Fasogbon SA, Emmanuel CJ, Nnorom SC, Olayera AB, Owolabi OD, et al. The effect of *Mucuna pruriens* aqueous leaf extract on haematological parameters of Wistar rats. Am J Health Med Nurs Pract 2020;5(2):48–66. [CrossRef]
20. Rifai N, Horvath AR, Wittwer CT. Tietz textbook of clinical chemistry and molecular diagnostics. 6th ed. St. Louis (MO): Elsevier Health Sciences; 2017.
21. Ezeigwe OC, Alaebo PO, Enemchukwu BN, Chukwuemeka UV, Ifedilichukwu NH, Uhama KC, et al. Haematological and biochemical effects of a combination of leaves of *Telfairia occidentalis* and *Mucuna pruriens* in male rats. Int J Biosci 2020;16(1):139–49.
22. Bruce RD. An up-and-down procedure for acute toxicity testing. Fundam Appl Toxicol 1985;5(1):151–7. [CrossRef]
23. Ochei J, Kolhatkar A. Medical laboratory science: Theory and practice. 7th ed. New Delhi: Tata McGraw-Hill, 2008. p. 273–83.
24. Pandey K, Meena AK, Jain A, Singh R. Molecular mechanism of phenylhydrazine-induced haematotoxicity: A review. Am J Phytomed Clin Ther 2014;2:390–4.
25. Perutz MF. The haemoglobin molecule. Proc R Soc Lond B Biol Sci 1969;173(1033):113–40. [CrossRef]
26. Siti MM, Mazalan N, Humaimi H, Mahmood MA, Abdul Razak MA. Automated red blood cells counting in peripheral blood smear image using circular Hough transform. In Proceedings of the 2013 International Conference on Advanced Informatics: Concepts, Theory and Applications (AIMS), 2013. p. 320–4. [CrossRef]
27. Adepoju GKA, Odubena OO. Effect of *mucuna pruriens* on some haematological and biochemical parameters. J Med Plants Res 2009;3(2):73–6.

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Research Article

Towards a predicted anti-aging molecular targets of asiaticoside based on bioinformatics analysis

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Abstract

Objectives: Human skin, the largest organ, serves as a critical barrier against environmental damage and microbial invasion. The skin aging process leads to collagen degradation, reduced elasticity, and wrinkle formation, influenced by intrinsic and extrinsic factors. This process has driven significant interest in the anti-aging market, which is expected to grow to $44.5 billion by 2030. Asiaticoside (AS) has exhibited anti-aging properties by promoting collagen synthesis and fibroblast proliferation.

Methods: This study employed bioinformatics analyses to identify molecular targets and pathways modulated by AS in skin aging. The gene databases were extracted from PubMed (www.ncbi.nlm.nih.gov), OMIM (www.omim.org), and GeneCards (www.genecards.org). Protein-protein interaction (PPI) networks and CytoHubba algorithms (MCC, DMNC, MNC) identified ten key genes implicated in the skin aging cascade. To validate the results, molecular docking was conducted to assess AS's binding affinity to these targets.

Results: This study identified IL-1β, JUN, TGF-β1, CCL-2, MMP-9, STAT-3, MAPK-3, CXCL-8, MMP-2, and KDR as potentially targeted by AS in the skin aging cascade. Molecular docking revealed a strong binding affinity of AS with MMP-9 (-8.16 kcal/mol), indicating its role in inhibiting ECM degradation.

Conclusion: This study highlights AS's potential as a promising anti-aging agent by targeting key proteins and pathways, paving the way for further therapeutic exploration. This prediction of molecular pathways should be further verified by *in vitro* and *in vivo* experiments.

Keywords: Asiaticoside, bioinformatics, molecular docking, pathway, skin aging

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Skin, the outermost and largest organ, serves as a protective layer for underlying tissue from microbial infection and contributes as an essential barrier against environmental damage [1–5]. The human skin is the first organ that exhibits obvious signs of aging, undergoing progressive changes in both morphology and physiology with age [6]. Awareness about skin aging has expanded lately as society becomes more conscious of beauty. Thus, numerous studies on the factors and strategies to slow skin aging have gained popularity in cosmetic medicine nowadays [7]. The global facial rejuvenation market is predicted to elevate significantly from $24.6 billion to approximately $44.5 billion by 2030 due to the increase in aging populations. Thus, technological innovations have greatly improved public interest in beauty and skin health, and attempts to delay skin aging are growing rapidly [8].

Skin aging refers to a natural, multifaceted, and complicated biological degenerative process [6, 7, 9, 10]. Three skin layers—epidermis, dermis, and subcutaneous—experience degenerative alterations due to aging, with dermal changes being the most obvious [11]. Skin aging is identified by features including skin laxity, wrinkles, elasticity loss, and a rough-looking texture [10]. Its aging process is accelerated by

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a combination of endogenous and exogenous factors more than in any other body organ [6, 12, 13]. The endogenous factors are characterized by a reduced ability to regenerate, decreased stratum corneum permeability, epidermal atrophy that mostly affects the stratum spinosum, as well as a reduction in fibroblast and collagen levels in the dermis [5, 12]. Collagen is a protein that provides tensile strength, firmness, and elasticity and supports skin integrity [14]. Exogenous factors, mainly resulting from exposure to ultraviolet (UV) rays, lead to progressive skin damage and play a role in the aging process known as photoaging [5, 15–17]. Chronic UV exposure on human skin activates the expression of matrix metalloproteases (MMPs), impacting collagen and elastin fibers in the dermis and ultimately resulting in solar elastosis. Both endogenous and exogenous factors decrease collagen, the primary factor associated with aging skin, which encourages extracellular matrix (ECM) degradation, skin laxity, deep wrinkle formation, and hyperpigmentation [10, 16, 18]. The skin aging process increases dryness, dullness, coarseness, sagging, and loss of elasticity due to a decrease in skin surface hydration [19, 20].

The utilization of biologically active compounds continues to be a rising trend in the 21st century, marked by the growth of the global natural cosmetics market [21]. Biologically active compounds with pharmaceutical properties, often referred to as cosmeceuticals, represent the latest advancement in beauty care products aimed at reducing wrinkles [22]. There are abundant botanical products that have been clinically proven to prevent the skin aging process. Among them, *Centella asiatica* contains natural products such as asiaticoside, madecassoside, asiatic acid, and madecassic acid [10, 23]. Asiaticoside and madecassoside are the two major terpenoid glycosides that have demonstrated anti-skin-aging effects [24, 25].

Asiaticoside (AS) (Fig. 1a) is a major pentacyclic triterpene glycoside (saponin) with similar sugar chains (Glu-Glu-Rha) bonded to its carboxyl groups [10, 26, 27]. Asiaticoside (AS) is synthesized through glycosylation followed by a rhamnosylation reaction of asiatic acid, catalyzed by UDP-glucosyltransferases (UGTs), which initially attach a glucose molecule to the carboxyl group at C-28 [27]. It increased normal human skin cell migration, adhesion, and proliferation [28]. In addition, AS, an active main secondary metabolite in *Centella asiatica*, induces anti-aging properties by promoting collagen levels and encouraging the growth of normal dermal fibroblasts [29–31]. Nevertheless, it is commonly used for cosmetic purposes in topical applications [32]. Previous studies have proven that AS possesses anti-aging properties by inducing collagen synthesis in dermal fibroblasts via the activation of TGF-β signaling pathways [33]. However, the precise mechanisms by which it interferes with skin aging at the molecular level remain uncertain. Since asiaticoside and madecassoside are major biomarkers of triterpenoid glycosides in *Centella asiatica* [34, 10], this study further analyzed both compound combinations for skin anti-aging.

This investigation outlined a molecular pathway related to bioinformatics assessments of AS's effects on skin aging. Over the past few years, bioinformatics analysis has been widely used for generating diverse datasets that analyze protein and gene expression levels, identifying various genes involved in pathways associated with skin aging [20]. Furthermore, bioinformatics assists in determining the molecular mechanisms underlying specific clinical alterations rapidly and precisely [7]. As a result, 10 top genes were ranked as the most influential genes using three network scoring methods: MCC (Maximal Clique Centrality), DMNC (Density of Maximum Neighborhood Component), and MNC (Maximum Neighborhood Component). These algorithms measure centrality by predicting and exploring the distance from the direct neighborhood of a vertex [20, 35].

This study employs molecular docking and simulation approaches for protein-ligand interactions to facilitate the discovery of innovative skin aging treatments. Thus, AS interacts with key molecular pathways associated with skin aging, which can be computationally predicted and validated by molecular docking and bioinformatics tools. Asiaticoside, by acting on MMP-2/9, plays a crucial role in the degradation of the extracellular matrix, making it a promising agent for skin rejuvenation. Furthermore, the combination of asiaticoside and madecassoside targeted metabolic enzymes such as CYP and UGT to protect the skin aging process from environmental oxidative stress. In conclusion, our research has outlined how asiaticoside targets various molecular pathways such as interleukins, growth regulators, metabolic enzymes, and matrix metalloproteinases, all of which are involved in inhibiting skin aging activities. Furthermore, this prediction of molecular pathways should be further verified by *in vitro* and *in vivo* experiments.

Materials and Methods

Data mining and collection

Key proteins and genes involved in the skin aging mechanism were retrieved from public biomedical databases, including PubMed (www.ncbi.nlm.nih.gov), OMIM (www.omim.org), and GeneCards (www.genecards.org), as a preliminary step in the analysis. The targets of asiaticoside, encompassing both direct and indirect influences on these biomolecules, were identified via www.stitch.embl.de. An interactive Venn diagram tool (www.interactivenn.net) was applied to identify the specific proteins and genes influenced by asiaticoside in relation to skin aging [36].

Construction of protein interaction networks and gene clustering

The construction of a protein-protein interaction (PPI) network and gene clustering involved mapping the dynamic, complex interactions among multiple proteins. Direct and indirect protein interactions were extracted using STRING-DB v11.5 (https://string-db.org), forming the basis of the interaction network. Subsequently, gene analysis was performed with Cytoscape 3.10.1 (https://cytoscape.org/), a platform designed to visualize molecular interaction networks effectively [20].

Analysis of hub gene expression levels

The study employed MNC and Degree algorithms from the CytoHubba plugin to identify the top 10 genes with the

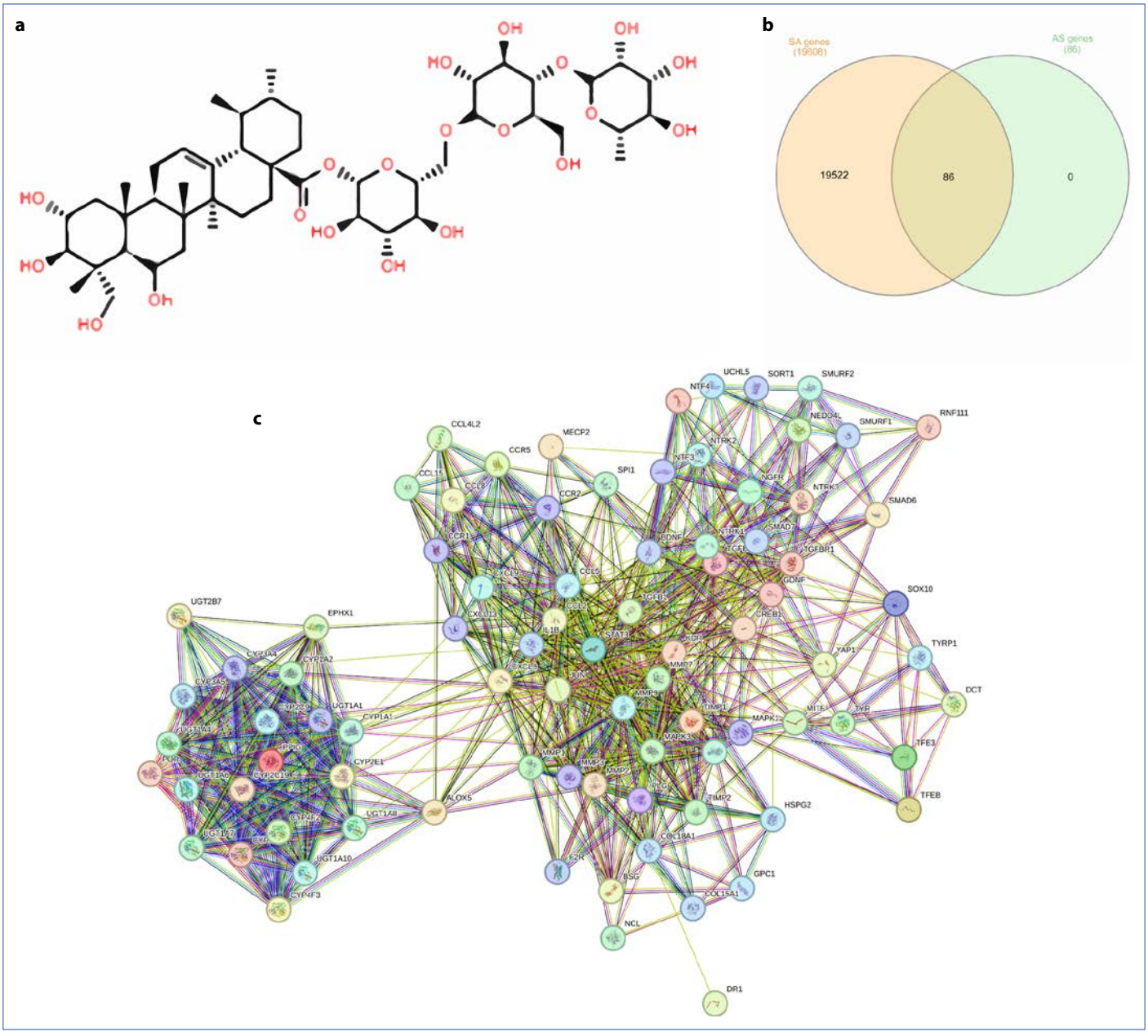

Figure 1. Asiaticoside's top target proteins and genes related to skin aging. (a) Asiaticoside's structure, (b) venn diagram of asiaticoside (AS) and skin aging (SA) interfered genes, (c) protein-protein interaction (PPI) network of the intersecting genes.

highest correlation within the PPI network. These genes, identified as hub genes, are closely associated with skin aging. The bioinformatics analysis was conducted on a system equipped with an 11th Gen Intel Core i3-1115G4 processor at 3.00 GHz and 8 GB of RAM.

Molecular docking

The structures IL1B (6Y8I), MMP9 (1GKC), and CYP3A5 (7LAD) were sourced from the RCSB Protein Data Bank (www.rcsb.org), and the ligand preparation was carried out using BIOVIA Discovery Studio 2021. The study utilized the native ligand as a control, followed by re-docking using AS. The ligand was protonated with Gasteiger charges, while Kollman charges were assigned to the macromolecule using AutoDockTools 1.5.7. The AS compounds were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov). Molecular docking employed a genetic algorithm with 100 GA runs. For the 6Y8I complex, the ligand was positioned at coordinates x=7.425, y=25.105, z=7.064, and for the 1GKC complex at x=65.607, y=31.083, z=117.697, using a grid box size of 40×40×40 and a spacing of 0.375 Å. The complexes were visualized in 3D and superimposed post-docking using BIOVIA Discovery Studio 2021. Docking accuracy was evaluated by calculating the RMSD value, which was 2.0 Å. The results of docking were applied to predict binding energies and protein-ligand interactions.

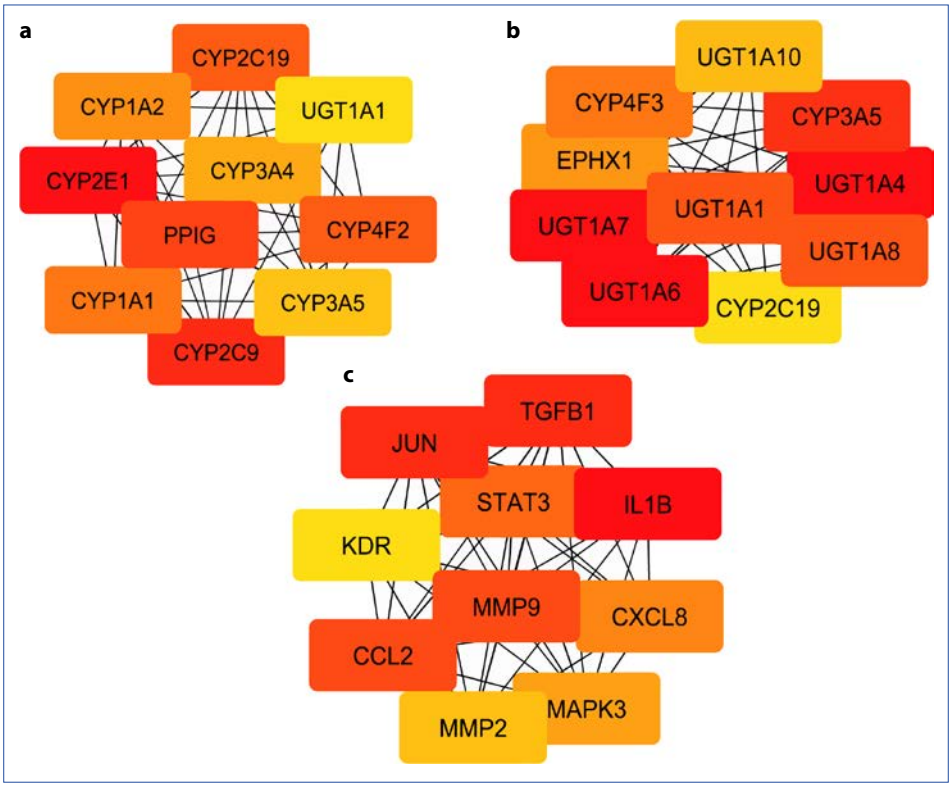

Figure 2. The clustering of the top 10 genes of AS related to skin aging according to MCC (a), DMNC (b), and MNC algorithm (c) in CytoHubba.

AS: Asiaticoside; MCC: Maximal clique centrality; DMNC: Density of maximum neighborhood component; MNC: Maximum neighborhood component.

Results

AS (Fig. 1a) is a saponin glycoside with sugar molecules (glucose-glucose-rhamnose) that are attached to the triterpene group [37]. Using the specified screening criteria, two data sets were generated through the Venn diagram tool. A total of 19,608 genes involved in the skin aging process were compared with 68 genes that interacted with AS. Venn diagram analysis identified 86 genes associated with asiaticoside that are linked to skin aging (Fig. 1b). The PPI network showed that 86 gene targets of AS interact with each other to produce two major interconnected networks (Fig. 1c). Since asiaticoside coexists with madecassoside in plant extract, we further conducted bioinformatics and molecular docking analysis of both compounds in the skin aging process.

Madecassoside (MS) is a triterpenoid saponin (Appendix 1a), which has been studied for its anti-inflammatory and wound-healing properties [10]. AS, in combination with MS, targets 21 genes related to the skin aging process, all of which are metabolic enzymes such as cytochrome P450 (CYP), arachidonate 5-lipoxygenase (ALOX5), UDP-glucuronosyltransferases (UGTs), cytochrome P450 oxidoreductase (POR), and peptidylprolyl isomerase G (PPIG) (Appendix 1b, c). Furthermore, the MCC, DMNC, and MNC methodologies quantify gene interactions based on interaction degree, with each approach identifying the highest-ranking genes within the top 10 results of the analysis. The ranks, shown in Figure 2, sequence the genes most affected by AS that contribute to skin aging. There was only one independent cluster based on each algorithm. The genes targeted by AS with the highest scores based on the MCC algorithm are CYP2E1 and CYP2C9 (Fig. 2a). Meanwhile, based on the DMNC algorithm: UGT1A7, UGT1A6, UGT1A4, and CYP3A5 were identified (Fig. 2b), and based on the MNC algorithm, there are IL-1β, JUN, and TGF-β1 (Fig. 2c). The biological functions of the genes related to skin aging, based on MNC data, are presented and analyzed in Table 1. When combined with MS, the top target genes based on MCC, DMNC, and MNC are subclasses of CYPs (CYP3A5, CYP1A2) (Appendix 2).

Molecular docking studies were conducted to predict the potential binding of AS, and further studies were carried out to investigate the relationship between anti-aging-related genes and AS. We selected IL-1B and MMP-9 as target genes for molecular docking with AS. MMP-9 and AS showed eight H-bonds to Pro421, His401, His411, Leu397, Leu418, Glu402, Leu188, and Ala189 (Fig. 3). UGTs are seen to have multiple hydrophobic bonds with Leu110 at IL-1B and Leu188, Val398, and Tyr423 at MMP9. In this process, AS showed lower binding energy at MMP-9, which is –8.16 kcal/mol, compared to –5.57 kcal/mol at IL-1B (Table 2), where AS has Van der Waals interactions with Asp12, Asn108, and Lys109 on IL-1B, as well as with Phe110, Ala191, His190, His405, Tyr420, Met422, Leu187,

Table 1. Top 10 proteins network interaction ranked by MNC algorithm

No	Gene symbol	Gene/protein name/abbreviation	Biological function related to skin aging	Reference
1	IL-1β	Interleukin 1β	IL-1β is an inflammatory mediator that is induced by main mediators in the inflammatory responses (CCL-2), activating signaling activities of STAT-3. The maturation and release of IL-1β is regulated by an inflammatory signaling platform called inflammasome.	[70–74]
2	JUN	c-Jun	Activated by ERK pathways. Combines with c-Fos to form the transcription factor AP-1 which stimulates MMP-2/9 transcription. Increased MMP transcription accelerates the degradation of collagen.	[39, 45]
3	TGF-β1	Transforming Growth Factor β1	TGF-β1 is a one of TGF-β isoform that induced Smad 2 and Smad 3 phosphorylation which function as a transcriptional activator to induced MMP-2/ 9 transcription.	[50, 54, 58]
4	CCL-2	Chemokine (C-C motif) ligand 2	Also known as monocyte chemotactic/chemoattractant protein 1 (MCP). CCL-2 is an inflammatory chemokine secreted by macrophage that induces activation to promote inflammation after binding to its ligand CCR-2. CCL-2 activates a series of downstream signals such as JAK which then activates phosphorylation STAT 3/5 which then activates phosphorylation IL-1β/CXCL-8.	[75–77]
5	MMP-9	Matrix metalloproteinase 9	MMP-9 is known as gelatinase B. The expression of MMP-9 is stimulated by AP-1 and produced by keratinocytes. MMP-9 can degrade gelatin types I and V, collagen types IV and V, fibronectin in dermal fibroblast cells located in the ECM thereby stimulating skin aging.	[18, 44, 52, 78–81]
6	STAT-3	Signal Transducer and Activator of Transcription 3	Activated by CCL-2 which then lead to upregulation expression of MMP-2/9. The STAT-3 pathway is activated in response to several cytokines, including IL-1β and CXCL-8.	[66, 73, 76, 82]
7	MAPK-3	Mitogen-Activated Protein Kinase 3	Also known as extracellular signal-regulated kinases (ERKs), it is present in the cytoplasm and translocated into the nucleus. MAPK-3 (JNK, ERK, and p38) transfers extracellular signals to the nucleus, thereby activating transcription factors and inducing AP-1 as a downstream activator of MAPK, which then induced and regulates the transcription of MMP 2/9.	[15, 17]
8	CXCL-8	Chemokine (C-X-C motif) ligand 8	Also known as IL-8, activated by CCL-2 signaling pathway which then activates the phosphorylation of JAKs and STAT3.	[67, 77, 83, 84]
9	MMP-2	Matrix Metalloproteinase 2	MMP-2 is known as gelatinase A. The expression of MMP-2 is stimulated by AP-1 and produced by keratinocytes. MMP-2 can degrade gelatin type I, collagen types IV, V, VII, X in dermal fibroblast cells located in the ECM thereby stimulating skin aging.	[18, 42, 52, 78–81]
10	KDR	Kinase insert Domain Receptor	Also referred to as VEGFR-2, VEGF receptor is bound by VEGF-A, activating downstream pathways like MAPK1/3, which then regulates transcription factors AP-1 (c-Jun, c-Fos).	[46, 85, 86]

MNC: Maximum neighborhood component.

Tyr393, and Gly186 on MMP9 (Fig. 3). This study is considered valid based on the calculation of RMSD control, where the value for IL1B was 1.74 Å and for MMP9 was 1.65 Å. These results allow the specific binding of AS to MMP9, where the genes play important roles in the anti-aging pathway.

Furthermore, we conducted molecular docking of AS and MS to CYP3A5 as one of the possible molecular targets based on MCC, DMNC, and MNC algorithms. AS indicated a lower binding energy of –11.82 kcal/mol than MS to CYP3A5 (–10.43 kcal/mol). AS has Van der Waals interactions with Leu108, Ser107, Leu120, Phe220, Gly306, Thr309, Thr310, Val313, Phe367, Pro368, Ala447, Met451, and Leu481. It also formed hydrogen bonds with Arg106, Phe213, Val369, and Glu374. On the other hand, MS interacts with CYP3A5 through hydrogen bonding at Arg106, Ser107, Gly109, Ala305, Thr309, Glu308, Phe304, and Phe434. It binds considerably to Arg105, Leu108, Ser119, Leu120, Phe210, Ile303, Gly306, Tyr307, Val369, Ala370, Arg372, Leu373, Glu374, Arg375, Pro433, Arg439, Asn440, Cys441, Gly435, and Leu481 through Van der Waals interactions (Appendix 3, 4). These findings are validated by confirming the binding energy of clobetasol propionate as the control, with a binding energy value of 8.11 kcal/mol.

Through a literature review, this study predicted the molecular cascade pathway illustrated in Figure 4. TGF-β1 (Transforming Growth Factor-β1), VEGFA (Vascular Endothelial Growth Factor A), EGF (Epidermal Growth Factor), and CCL-2 (Chemokine (C-C motif) ligand 2) bind to their respective receptors, triggering a molecular cascade within the cytoplasm. This cascade eventually leads to the phosphorylation of MMP-2/9 in the ECM, resulting in collagen degradation. AS is predicted to interact with certain molecules, inhibiting skin aging.

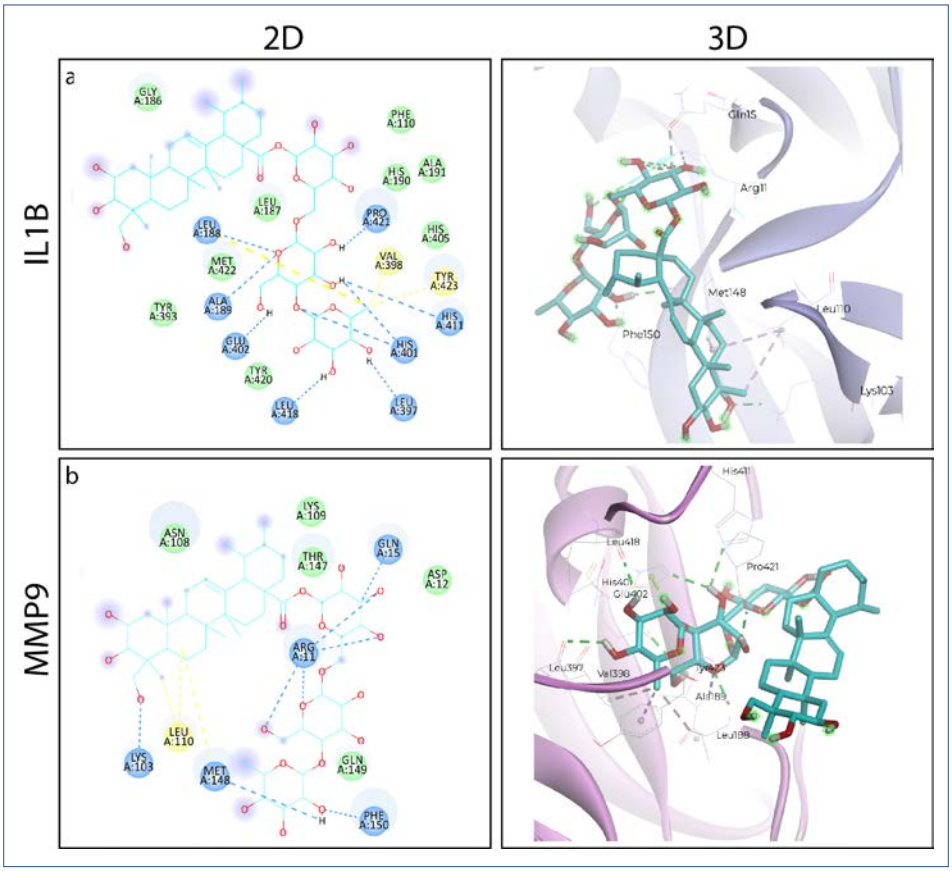

Figure 3. The binding poses of AS in IL1B and MMP-9 binding pocket in 2D and 3D view. Yellow, red, and white indicated carbon, oxygen, and hydrogen atoms. Yellow, blue, and green indicated hydrophobic bond, hydrogen bond, van der waals interaction.

IL1B: Interleukin 1β; MMP: Matrix metalloproteinase; AS: Asiaticoside.

Discussion

This study is the first to explore AS's impact on molecular pathways associated with skin aging, examining upstream and downstream elements within these routes (Fig. 4). The gene targets obtained in this study differed from previous bioinformatics studies of AS in skin aging due to the use of different databases such as PharmMapper, SwissTargetPrediction, CTD, and BATMAN, as well as analysis tools. While Huang and colleagues' study demonstrated that AS targeted apoptosis and inflammation-related signaling pathways [38], our findings suggest that AS targeted both inflammation-related signaling pathways and ECM degradation enzymes.

Skin aging is a complex biological phenomenon linked to the increased expression of genes that contribute to ECM breakdown [11, 39]. Comprising an adaptable, three-dimensional network of macromolecules, the ECM provides both biochemical and structural support to neighboring cells, while its specialized biochemical and biomechanical properties regulate key physiological activities such as cell growth, migration, and homeostasis [40, 41]. One of the cell types that secrete components in the ECM is glycoproteins, which contain proteoglycans, collagen, fibronectin, elastin, and laminins [42].

As the predominant structural protein within the extracellular matrix, collagen is present in substantial quantities and is susceptible to degradation, which then triggers the downregulation of the ECM [43]. Collagen degradation is primarily driven by extrinsic factors, particularly UV radiation [11, 14, 41, 44, 45]. However, even in the absence of sunlight exposure, aging leads to a decline in collagen production, attributed to genetically regulated apoptosis, mitochondrial dysfunction, and diminished antioxidant defense mechanisms [46]. These factors lead to an upregulation of enzymes that break down the ECM [14, 41]. This is due to differences in MMP concentrations and the four protease inhibitors, known as tissue inhibitors of matrix metalloproteinases (TIMPs), that modulate MMP activity [47]. Specifically, MMP expression increases, while the expression of TIMPs decreases [6, 48]. MMPs represent an extensive group of zinc-dependent endopeptidases capable of breaking down ECM proteins [11, 14, 45, 49, 50]. Among them, MMP-2 and MMP-9 are key ECM enzymes essential for ECM degradation, a process driven by endopeptidase activity [11, 40, 48, 51].

The activation of MMPs is triggered by various factors such as TGF-β, VEGF, and epidermal growth factor (EGF) [52]. EGF binds to its receptor (EGFR) on the cell surface and then combines with the Src homology 2 (SH2) domain of the growth-factor-

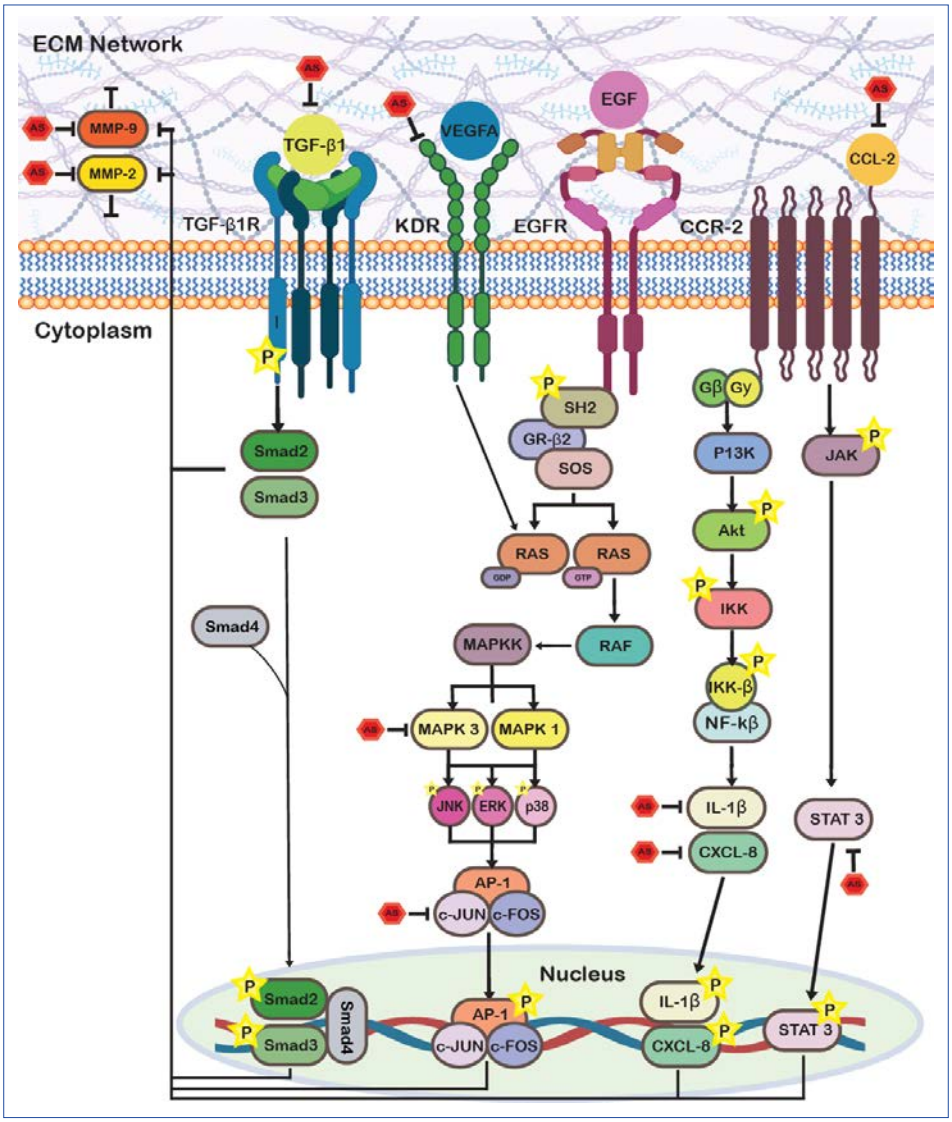

Figure 4. Predicted molecular cascade of AS in skin aging.

IL-1β: Interleukin 1β; JUN: c-Jun; TGF-β1: Transforming growth factor β1; CCL-2: Chemokine (C-C motif) ligand 2; MMP-9: Matrix metalloproteinase 9; STAT-3: Signal transducer and activator of transcription 3; MAPK-3: Mitogen-activated protein kinase 3; CXCL-8: Chemokine (C-X-C motif) ligand 8; MMP-2: Matrix metalloproteinase 2; KDR: Kinase insert domain receptor; AS: Asiaticoside.

receptor-binding protein 2 (GRB2). Simultaneously, GRB2 binds to the ornithine conversion factor, Son of Sevenless (SoS), promoting the activation of the Rat Sarcoma Virus (RAS) protein, a small GTPase. Upon activation, RAS recruits and activates downstream RAF (Rapidly Accelerated Fibrosarcoma) kinases [52, 53]. Subsequently, the activated RAF phosphorylates and triggers mitogen-activated protein kinase kinase (MAPKK) signaling [46]. Activated MAPKK phosphorylates mitogen-ac-

Table 2. Molecular docking of asiaticoside with IL-1β (6Y8I) and MMP-9 (1GKC)

Target protein	Binding energy (kcal/mol)	H-bond residues	Hydrophobic residues	Van der Waals residues
IL-1β	-5.57	Phe150, Met148, Arg11, Lys103, Thr147, Gln15, Gln149	Leu110	Asp12, Asn108, Lys109
MMP-9	-8.16	Pro421, His401, His411, Leu397, Leu418, Glu402, Leu188, Ala189	Val398, Tyr423	Phe110, Ala191, His190, His405, Tyr420, Met422, Leu187, Tyr393, Gly186

IL-1β: Interleukin 1β; MMP-9: Matrix metalloproteinase 9; Phe: Phenylalanine; Met: Methionine; Arg: Arginine; Lys: Lysine; Gln: Glutamine; Leu: Leucine; Asp: Aspartatic acid; Asn: Asparagine; Pro: Proline; His: Histidine; Glu: Glutamic acid; Val: Valine; Tyr: Tyrosine.

tivated protein kinases (MAPKs), such as MAPK-1/3, which subsequently phosphorylate the JNK, ERK, and p38 signaling pathways [41, 54]. This leads to the phosphorylation of the AP-1 complex (c-Fos and c-Jun), which translocates into the nucleus to directly regulate MMP-2/9 expression within the extracellular matrix (ECM) network [5, 39, 41, 44]. AP-1 indirectly suppresses collagen biosynthesis and promotes collagen degradation through multiple mechanisms. It alters the balance between MMPs and TIMPs, favoring MMP dominance. When MMPs prevail over TIMPs, collagen and other fibrillar structures undergo degradation [48]. RAS downstream signaling pathways are also activated by VEGFA binding to its receptor, KDR [55].

Meanwhile, TGF-β1, TGF-β2, and TGF-β3, the three isoforms of the transforming growth factor (TGF-β) subfamily, play distinct roles in various biological processes [56]. TGF-β plays a crucial role in regulating ECM synthesis and managing collagen breakdown through activation of the Smad signaling pathway [11]. TGF-β1 modulates the expression of several MMPs, including MMP-2 and MMP-9, contributing to extracellular matrix remodeling. TGF-β1 increases the activation of MMP-2/9 via phosphorylation of the transcription factors Smad-2/3 (canonical Smad signaling) facilitated by its receptors (TGF-βR1 and TGF-βR2), which assemble into homodimeric and heterodimeric complexes essential for signaling [48, 54, 57]. Smad-2/3 then complexes with Smad4 and translocates to the nucleus, inducing the expression of MMP-2/9 [50, 54, 58].

The upregulation of MMP-2/9 is further initiated by the activation of CCL-2 (MCP-1) [59–62]. CCL-2 plays a critical role in driving disease progression by enabling the attraction of immune cells like monocytes and macrophages to inflammatory sites, thereby enhancing immune cell infiltration and contributing to fibrotic tissue remodeling [62, 63]. Upon binding to its receptor, C-C motif chemokine receptor 2 (CCR-2), CCL-2 activates the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) and JAK/STAT pathways [62]. The PI3K/Akt pathway triggers NF-κB, which then moves into the cell nucleus to begin gene transcription, leading to the expression of factors like interleukin-1β (IL-1β) and chemokine (C-X-C motif) ligand 8 (CXCL-8), ultimately resulting in the production and release of these cytokines [41, 52, 59, 63–65]. CCL-2/CCR-2 signaling also activates the JAK/STAT pathway through the stimulation of Janus kinase 2 (JAK-2), which subsequently triggers downstream signaling cascades, including the activation of STAT3/5. This ultimately regulates the transcriptional activation of MMP-2/9, thereby exacerbating the detrimental effects on the skin [64, 66, 67].

Additionally, earlier research has shown that AS reduces TGF-β1 expression by decreasing its mRNA synthesis [10, 29]. Beyond its anti-fibrotic actions, AS also possesses potent anti-inflammatory effects by blocking IL-1β production, further supporting its potential therapeutic role in skin aging and fibrosis [68]. In Figure 4, AS has been shown to inhibit several key molecular targets, including MMP-2/9, CXCL-8, KDR, c-Jun, CCL-2, STAT3, and MAPK3, demonstrating its potential therapeutic role in mitigating skin damage and fibrosis by modulating both upstream and downstream components of these pathways.

Since AS and MS are terpenoid saponins, AS often coexists with MS in plant extract. Both of them inhibit metabolic enzymes related to skin aging, mainly CYPs and UGTs. During the aging process, oxidative stress contributes to the propagation of ROS and reduces enzymatic protection [69]. CYP subclasses are expressed in different skin layers and are responsible for several vitamin metabolisms, including retinoid acid, which contributes to skin aging. One of CYP's AS and MS targets is CYP3A5, which is primarily expressed in the basal layer of the skin epidermis [10]. To date, the findings of this study have initiated further research to confirm *in vitro* and *in vivo* skin aging experiments with the help of network pharmacology analysis through bioinformatics and molecular docking.

Conclusion

Based on bioinformatics analysis, asiaticoside (AS) has been identified to target a wide range of key proteins involved in skin aging. These proteins function collaboratively within various molecular pathways, enhancing the therapeutic potential of AS in combating extracellular matrix (ECM) degradation and inflammation. AS modulates both upstream and downstream signaling mechanisms, including those involving MMP-2/9, TGF-β1, IL-1β, CXCL-8, KDR, c-Jun, CCL-2, STAT3, and MAPK3, to inhibit processes that contribute to skin aging. These findings provide crucial foundational data for further investigation into AS's *in vitro* and *in vivo* activities. AS's ability to regulate multiple molecular targets positions it as a promising candidate for anti-aging therapy. Further exploration of its clinical efficacy is warranted.

Appendix files: https://jag.journalagent.com/ijmb/abs_files/IJMB-26122/IJMB-26122_(2)_IJMB-26122_Appendixes.pdf

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References

1. Lima TPL, Passos MF. Skin wounds, the healing process, and hydrogel-based wound dressings: A short review. J Biomater Sci Polym Ed 2021;32(14):1910–25. [CrossRef]
2. Su J, Li J, Liang J, Zhang K, Li J. Hydrogel preparation methods and biomaterials for wound dressing. Life 2021;11(10):1–22. [CrossRef]
3. Firlar I, Altunbek M, McCarthy C, Ramalingam M, Camci-Unal G. Functional hydrogels for treatment of chronic wounds. Gels 2022;8(2):1–23. [CrossRef]

4. Lai-Cheong JE, McGrath JA. Structure and function of skin, hair and nails. Medicine 2021;49(6):337–42. [CrossRef]
5. Lee H, Hong Y, Kim M. Structural and functional changes and possible molecular mechanisms in aged skin. Int J Mol Sci 2021;22(22):12489. [CrossRef]
6. Zouboulis CC, Ganceviciene R, Liakou AI, Theodoridis A, Elewa R, Makrantonaki E. Aesthetic aspects of skin aging, prevention, and local treatment. Clin Dermatol 2019;37(4):365–72. [CrossRef]
7. Xiao X, Feng H, Liao Y, Tang H, Li L, Li K, et al. Identification of key circadian rhythm genes in skin aging based on bioinformatics and machine learning. Aging 2023;15(20):11672–89. [CrossRef]
8. Griffiths TW, Watson REB, Langton AK. Skin ageing and topical rejuvenation strategies. BJD 2023;189(1):l17–l23. [CrossRef]
9. Bjørklund G, Shanaida M, Lysiuk R, Butnariu M, Peana M, Sarac I, et al. Natural compounds and products from an anti-aging perspective. Molecules 2022;27(20):7084. [CrossRef]
10. Tan SC, Bhattamisra SK, Chellappan DK, Candasamy M. Actions and therapeutic potential of madecassoside and other major constituents of *centella asiatica*: A review. Appl Sci 2021;11(18):8475. [CrossRef]
11. Shin JW, Kwon SH, Choi JY, Na JI, Huh CH, Choi HR, et al. Molecular mechanisms of dermal aging and antiaging approaches. Int J Mol Sci 2019;20(9):2126. [CrossRef]
12. He X, Wan F, Su W, Xie W. Research progress on skin aging and active ingredients. Molecules 2023;28(14):1–28. [CrossRef]
13. Ng JY, Chew FT. A systematic review of skin ageing genes: Gene pleiotropy and genes on the chromosomal band 16q24.3 may drive skin ageing. Sci Rep 2022;12:13099. [CrossRef]
14. Reilly DM, Lozano J. Skin collagen through the lifestages: Importance for skin health and beauty. Plast Aesthet Res 2021;8:2. [CrossRef]
15. Qian H, Shan Y, Gong R, Lin D, Zhang M, Wang C, et al. Mechanism of action and therapeutic effects of oxidative stress and stem cell-based materials in skin aging: Current evidence and future perspectives. Front Bioeng Biotechnol 2023;10:1082403. [CrossRef]
16. Ke Y, Wang XJ. TGFβ signaling in photoaging and UV-induced skin cancer. JID 2021;141(4):1104–10. [CrossRef]
17. Fang M, Lee HM, Oh S, Zheng S, Bellere AD, Kim M, et al. Rosa davurica inhibits skin photoaging via regulating MAPK/AP-1, NF-κB, and Nrf2/HO-1 signaling in UVB-irradiated HaCaTs. Photochem Photobiol Sci 2022;21:2217–30. [CrossRef]
18. Kim M, Park HJ. Molecular mechanisms of skin aging and age-related diseases. InTech 2016;57–74. [CrossRef]
19. Yulianti L, Mardliyati E, Bramono K, Freisleben HJ. Time- and dose-dependent effects of *Centella asiatica* ethanolic extract encapsulated into chitosan nanoparticles on collagen III synthesis and proliferation in human dermal fibroblasts. J Pharm Biomed Sci 2016;6(5):315–27. [CrossRef]
20. Hu W, Jing Y, Yu Q, Huang N. Differential gene screening and bioinformatics analysis of epidermal stem cells and dermal fibroblasts during skin aging. Sci Rep 2022;12:12019. [CrossRef]
21. Ferreira MS, Magalhães MC, Oliveira R, Sousa-Lobo JM, Almeida IF. Trends in the use of botanicals in anti-aging cosmetics. Molecules 2021;26(12):3584. [CrossRef]
22. Shah F, Sarheed O, Ramesh KVR. A prospective study of knowledge and perception towards the efficacy of anti-aging cosmetics among the female population of Ras Al Khaimah, UAE. JCDSA 2017;7(3):275–89. [CrossRef]
23. Min DH, Yu YB, Kim TH, Kim H, Lee S. Pharmacological effects of pentacyclic triterpenoids isolated from *Centella asiatica*. Hortic Environ Biotechnol 2024;65(2):189–97. [CrossRef]
24. Bandopadhyay S, Mandal S, Ghorai M, Jha NK, Kumar M, Radha, et al. Therapeutic properties and pharmacological activities of asiaticoside and madecassoside: A review. J Cell Mol Med 2023;27(5):593–608. [CrossRef]
25. Jiang H, Zhou X, Chen L. Asiaticoside delays senescence and attenuates the generation of ROS in UV exposure cells through regulation of the TGF β1/Smad pathway. Exp Ther Med 2022;24(5):667. [CrossRef]
26. Da Rocha PBR, Souza BS, Andrade LM, Dos Anjos JLV, Mendanha SA, Alonso A, et al. Enhanced asiaticoside skin permeation by *centella asiatica*-loaded lipid nanoparticles: Effects of extract type and study of stratum corneum lipid dynamics. J Drug Del Sci Tech 2019;50:305–12. [CrossRef]
27. Kim OT, Jin ML, Lee DY, Jetter R. Characterization of the asiatic acid glucosyltransferase, UGT73AH1, involved in asiaticoside biosynthesis in *centella asiatica* (L.) Urban. Int J Mol Sci 2017;18(12):2630. [CrossRef]
28. Park KS. Pharmacological effects of *centella asiatica* on skin diseases: Evidence and possible mechanisms. eCAM 2021;2021:1–8. [CrossRef]
29. Saeidinia A, Keihanian F, Lashkari AP, Lahiji HG, Mobayyen M, Heidarzade A, et al. Partial-thickness burn wounds healing by topical treatment: A randomized controlled comparison between silver sulfadiazine and centiderm. Medicine 2017;96(9):e6168. [CrossRef]
30. Ranjith GP, Jisha S, Hemanthakumar AS, Saji CV, Shenoi RA, Sabu KK. Impact of potential stimulants on asiaticoside and madecassoside levels and expression of triterpenoid-related genes in axenic shoot cultures of *centella asiatica* (L.) Urb. Phytochemistry 2021;186:112735. [CrossRef]
31. Pamornpathomkul B, Rangsimawong W, Rojanarata T, Opanasopit P, Chaiyodsilp C, Ngawhirunpat T. Lipid-based nanocarriers to enhance skin permeation and antioxidant activity of *centella asiatica* extract. MATEC Web Conf 2018;192:1–4. [CrossRef]
32. Da Rocha PBR, Souza BS, Andrade LM, Marreto RN, Lima EM, Taveira SF. Development of a high-performance liquid chromatographic method for asiaticoside quantification in different skin layers after topical application of a *Centella asiatica* extract. Planta Med 2017;83(18):1431–7. [CrossRef]
33. Khotimah H, Setiawan A, Rita CI, Mardiyah M, Ali A, Sukatman MP, et al. In silico studies of natural compounds of *Centella asiatica* as anti-aging and wound healing agents. AIP Conf Proc 2021;2353:030031–9. [CrossRef]
34. Aziz ZA, Davey MR, Power JB, Anthony P, Smith RM, Lowe KC. Production of asiaticoside and madecassoside in *centella asiatica in vitro* and *in vivo*. Biol Plant 2007;51:34–42. [CrossRef]
35. Chin CH, Chen SH, Wu HH, Ho CW, Ko MT, Lin CY. cytoHubba: Identifying hub objects and sub-networks from complex interactomes. BMC Syst Biol 2014;8(Suppl 4):S11. [CrossRef]

36. Hartini YS, Maharani BA, Widyantara KA, Saputra BW, Setyaningsih D, Setiawati A. Molecular cascade of neolignan as a natural anti-diabetics agent: A bioinformatics approach. TJNPR 2023;7(11):5188–94. [CrossRef]
37. Azis HA, Taher M, Ahmed AS, Sulaiman WMAW, Susanti D, Chowdhury SR, et al. *In vitro* and *in vivo* wound healing studies of methanolic fraction of *Centella asiatica* extract. J S Afr Bot 2017;108:163–74. [CrossRef]
38. Huang J, Gong Y, Liu K, Chen J, Zhou X. Anti-photoaging properties of asiaticoside in ultraviolet A-irradiated human dermal fibroblasts by activating the PI3K-Akt pathway and inhibiting the NF-κB pathway. Explor Res Hypothesis Med 2023;8(4):319–37. [CrossRef]
39. Philips N, Ding X, Kandalai P, Marte I, Krawczyk H, Richardson R. The beneficial regulation of extracellular matrix and heat shock proteins, and the inhibition of cellular oxidative stress effects and inflammatory cytokines by 1α, 25 dihydroxyvitamind3 in non-irradiated and ultraviolet-radiated dermal fibroblasts. Cosmetics 2019;6(3):46. [CrossRef]
40. Popova NV, Jücker M. The functional role of extracellular matrix proteins in cancer. Cancers 2022;14(1):238. [CrossRef]
41. Freitas-Rodríguez S, Folgueras AR, López-Otín C. The role of matrix metalloproteinases in aging: Tissue remodeling and beyond. BBA Mol Cell Res 2017;1864(11):2015–25. [CrossRef]
42. Dzobo K, Dandara C. The extracellular matrix: Its composition, function, remodeling, and role in tumorigenesis. Biomimetics 2023;8(2):1–39. [CrossRef]
43. Zhou H, Li W, Pan L, Zhu T, Zhou T, Xiao E, et al. Human extracellular matrix (ECM)-like collagen and its bioactivity. Regen Biomater 2024;11:1–13. [CrossRef]
44. Zhang S, Duan E. Fighting against skin aging: The way from bench to bedside. Cell Transplant 2018;27(5):729–38. [CrossRef]
45. Pittayapruek P, Meephansan J, Prapapan O, Komine M, Ohtsuki M. Role of matrix metalloproteinases in photoaging and photocarcinogenesis. Int J Mol Sci 2016;17(6):868. [CrossRef]
46. Shiragannavar VD, Karunakara SH, Puttahanumantharayappa LD, Sannappa Gowda NG, Santhekadur PK. Unraveling key signaling pathways altered in hepatocellular carcinoma. Gene Expr 2023;22(1):28–40.
47. Wang X, Khalil RA. Matrix metalloproteinases, vascular remodeling, and vascular disease. Adv Pharmacol 2018;8:241–330. [CrossRef]
48. Kammeyer A, Luiten RM. Oxidation events and skin aging. ARR 2019;21:16–29. [CrossRef]
49. Nessler MB, Puchała J, Chrapusta A, Nessler K, Drukała J. Levels of plasma matrix metalloproteinases (MMP-2 and MMP-9) in response to INTEGRA® dermal regeneration template implantation. Med Sci Monit 2014;20:91–6. [CrossRef]
50. March JT, Golshirazi G, Cernisova V, Carr H, Leong Y, Lu-Nguyen N, et al. Targeting TGFβ signaling to address fibrosis using antisense oligonucleotides. Biomedicines 2018;6(3):1–21. [CrossRef]
51. Chen B, Li R, Yan N, Chen G, Qian W, Jiang HL, et al. Astragaloside IV controls collagen reduction in photoaging skin by improving transforming growth factor-β/Smad signaling suppression and inhibiting matrix metalloproteinase-1. Mol Med Rep 2015;1(5):3344–8. [CrossRef]
52. Quintero-Fabián S, Arreola R, Becerril-Villanueva E, Torres-Romero JC, Arana-Argáez V, Lara-Riegos J, et al. Role of matrix metalloproteinases in angiogenesis and cancer. Front Oncol 2019;9:1370. [CrossRef]
53. Wang D, Liu G, Meng Y, Chen H, Ye Z, Jing J. The configuration of GRB2 in protein interaction and signal transduction. Biomolecules 2024;14(3):259. [CrossRef]
54. Cheruku HR, Mohamedali A, Cantor DI, Tan SH, Nice EC, Baker MS. Transforming growth factor-β, MAPK and Wnt signaling interactions in colorectal cancer. EuPA 2015;8:104–15. [CrossRef]
55. Qi S, Deng S, Lian Z, Yu K. Novel drugs with high efficacy against tumor angiogenesis. Int J Mol Sci 2022;23(13):6934. [CrossRef]
56. Krstic J, Santibanez JF. Transforming growth factor-beta and matrix metalloproteinases: Functional interactions in tumor stroma-infiltrating myeloid cells. TSWJ 2014;2014:1–14. [CrossRef]
57. Jung HY, Shin JC, Park SM, Kim NR, Kwak W, Choi BH. Pinus densiflora extract protects human skin fibroblasts against UVB-induced photoaging by inhibiting the expression of MMPs and increasing type I procollagen expression. Toxicol Rep 2014;1:658–66. [CrossRef]
58. Lian GY, Wang QM, Mak TSK, Huang XR, Yu XQ, Lan HY. Inhibition of tumor invasion and metastasis by targeting TGF-β-Smad-MMP2 pathway with asiatic acid and naringenin. Mol Ther Oncolytics 2021;20:277–89. [CrossRef]
59. Cambier S, Gouwy M, Proost P. The chemokines CXCL8 and CXCL12: molecular and functional properties, role in disease and efforts towards pharmacological intervention. Cell Mol Immunol 2023;20:217–51. [CrossRef]
60. Liu JF, Chen PC, Chang TM, Hou CH. Monocyte chemoattractant protein-1 promotes cancer cell migration via c-Raf/MAPK/AP-1 pathway and MMP-9 production in osteosarcoma. J Exp Clin Cancer Res 2020;39:254. [CrossRef]
61. Li S, Lu J, Chen Y, Xiong N, Li L, Zhang J, et al. MCP-1-induced ERK/GSK-3β/Snail signaling facilitates the epithelial-mesenchymal transition and promotes the migration of MCF-7 human breast carcinoma cells. Cell Mol Immunol 2017;14:621–30. [CrossRef]
62. Guo D, Zhu W, Qiu H. C-C motif chemokine ligand 2 and chemokine receptor 2 in cardiovascular and neural aging and aging-related diseases. Int J Mol Sci 2024;25(16):8794. [CrossRef]
63. Shibuya R, Ishida Y, Hanakawa S, Kataoka TR, Takeuchi Y, Murata T, et al. CCL2-CCR2 signaling in the skin drives surfactant-induced irritant contact dermatitis through IL-1β-mediated neutrophil accumulation. JID 2022;142(3):571–82. [CrossRef]
64. Fuller B. Role of PGE-2 and other inflammatory mediators in skin aging and their inhibition by topical natural anti-inflammatories. Cosmetics 2019;6(1):6. [CrossRef]
65. Latronico T, Petraglia T, Sileo C, Bilancia D, Rossano R, Liuzzi GM. Inhibition of MMP-2 and MMP-9 by dietary antioxidants in THP-1 macrophages and sera from patients with breast cancer. Molecules 2024;29(8):1718. [CrossRef]

66. Zhang F, Wang Z, Fan Y, Xu Q, Ji W, Tian R, et al. Elevated STAT3 signaling-mediated upregulation of MMP-2/9 confers enhanced invasion ability in multidrug-resistant breast cancer cells. Int J Mol Sci 2015;16(10):24772–90. [CrossRef]
67. Ma JH, Qin L, Li X. Role of STAT3 signaling pathway in breast cancer. Cell Commun Signal 2020;83:3. [CrossRef]
68. Wang L, Guo T, Guo Y, Xu Y. Asiaticoside produces an antidepressant-like effect in a chronic unpredictable mild stress model of depression in mice, involving reversion of inflammation and the PKA/pCREB/BDNF signaling pathway. Mol Med Rep 2020;22(3):2364–72. [CrossRef]
69. Papaccio FD, D'Arino A, Caputo S, Bellei B. Focus on the contribution of oxidative stress in skin aging. Antioxidants 2022;11(6):1121. [CrossRef]
70. Kersten K, Coffelt SB, Hoogstraat M, Verstegen NJM, Vrijland K, Ciampricotti M, et al. Mammary tumor-derived CCL2 enhances pro-metastatic systemic inflammation through upregulation of IL-1β in tumor-associated macrophages. Oncoimmunology 2017;6(8):1–14. [CrossRef]
71. Mantsounga CS, Lee C, Neverson J, Sharma S, Healy A, Berus JM, et al. Macrophage IL-1β promotes arteriogenesis by autocrine STAT3- and NF-κB-mediated transcription of pro-angiogenic VEGF-A. Cell Rep 2022;38(5):1–12. [CrossRef]
72. Guo H, Liu H, Jian Z, Cui H, Fang J, Zuo Z, et al. Nickel induces inflammatory activation via NF-kB, MAPKs, IRF3 and NLRP3 inflammasome signaling pathways in macrophages. Aging 2019;11(23):11659–72. [CrossRef]
73. Samavati L, Rastogi R, Du W, Hüttemann M, Fite A, Franchi L. STAT3 tyrosine phosphorylation is critical for interleukin-1β and interleukin-6 production in response to lipopolysaccharide and live bacteria. Mol Immunol 2009;46(8–9):1867–77. [CrossRef]
74. Ahmed IA, Mikail MA, Zamakshshari NH, Mustafa MR, Hashim NM, Othman R. Trends and challenges in phytotherapy and phytocosmetics for skin aging. Saudi J Biol Sci 2022;29(8):103363. [CrossRef]
75. Thomas D, Apovian C. Macrophage functions in lean and obese adipose tissue. Metab Clin Exp 2017;72:120–43. [CrossRef]
76. Xu M, Wang Y, Xia R, Wei Y, Wei X. Role of the CCL2-CCR2 signaling axis in cancer: Mechanisms and therapeutic targeting. Cell Prolif 2021;54(10):1–17. [CrossRef]
77. Chen X, Guo W, Chang Y, Chen J, Kang P, Yi X, et al. Oxidative stress-induced IL-15 trans-presentation in keratinocytes contributes to CD8+ T cells activation via JAK-STAT pathway in vitiligo. Free Radic Biol Med 2019;139:80–91. [CrossRef]
78. Napoli S, Scuderi C, Gattuso G, Bella VD, Candido S, Basile MS, et al. Functional roles of matrix metalloproteinases and their inhibitors in melanoma. Cells 2020;9(5):1–24. [CrossRef]
79. Boukhedouni N, Martins C, Darrigade AS, Drullion C, Rambert J, Barrault C, et al. Type-1 cytokines regulate MMP-9 production and E-cadherin disruption to promote melanocyte loss in vitiligo. JCI Insight 2020;5(11):e133772. [CrossRef]
80. Umbayev B, Askarova S, Almabayeva A, Saliev T, Masoud AR, Bulanin D. Galactose-induced skin aging: The role of oxidative stress. Oxid Med Cell 2020;2020:23–6. [CrossRef]
81. Das S, Amin SA, Jha T. Inhibitors of gelatinases (MMP-2 and MMP-9) for the management of hematological malignancies. Eur J Med Chem 2021;223:113623. [CrossRef]
82. Pandey G, Kuykendall AT, Reuther GW. JAK2 inhibitor persistence in MPN: uncovering a central role of ERK activation. Blood Cancer J 2022;12(13):1–13. [CrossRef]
83. Favaro F, Luciano-Mateo F, Moreno-Caceres J, Hernández-Madrigal M, Both D, Montironi C, et al. TRAIL receptors promote constitutive and inducible IL-8 secretion in non-small cell lung carcinoma. Cell Death Dis 2022;13(12):1–12. [CrossRef]
84. Campbell LM, Maxwell PJ, Waugh DJJ. Rationale and means to target pro-inflammatory interleukin-8 (CXCL8) signaling in cancer. Pharmaceuticals 2013;6(8):929–59. [CrossRef]
85. Cherukupalli S, Karpoormath R, Chandrasekaran B, Hampannavar GA, Thapliyal N, Palakollu VN. An insight on synthetic and medicinal aspects of pyrazolo[1,5-a]pyrimidine scaffold. Eur J Med Chem 2017;126:198–352. [CrossRef]
86. Yadav PS, Papaioannou G, Kobelski MM, Demay MB. Phosphate-induced activation of VEGFR2 leads to caspase-9-mediated apoptosis of hypertrophic chondrocytes. iScience 2023;26(9):1–15. [CrossRef]

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Research Article

Reducing oxidative stress and enhancing antioxidant defenses for faster healing in diabetic wounds: The role of topical metformin

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Abstract

Objectives: Hyperglycemia, one of the most important metabolic indicators of diabetes, causes increased oxidative stress and inflammation both systemically and locally at the tissue level, particularly in chronic wound sites where healing is impaired. Increased oxidative stress products are controlled by the body's antioxidant capacity. Oxidative damage develops as a result of excessive production or improper quenching of reactive oxygen species (ROS) and is an important cause of non-healing chronic wounds. We aimed to accelerate wound healing by increasing the antioxidant capacity of oxidative damage caused by diabetes by applying metformin, which is routinely used orally, topically on wounds.

Methods: For this purpose, we applied metformin on diabetic and non-diabetic wounds for 14 days and measured oxidative stress markers malondialdehyde (MDA), total antioxidant status (TAS), total oxidant status (TOS) levels, and antioxidant levels of glutathione (GSH) and catalase (CAT) in wound samples obtained by biopsy using ELISA technique.

Results: The markers of oxidative stress increased in untreated diabetic rats because of hyperglycemia, the most important clinical marker of diabetes, compared to non-diabetic rats. In contrast, our results showed that metformin administration decreased oxidative stress markers and increased antioxidant levels compared to controls.

Conclusion: As a result, it has been revealed that topically applied metformin can minimize oxidative damage caused by hyperglycemia by increasing antioxidant capacity, especially in diabetic wounds, and thus wounds heal faster by controlling oxidative stress in wound healing.

Keywords: Antioxidant, diabetes mellitus, metformin, oxidative stress, wound healing

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Diabetes is a metabolic disorder characterized by hyperglycemia due to insulin resistance, insufficient insulin secretion, or both [1]. It has been shown that hyperglycemia acts through pathways that include the polyol/aldose reduction pathway, the advanced glycation end product (AGE) pathway, the reactive oxygen species (ROS) pathway, and the protein kinase-C (PKC) pathway [2]. These pathways produce oxidant and inflammatory mediators that cause damage both locally in tissues and systemically. Inflammatory and oxidant pathways contribute to the pathogenesis of diabetic complications. Hyperglycemia is known to cause intracellular oxidative stress, and ROS that result from oxidative stress cause more damage to cells and delay healing in chronic or impaired acute wounds [3–5].

During wound healing, inflammatory cells, including macrophages, neutrophils, endothelial cells and fibroblasts, produce active oxygen and free radicals [6]. While adequate amounts of free radicals promote wound healing, too much

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active oxygen suppresses the migration and proliferation of repairing cells, inhibits extracellular matrix synthesis, and ultimately delays wound healing [7, 8]. Experimental and clinical studies have shown that delay in diabetic wounds increases cellular damage by excessive ROS production via the glucose autooxidation pathway, and this delayed wound healing is associated with oxidative stress [9–14].

In recent years, a variety of agents have been introduced to reduce the oxidant level and accelerate the wound healing process by increasing the antioxidant level [15–24]. Metformin, a biguanide derivative has been used for over 60 years in the treatment of the early stages of type 2 diabetes due to its ability to lower plasma glucose levels. Studies have been conducted to show its effectiveness against other diseases, including cancer (breast cancer, endometrial cancer, bone cancer, colorectal cancer and melanoma), obesity, liver diseases, cardiovascular diseases kidney diseases and even ageing, but the mechanisms underlying these different benefits remain unclear [25]. *In vitro* and *in vivo* studies have shown that metformin has a protective effect against oxidative damage caused by hyperglycemia and inhibits the expression of pro-inflammatory cytokines [26–28]. However, studies have yet to identify the effect of metformin on oxidative stress in wound healing.

It has been reported that a decrease in antioxidant levels and an increase in oxidant levels are important causes of delayed wound healing in diabetes [29, 30]. In this study, we investigated how to reverse the oxidative stress effects of hyperglycemia, one of the main complications of diabetes, on wounds on the 3rd, 7th and 14th days, which are the critical days in the wound healing process, by using topical metformin with its possible antioxidative effects. These days represent key stages in the wound healing process: inflammation (day 3), proliferation (day 7), and remodeling (day 14). During these phases, oxidative stress levels and antioxidant defenses exhibit dynamic changes. Day 3 is marked by high oxidative stress levels due to inflammatory cell activity, day 7 shows increasing antioxidant defenses supporting tissue regeneration, and by day 14, oxidative stress declines significantly while antioxidant systems peak to facilitate wound remodeling and closure [5, 6]. Current literature suggests that metformin may regulate inflammatory processes by suppressing important markers of oxidative stress, but the specific mechanisms in wound healing are still not fully elucidated. Our study shows that metformin strengthens antioxidant defense mechanisms by reducing oxidative stress load and thus accelerates wound healing. Thus, by applying metformin, which is known for its oral use, locally on the wound, we presented the potential to reduce the level of oxidative stress biomolecules and accelerate the wound healing process by increasing the level of antioxidative biomolecules. These findings emphasize that metformin may play a critical role not only in glucose regulation but also in cellular stress responses and offer a new treatment approach for wound healing. Topical use of metformin stands out as a therapeutic alternative for the wound healing process, especially with the advantage of avoiding systemic side effects.

Materials and Methods

Study design

An application was made to the Animal Experiments Local Ethics Committee of Bezmialem Vakif University to investigate the oxidative mechanism biomarker levels in tissues from experimental animals obtained in another research project. Approval was granted on 06.07.2022 according to committee decision E.69264. The study was designed in accordance with the Helsinki Declaration.

Tissue samples were taken from a previous study conducted by our group [31]. In the study, CG defines the control group, CT defines the healthy treatment group administered metformin, DCG defines the diabetic control group, and DTG defines the diabetic treatment group administered metformin. Each experimental group consisted of 6 rats. Physiological saline was applied to the control wounds and 3mM metformin was applied to the treatment groups by being absorbed into surgical sponges for 14 days. The absorption of metformin was standardized separately by pre-soaking the sponges in a metformin solution of defined concentration and volume, ensuring consistent dosing across all treatment groups. Levels of oxidative and antioxidative markers were investigated in wound tissues taken on days 0, 3, 7, and 14.

Tissue sampling and homogenization

Tissue samples from rats (n=24) were weighed, and then placed in polypropylene tubes; 1 mL of phosphate buffer (PBS, pH 7.4) was added to the samples of equal weight. Homogenization was carried out via homogenizer at 30 m/s for 5 minutes (FastPrep-24 homogenizer, MP Biomedicals, New Zealand). The homogenates were centrifuged at 10,000 x rpm for 15 minutes. Total protein quantitation was measured using the Bradford method [32]. All homogenates were stored at -80°C.

Oxidative stress marker assays

Total Antoxidant Status (TAS) and Total Oxidant Status (TOS) measurements

TAS and TOS levels were measured by spectrophotometry using commercial kits (Rel Assay, Türkiye). Antioxidants in the sample converted the dark blue-green ABTS radical to its colourless reduced form. The 660 nm absorbance change (Perkin Elmer, 1420 Victor 3 instrument, USA) was related to the total antioxidant level of the sample. Total antioxidant activity was expressed in mmol Trolox eq/L of samples. Oxidants present in the sample oxidized the ferrous ion chelator complex to ferric ion. The oxidation reaction is prolonged by enhancer molecules, which are abundantly present in the reaction medium. The ferric ion formed a colour complex with chromogen in an acidic medium. The colour intensity was related to the total amount of oxidant molecules present in the sample. Total oxidant activity was expressed in terms of μm H_2O_2 eq/L.

Determination of oxidative stress index (OSI)

The TOS to TAS ratio was used to calculate the oxidative stress index (OSI). The resulting TAS unit was converted to

mmol/l, and the OSI value was calculated according to the following formula [33]:

OSI (arbitrary unit) = TOS (μmol H_2O_2 eq/L) / TAS (mmol Trolox eq/L)

Catalase (CAT), Glutathione (GSH), and Malondialdehyde (MDA) measurements

Homogenates were thawed and Rat CAT ELISA kit (Mybiosource inc., USA, Cat Num: MBS726781), Rat GSH ELISA kit (Mybiosource inc., USA, Cat Num: MBS1600118) and Rat MDA ELISA kit (Mybiosource inc., USA, Cat Num: MBS738685 were used to quantitatively measure tissue sample levels. Briefly; tissue homogenates and standards were added to wells precoated with Anti-Rat monoclonal antibody before incubation; then biotin was added to all wells with Streptavidin-HRP to form the immune complex. Subsequent incubation and washing removed the uncombined enzyme. Chromogen Solutions A and B were added for the colour of the liquid changes into the blue in plates. At the effect of acid, the colour finally became yellow. A standard automated plate reader at 450 nm (Perkin Elmer, 1420 Victor3) was used to read optical density. The detection range of kits is between 0–50 ng/mL for CAT; 20–1600 mg/mL for glutathione and 0–1000 ng/mL for MDA. Samples were prepared at 4°C. Additionally, antioxidant and oxidant measurements were taken from all tissues simultaneously and without waiting, immediately after the homogenization of the tissues.

Statistical analysis

Statistical analysis was performed by using the software GraphPad Prism 8 version 8.4.3. The experimental data were expressed as mean ± standard deviation (SD). Statistical differences were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey test for pairwise comparisons. $p<0.05$ was considered statistically significant.

Results

TAS and TOS levels

TAS levels were measured on days 3, 7 and 14 of the wound healing process (Table 1). On day 3, no significant difference was found between the treatment groups of the control and diabetic groups. When the 7th-day TAS results were evaluated, the DTG group did show a significant increase compared to its control (DCG) (p=0.0011). Results obtained on the 14th day of wound healing were similar to results obtained on the 7th day. The DTG group showed a significant increase (p=0.0001) compared to its control, while the CT group showed no significant difference compared to its control.

TOS levels also showed significant changes over the healing period (Table 1). On day 3, the CT group and DTG group significantly decreased compared to their respective controls (p<0.0001, p<0.0001, respectively). Similar to the results of the 3rd day, the CT and DTG groups showed a significant decrease compared to their controls on the 7th day (p=0.001, p=0.0275, respectively). On the 14th day, unlike the other treatment days, only the diabetic treatment group showed significant improvement on day 14, highlighting the prolonged effect of metformin in diabetic wounds (p=0.0169).

Oxidative stress index (OSI)

The oxidative stress index (OSI) on the 3rd, 7th and 14th days of wound healing was recorded for the groups (Table 1, Fig. 1). On the 3rd and 7th days, the OSI level in the CT and DTG groups showed significance compared to the controls (p=0.0009, p=0.0007, p=0,0456, p=0.0002 respectively). While the CT group did not show a significant difference compared to the control on the 14th day, the DTG group continued its significant decrease compared to the control on the last day of treatment (p<0.0001).

MDA levels

MDA levels were recorded on the 3rd, 7th and 14th days of wound healing (Table 1, Fig. 1). When the CT group was compared to its control and the diabetic treatment group was compared to its control, both were found to be significantly reduced on the 3rd and 7th days (p<0.0001, p<0.0001, p=0.001, p=0.0273, respectively). A significant decrease was found comparing the diabetic treatment group on the 14th day to its control (p=0.0168). On day 14, the CT group did not show significant differences compared to its control, indicating limited effects of metformin in non-diabetic wounds.

Glutathione levels

Glutathione values (Table 1, Fig. 1) in the CT group were not significantly different compared to their controls in each other every three days. The metformin-treated diabetic group increased significantly on days 3, 7, and 14 compared to its control (p=0.0054, p<0.0001, p<0.0001, respectively).

CAT levels

The catalase values (Table 1, Fig. 1) in the health treatment group showed no significant difference compared to their controls in each other every 3 days. The diabetic group treated with metformin increased significantly on days 3, 7, and 14 compared to its control (p=0.0054, p<0.0001, p<0.0001, respectively).

Discussion

Oxidative stress arises from an imbalance between the production of reactive oxygen species (ROS) and the compensatory mechanisms of the endogenous antioxidant system. Antioxidant enzymes serve as the primary defense against the detrimental effects of free radicals. Key enzymes in this process include superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glucose-6-phosphate dehydrogenase, which are abundant in the skin and play a crucial role in counteracting the harmful effects of excess ROS during the wound healing process [34]. In diabetic individuals, this antioxidant activity diminishes further as oxidative stress escalates, thereby increasing susceptibility to the damaging effects of free radicals [35]. In our study, catalase (CAT) for enzymatic antioxidant defense,

Table 1. TAS, TOS, calculated OSI, MDA, GSH and CAT levels of biopsy samples taken on days 0, 3, 7 and 14

Parameters	Days	Groups				
		CG	CT	DCG	DTG	p
Total oxidant status (TOS)	Day 0	6.953±0.8431		9.093±0.8624***		0.0002
	Day 3	9.38±0.4812	7.343±0.5698†	10.28±0.467§	8.359±0.63†,¶	<0.0001
	Day 7	9.655±0.6158	8.327±0.2775†	9.958±0.4901	9.058±0.5704†	<0.0001
	Day 14	8.852±0.8525	7.978±0.05675	9.522±0.467	8.528±0.2562†	0.0169
Total antioxidant status (TAS)	Day 0	1.101±0.04483		0.845±0.04806****		<0.0001
	Day 3	0.9517±0.07468	1.03±0.07127	0.77±0.09165§	0.7886±0.085¶	<0.0001
	Day 7	0.8433±0.06088	0.9233±0.8618	0.6917±0.0801§	0.9067±0.098†	0.0003
	Day 14	0.8833±0.07339	0.946±0.05727	0.6417±0.088§	0.8967±0.092†	<0.0001
Oxidative stress index (OSI)	Day 0	6.34±0.8751		10.75±0.6992****		<0.0001
	Day 3	9.917±1.033	7.162±0.853†	13.52±1.726§	10.7±1.578†,¶	<0.0001
	Day 7	11.57±1.483	9.09±0.8337†	14.63±2.244§	10.08±1.082†	<0.0001
	Day 14	10.03±0.9919	8.446±0.4998	15.1±2.37§	9.614±1.143†	<0.0001
Malondialdehyde (MDA)	Day 0	387.2±46.95		506.2±47.99***		0.0002
	Day 3	522.1±26.82	408.7±31.64†	572.4±25.88§	465.3±35.24†,¶	<0.0001
	Day 7	537.6±34.21	463.5±15.38†	554.4±27.32	504.3±31.81†	<0.0001
	Day 14	492.6±47.41	444.1±3.248	530.1±26.04	474.8±14.2†	0.0008
Glutathione (GSH)	Day 0	1205±209.7		755.5±244.6**		0.0016
	Day 3	880.7±207.9	985.1±190.4	474.5±70.45§	783.1±67.65†	<0.0001
	Day 7	764.2±107.9	771.3±106.4	318.2±91.25§	707.5±107.6†	<0.0001
	Day 14	751.6±39.36	828.4±41.11	442.2±46.63§	827.1±71.9†	<0.0001
Catalase (CAT)	Day 0	45.19±8.499		26.98±9.912**		0.0016
	Day 3	32.06±8.428	36.29±7.713	15.59±2.856§	28.1±2.741†	<0.0001
	Day 7	27.34±4.373	27.62±4.315	9.26±3.699§	25.04±4.362†	<0.0001
	Day 14	26.83±1.595	29.94±1.666	14.02±2.236§	29.48±3.25†	<0.0001

Significant levels of non-diabetic and diabetic groups treated with metformin compared to their controls on days 3, 7, and 14 are shown with the † symbol. In addition, the results of the non-diabetic and diabetic control groups without treatment were evaluated with the symbol § among themselves on the 3rd, 7th, and 14th days, and the results of the non-diabetic and diabetic treatment groups treated with metformin were evaluated with the symbol ¶ among themselves on the 3rd, 7th, and 14th days. The comparison of the diabetic groups to the non-diabetic group on day 0, which is the day the wound first opened, is marked with the * symbol (*p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001). CG: Untreated non-diabetic group; CT: Non-diabetic group treated with metformin; DCG: Untreated diabetic group; DTG: Diabetic group treated with metformin.

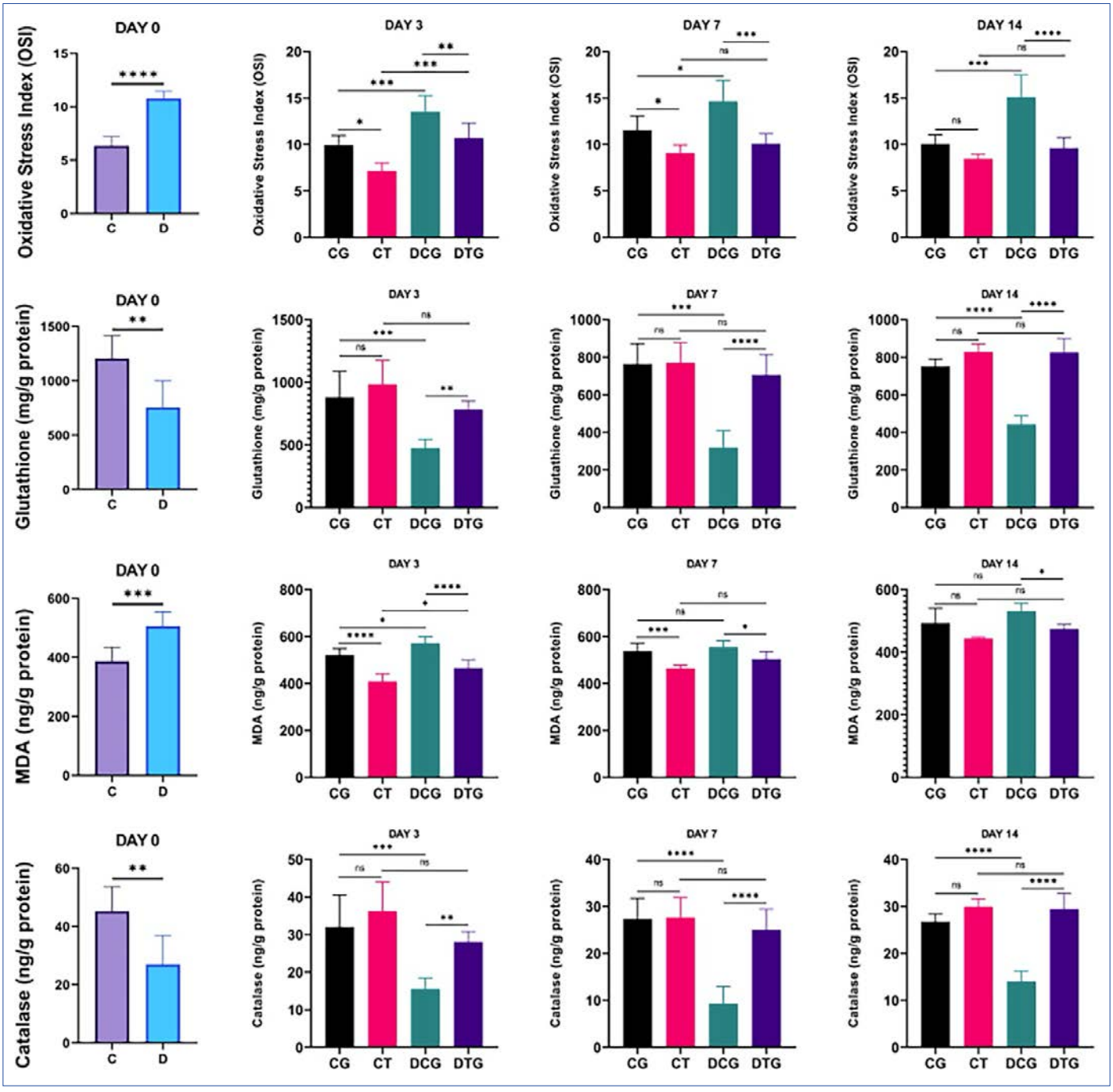

Figure 1. Comparative display of calculated OSI, MDA, GSH and CAT levels of biopsy samples taken on days 0, 3, 7 and 14.

ns: No significant; *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001. CG: Untreated non-diabetic group; CT: Non-diabetic group treated with metformin; DCG: Untreated diabetic group; DTG: Diabetic group treated with metformin; OSI: Oxidative stress index; MDA: Malondialdehyde; GSH: Glutathione; CAT: Catalase.

glutathione (GSH) for non-enzymatic antioxidant capacity, and malondialdehyde (MDA) for lipid peroxidation were selected as they represent the key mechanisms in the oxidative stress pathway. CAT and GSH are primary antioxidants that neutralize reactive oxygen species (ROS) and protect cellular structures, while MDA is a well-known marker of lipid peroxidation indicating oxidative damage to cell membranes [24]. Collectively, these markers provide a comprehensive view of the oxidative-antioxidative balance in wound tissues.

Metformin, an established medication for type 2 diabetes, has garnered attention for its antioxidant properties, particularly through its activation of the AMP-activated protein kinase (AMPK) pathway. This activation enhances the expression of various antioxidant enzymes, thereby improving the body's ability to combat oxidative stress. The antioxidant effects of metformin are particularly significant in tissues where oxidative stress is prevalent, as it reduces reactive oxygen species (ROS) levels and improves overall antioxidant capacity [36,

37]. In both *in vitro* and *in vivo* studies, metformin has demonstrated a protective effect against oxidative damage induced by hyperglycemia and has been shown to inhibit the expression of pro-inflammatory cytokines [26–28]. While metformin is commonly administered orally, recent studies highlight the potential benefits of topical applications. Topical metformin has been shown to provide more effective localized healing that may exceed that achieved through oral administration, particularly in conditions such as diabetic wounds [38–40]. By delivering metformin directly to the affected tissue, this approach can effectively mitigate oxidative damage, enhance healing processes, and reduce systemic side effects associated with oral dosing. The localized application also might allow for higher concentrations of metformin at the site of injury, further promoting antioxidant activity and potentially accelerating the wound healing process. In summary, the antioxidant properties of metformin, combined with its effective topical application, present a promising avenue for enhancing therapeutic outcomes in oxidative stress-related conditions, including diabetic wounds. This approach not only utilizes metformin's inherent benefits but also maximizes its efficacy by targeting the specific areas of need directly. Our findings indicate that the topical application of metformin effectively reduces oxidative stress in diabetic wounds while concurrently elevating antioxidant levels.

Antioxidants are recognized for their protective effects against free radicals. Total antioxidant status (TAS) analysis serves as an important measure of tissue antioxidant capacity. The synergistic effects of various antioxidants provide enhanced defense against reactive oxygen and nitrogen species compared to individual compounds. Additionally, the oxidative stress index (OSI)—the ratio of total oxidant status (TOS) to TAS—reflects the oxidative stress level and redox balance within tissues [41, 42]. In wound model studies, such as those using Aloe vera and Hypericum perforatum (HPO), significant increases in TAS and non-significant changes in TOS have been observed in diabetic treatment groups, alongside reduced OSI levels compared to diabetic controls [43]. Our study similarly shows significant decreases in TOS in both non-diabetic and diabetic scar tissues on the 3rd and 7th days post-treatment, with comparable trends on the 14th day specifically in the diabetic group. The relative decrease in TOS was more pronounced in the diabetic treatment group, reflecting their higher baseline oxidative stress levels. This indicates that the treatment was more effective in the diabetic cohort, likely due to the elevated oxidative stress associated with diabetes. Moreover, TAS results revealed notable increases in antioxidant levels in diabetic wounds treated with metformin on the 7th and 14th days compared to controls, while no significant differences were observed in non-diabetic wounds. Interestingly, total antioxidant levels were higher in the diabetic group receiving metformin during later stages of healing. OSI values, indicating oxidative stress, significantly decreased in both diabetic and non-diabetic treatment groups on the 3rd and 7th days, with significant differences persisting only in the diabetic treatment group on the 14th day. These findings emphasize the efficacy of topical metformin application in reducing oxidative stress specifically in diabetic wounds compared to non-diabetic wounds, highlighting its therapeutic potential in enhancing wound healing. The increased efficacy in diabetic wounds may be attributed to elevated oxidative stress levels in diabetes, which make the antioxidant effects of metformin more impactful.

High levels of reactive oxygen species (ROS) lead to lipid oxidation, resulting in malondialdehyde (MDA) accumulation, which serves as a well-established biomarker for oxidative stress and cell membrane damage [44, 45]. Research by Yang et al. [46] indicates that increased oxidative stress delays wound healing, underscoring the importance of limiting ROS formation in diabetic patients to facilitate repair processes. Our findings corroborate these observations, demonstrating that elevated oxidative stress adversely affects the antioxidant/oxidant ratio, thereby hindering wound healing. Lima et al. [47] reported that Galactomannan GM-DR, known for its anti-inflammatory properties, reduced MDA levels by 44% on the 2nd day compared to controls administered saline, with no changes observed on the 5th and 14th days [47]. Additionally, studies utilizing diabetic rat wound models have shown that topical applications of bilirubin and deferoxamine significantly lowered MDA levels from day 7 to day 19, while topical quercetin showed similar effects on days 3 and 14 [48, 49]. Consistent with our findings, Assar et al. [50] observed a notable decrease in MDA levels in groups treated with licorice extract compared to untreated controls [50]. In our study, we compared each diabetic treatment group with their respective control groups and observed significant reductions in MDA levels, particularly on the 3rd and 7th days. The decrease in MDA levels across all treatment days suggests that the treatment was effective, especially on the 3rd day, likely due to the inflammatory response integral to the wound healing process. This highlights the potential of targeting oxidative stress to enhance healing outcomes in diabetic wounds.

Catalase, an enzyme prevalent in most aerobic cells, is essential for mitigating oxidative stress by catalyzing the rapid decomposition of hydrogen peroxide (H_2O_2) through its peroxidative and catalytic activities [51]. In both acute and chronic wounds, the activity of enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase is often diminished due to elevated oxidative stress, which can overwhelm these antioxidants when released in significant quantities. Furthermore, high oxidative stress contributes to the depletion of non-enzymatic antioxidants, including vitamins E and C, as well as glutathione. This effect is more pronounced in chronic wounds compared to acute ones, indicating that antioxidant supplementation may help prevent cellular oxidative damage and enhance healing outcomes [52]. In a study involving diabetic rats, Afzali et al. [53] reported decreased levels of glutathione (GSH) alongside unaffected catalase activity and total antioxidant status (TAS) on the 7th day following treatment with acidic nitrite, which contradicted our findings. They also noted that these

parameters showed no significant changes on the 14th, 21st, and 28th days post-injury. Conversely, consistent with our results, other studies indicated that topical quercetin significantly increased CAT levels in diabetic wounds only on day 14 compared to controls, while bilirubin-deferoxamine treatment elevated CAT levels on days 3, 7, 14, and 19 compared to controls [48, 49]. Our study demonstrated that, although metformin treatment increased CAT levels in non-diabetic groups, the difference was not statistically significant. However, in the diabetic treatment group, there was a significant and consistent linear increase in CAT levels compared to controls across all biopsy days. This pronounced increase in the diabetic group suggests a substantial reduction in CAT levels due to diabetes, with metformin effectively reversing this decline and thereby widening the gap in CAT levels compared to the non-diabetic group. This reinforces the potential of metformin as a therapeutic agent to enhance antioxidant defenses in diabetic wound healing.

The intracellular redox buffer glutathione (GSH), a thiol antioxidant, plays a crucial role as a direct free radical scavenger, a co-substrate for glutathione peroxidase, and a cofactor for various enzymes [54, 55]. A significant decrease in this non-enzymatic antioxidant increases susceptibility to oxidative stress [56, 57]. When utilized to alleviate oxidative stress, there is a possibility that GSH levels may decrease in diabetic rats [58]. Lima et al. [47] reported that in rats treated with Galactomannan GM-DR, GSH levels increased by 25% on the 2nd day and 50% on the 5th day compared to controls administered saline, although no change was observed on the 7th day. Similarly, Assar et al. [50] demonstrated a significant increase in GSH content in rats treated with licorice extract, indicating an improved antioxidant status compared to untreated controls. These findings align closely with our results, as the GSH level in our study mirrored the CAT levels noted by Aneesha et al. [49], who found significantly elevated GSH levels in diabetic wounds treated with bilirubin-deferoxamine on the 3rd, 14th, and 19th days compared to the control group. Conversely, Kant et al. [48] investigated the effects of quercetin in a diabetic rat wound model and reported no significant differences in GSH levels across groups, except on day 14, where an increase was noted [48]. These contrasting findings emphasize the variability in GSH response depending on the treatment and underline the importance of maintaining adequate GSH levels to counteract oxidative stress during wound healing.

Metformin not only regulates glucose levels but also draws attention with its oxidative stress reducing effects. In recent studies, the antioxidative properties of metformin have been emphasised and these properties have been shown to strengthen cellular defence mechanisms. In recent years, the effects of systemic metformin on antioxidant enzymes and oxidative stress markers have been investigated in rodents with hyperglycaemia [59], diabetic and hepatocellular carcinoma (HCC) [60], diabetic kidney [61] and non-alcoholic fatty liver disease (NAFLD) [62] models. All these studies reveal that metformin both helps to alleviate the oxidative stress burden and is effective in maintaining cellular redox balance by increasing antioxidant activity. Thus, organ damage can be reversed. Although the current literature shows that metformin can regulate inflammatory processes by suppressing important markers of oxidative stress, the specific oxidative stress and antioxidant mechanisms in wound healing are still not fully clarified. These findings are consistent with the results of our study and show that topical application of metformin is effective in reducing oxidative stress and increasing antioxidant levels in diabetic wounds and fill this gap in the literature. In particular, the decrease in MDA levels, an important component of oxidative stress, and the increase in antioxidant enzymes confirm that metformin shows a protective effect against oxidative damage. While this study focused on CAT, GSH, and MDA, further studies are needed to evaluate the potential effects of metformin on other enzymes not directly involved in wound healing.

Studies on the effect of metformin on diabetic wounds have gained momentum in recent years, and its role on oxidative stress in particular has attracted great attention. However, there are still points to be clarified on this subject. Our study makes a significant contribution to this gap by focusing on the role of metformin in reducing oxidative stress in diabetic wounds. In particular, topical metformin application can increase antioxidant capacity in wounded tissues while avoiding systemic side effects, and this innovative approach makes a significant contribution to the literature. In addition, a new contribution of our study is that we present the effect of metformin on wound healing not only through biochemical parameters, but also by taking into account oxidative stress load. This allows us to better understand how metformin modulates not only glucose levels but also cellular stress responses. Although it is known in the literature that metformin accelerates wound healing, our study fills an important gap in this field by addressing in detail its effects on oxidative stress and antioxidant defense mechanisms.

Streptozotocin-induced diabetes is one of many factors that cause delayed wound healing because it increases oxidative stress at the wound site. In our previous study, we evaluated the wound healing process through macroscopic assessment and demonstrated that treatment with metformin resulted in faster healing in diabetic groups [31]. Our present study showed that topically applied metformin significantly decreased MDA and TOS levels in wounds, especially diabetic wounds, while it significantly increased the levels of enzymatic or non-enzymatic antioxidants such as TAS, glutathione, and catalase (Fig. 2). Metformin scavenges free radicals, which are more prevalent in the wounds of diabetic rats than in healthy tissue, increasing antioxidant activity at these sites. In conclusion, the results of our current study show that topical metformin application supports wound healing by increasing the capacity of antioxidants, especially in diabetic wounds, with the reduction of oxidants. Large-scale clinical trials of metformin are necessary before they can be recommended.

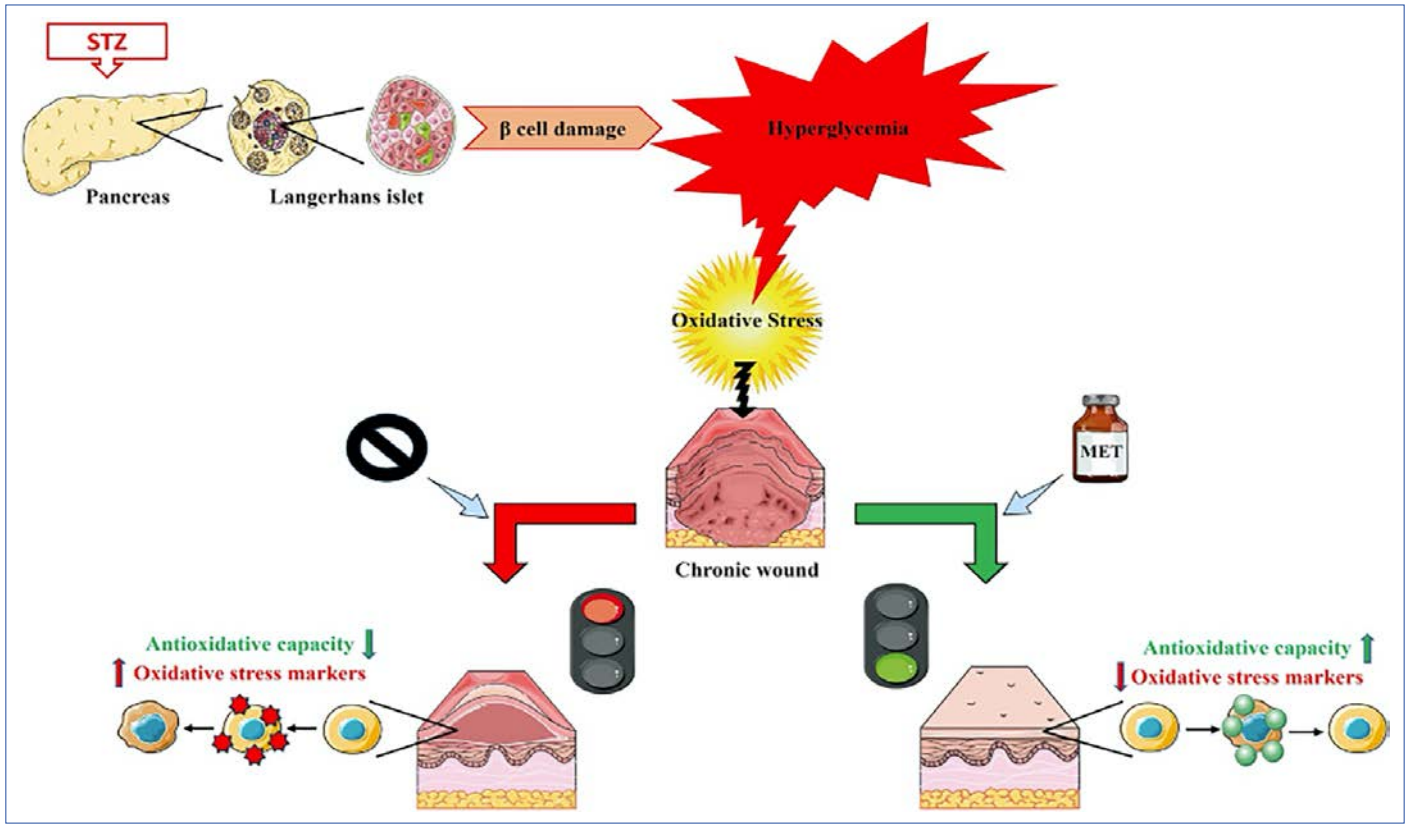

Figure 2. As a result of creating a full-thickness excisional wound model in rats in which we induced diabetes with STZ and treated them with topical metformin for fourteen days, we showed that topical metformin application increased antioxidant capacity by reducing oxidative stress biomarkers in the biopsy samples taken on days 0, 3, 7 and 14, thus improving the wound healing process.

STZ: Streptozotocin; MET: Metformin.

Ethics Committee Approval: The study was approved by The Bezmialem Vakif University Animal Experiments Local Ethics Committee (No: E.69264, Date: 06/07/2022).

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Conflict of Interest: The authors declare that there is no conflict of interest.

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References

1. American Diabetes Association. 2. Classification and diagnosis of diabetes: Standards of medical care in diabetes-2018. Diabetes Care 2018;41(Suppl 1):13–27. [CrossRef]
2. Marques-Vidal P, Schmid R, Bochud M, Bastardot F, von Kanel R, Paccaud F, et al. Adipocytokines, hepatic and inflammatory biomarkers and incidence of type 2 diabetes The CoLaus study. PLoS One 2012;7(12):e51768. [CrossRef]
3. Kengne AP, Batty GD, Hamer M, Stamatakis E, Czernichow S. Association of C-reactive protein with cardiovascular disease mortality according to diabetes status: Pooled analyses of 25,979 participants from four U.K. prospective cohort studies. Diabetes Care 2012;35(2):396–403. [CrossRef]
4. Schiekofer S, Andrassy M, Chen J, Rudofsky G, Schneider J, Wendt T, et al. Acute hyperglycemia causes intracellular formation of CML and activation of ras, p42/44 MAPK, and nuclear factor kappaB in PBMCs. Diabetes 2003;52(3):621–33. [CrossRef]
5. Schäfer M, Werner S. Oxidative stress in normal and impaired wound repair. Pharmacol Res 2008;58(2):165–71. [CrossRef]
6. Sorg H, Tilkorn DJ, Hager S, Hauser J, Mirastschijski U. Skin wound healing: An update on the current knowledge and concepts. Eur Surg Res 2017;58(1–2):81–94. [CrossRef]
7. Dunnill C, Patton T, Brennan J, Barrett J, Dryden M, Cooke J, et al. Reactive oxygen species (ROS) and wound healing: The functional role of ROS and emerging ROS-modulating technologies for augmentation of the healing process. Int Wound J 2017;14(1):89–96. [CrossRef]
8. André-Lévigne D, Modarressi A, Pepper MS, Pittet-Cuénod B. Reactive oxygen species and NOX enzymes are emerging as key players in cutaneous wound repair. Int J Mol Sci 2017;18(10):2149. [CrossRef]

9. Ren J, Yang M, Xu F, Chen J, Ma S. Acceleration of wound healing activity with syringic acid in streptozotocin-induced diabetic rats. Life Sci 2019;233:116728. [CrossRef]
10. Deng L, Du C, Song P, Chen T, Rui S, Armstrong DG, et al. The role of oxidative stress and antioxidants in diabetic wound healing. Oxid Med Cell Longev 2021;2021:8852759. [CrossRef]
11. Dworzański J, Strycharz-Dudziak M, Kliszczewska E, Kiełczykowska M, Dworzańska A, Drop Bet al. Glutathione peroxidase (GPx) and superoxide dismutase (SOD) activity in patients with diabetes mellitus type 2 infected with Epstein-Barr virus. PLoS One 2020;15(3):e0230374. [CrossRef]
12. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature 2001;414(6865):813–20. [CrossRef]
13. Kowluru RA, Chan PS. Oxidative stress and diabetic retinopathy. Exp Diabetes Res 2007;2007:43603. [CrossRef]
14. Cano Sanchez M, Lancel S, Boulanger E, Neviere R. Targeting oxidative stress and mitochondrial dysfunction in the treatment of impaired wound healing: A systematic review. Antioxidants Basel 2018;7(8):98. [CrossRef]
15. Parihar A, Parihar MS, Milner S, Bhat S. Oxidative stress and anti-oxidative mobilization in burn injury. Burns 2008;34(1):6–17. [CrossRef]
16. Serarslan G, Altuğ E, Kontas T, Atik E, Avci G. Caffeic acid phenethyl ester accelerates cutaneous wound healing in a rat model and decreases oxidative stress. Clin Exp Dermatol 2007;32(6):709–15. [CrossRef]
17. Ahmed OM, Mohamed T, Moustafa H, Hamdy H, Ahmed RR, Aboud E. Quercetin and low-level laser therapy promote wound healing process in diabetic rats via structural reorganization and modulatory effects on inflammation and oxidative stress. Biomed Pharmacother 2018;101:58–73. [CrossRef]
18. Vendidandala NR, Yin TP, Nelli G, Pasupuleti VR, Nyamathulla S, Mokhtar SI. Gallocatechin-silver nanoparticle impregnated cotton gauze patches enhance wound healing in diabetic rats by suppressing oxidative stress and inflammation via modulating the Nrf2/HO-1 and TLR4/NF-κB pathways. Life Sci 2021;286:120019. [CrossRef]
19. Gürünlüoğlu K, Demircan M, Taşçı A, Üremiş MM, Türköz Y, Bağ HG et al. The effects of two different burn dressings on serum oxidative stress indicators in children with partial burn. J Burn Care Res 2019;40(4):444–50. [CrossRef]
20. Kaltalioglu K, Coskun-Cevher S, Tugcu-Demiroz F, Celebi N. PDGF supplementation alters oxidative events in wound healing process: A time course study. Arch Dermatol Res 2013;305(5):415–22. [CrossRef]
21. Gökşen S, Balabanlı B, Coşkun-Cevher Ş. Application of platelet derived growth factor-BB and diabetic wound healing: The relationship with oxidative events. Free Radic Res 2017;51(5):498–505. [CrossRef]
22. Ersel M, Uyanikgil Y, Karbek Akarca F, Ozcete E, Altunci YA, Karabey F, et al. Effects of silk sericin on incision wound healing in a dorsal skin flap wound healing rat model. Med Sci Monit 2016;22:1064–78. [CrossRef]
23. Ayyildiz A, Ayyildiz SN, Benli E, Erdem H, Cirrik S, Noyan T, et al. The effect of oral and intraurethral trimetazidine use on urethral healing. Iran J Basic Med Sci 2016;19(9):932–9.
24. Kumar D, Jena GR, Ram M, Lingaraju MC, Singh V, Prasad R, et al. Hemin attenuated oxidative stress and inflammation to improve wound healing in diabetic rats. Naunyn Schmiedebergs Arch Pharmacol 2019;392(11):1435–45. [CrossRef]
25. Lv Z, Guo Y. Metformin and its benefits for various diseases. Front Endocrinol (Lausanne) 2020;11:191. [CrossRef]
26. Schuiveling M, Vazirpanah N, Radstake TRDJ, Zimmermann M, Broen JCA. Metformin, a new era for an old drug in the treatment of immune mediated disease? Curr Drug Targets 2018;19(8):945–59. [CrossRef]
27. Zhao P, Sui BD, Liu N, Lv YJ, Zheng CX, Lu YB, et al. Anti-aging pharmacology in cutaneous wound healing: Effects of metformin, resveratrol, and rapamycin by local application. Aging Cell 2017;16(5):1083–93. [CrossRef]
28. El-Bahy AAZ, Aboulmagd YM, Zaki M. Diabetex: A novel approach for diabetic wound healing. Life Sci 2018;207:332–9. [CrossRef]
29. Rasik AM, Shukla A. Antioxidant status in delayed healing type of wounds. Int J Exp Pathol 2000;81(4):257–63. [CrossRef]
30. Guo S, Dipietro LA. Factors affecting wound healing. J Dent Res 2010;89(3):219–29. [CrossRef]
31. Tombulturk FK, Todurga-Seven ZG, Huseyinbas O, Ozyazgan S, Ulutin T, Kanigur-Sultuybek G. Topical application of metformin accelerates cutaneous wound healing in streptozotocin-induced diabetic rats. Mol Biol Rep 2022;49(1):73–83. [CrossRef]
32. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54. [CrossRef]
33. Demirbag R, Gur M, Yilmaz R, Kunt AS, Erel O, Andac MH. Influence of oxidative stress on the development of collateral circulation in total coronary occlusions. Int J Cardiol 2007;116(1):14–9. [CrossRef]
34. Sinclair AJ, Barnett AH, Lunec J. Free radicals and antioxidant systems in health and disease. Br J Hosp Med 1990;43(5):334–44.
35. Atli T, Keven K, Avci A, Kutlay S, Turkcapar N, Varli M, et al. Oxidative stress and antioxidant status in elderly diabetes mellitus and glucose intolerance patients. Arch Gerontol Geriatr 2004;39(3):269–75. [CrossRef]
36. Buczyńska A, Sidorkiewicz I, Krętowski AJ, Adamska A. Examining the clinical relevance of metformin as an antioxidant intervention. Front Pharmacol 2024;15:1330797. [CrossRef]
37. Forouzandeh F, Salazar G, Patrushev N, Xiong S, Hilenski L, Fei B, et al. Metformin beyond diabetes: Pleiotropic benefits of metformin in attenuation of atherosclerosis. J Am Heart Assoc 2014;3(6):e001202. [CrossRef]
38. Han X, Tao Y, Deng Y, Yu J, Sun Y, Jiang G. Metformin accelerates wound healing in type 2 diabetic db/db mice. Mol Med Rep 2017;16(6):8691–8. [CrossRef]
39. Ochoa-Gonzalez F, Cervantes-Villagrana AR, Fernandez-Ruiz JC, et al. Metformin induces cell cycle arrest, reduced proliferation, wound healing impairment *in vivo* and is associated to clinical outcomes in diabetic foot ulcer patients. PLoS One 2016;11(3):e0150900. [CrossRef]

40. Viollet B, Guigas B, Sanz Garcia N, Leclerc J, Foretz M, Andreelli F. Cellular and molecular mechanisms of metformin: An overview. Clin Sci Lond 2012;122(6):253–70. [CrossRef]
41. Erel O. A novel automated method to measure total antioxidant response against potent free radical reactions. Clin Biochem 2004;37(2):112–9. [CrossRef]
42. Rabus M, Demirbağ R, Sezen Y, Konukoğlu O, Yildiz A, Erel O, et al. Plasma and tissue oxidative stress index in patients with rheumatic and degenerative heart valve disease. Arch Turk Soc Cardiol 2008;36(8):536–40.
43. Guleken Z, Depciuch J, Ege H, İlbay G, Kalkandelen C, Ozbeyli D, et al. Spectrochemical and biochemical assay comparison study of the healing effect of the Aloe vera and Hypericum perforatum loaded nanofiber dressings on diabetic wound. Spectrochim Acta A Mol Biomol Spectrosc 2021;254:119639. [CrossRef]
44. Lorente L, Rodriguez ST, Sanz P, Abreu-González P, Díaz D, Moreno AM, et al. Association between pre-transplant serum malondialdehyde levels and survival one year after liver transplantation for hepatocellular carcinoma. Int J Mol Sci 2016;17(4):500. [CrossRef]
45. Halliwell B, Gutteridge JM. Free radicals in biology and medicine (5th edn). Clarendon Press: Oxford, UK; 2015. p. 329–341. [CrossRef]
46. Yang S, Xu M, Meng G, Lu Y. SIRT3 deficiency delays diabetic skin wound healing via oxidative stress and necroptosis enhancement. J Cell Mol Med 2020;24(8):4415–27. [CrossRef]
47. Lima IC, Castro RR, Adjafre BL, Sousa SHAF, de Paula DS, Alves APNN, et al. Galactomannan of Delonix regia seeds modulates cytokine expression and oxidative stress eliciting anti-inflammatory and healing effects in mice cutaneous wound. Int J Biol Macromol 2022;203:342–9. [CrossRef]
48. Kant V, Sharma M, Jangir BL, Kumar V. Acceleration of wound healing by quercetin in diabetic rats requires mitigation of oxidative stress and stimulation of the proliferative phase. Biotech Histochem 2022;97(6):461–72. [CrossRef]
49. Aneesha VA, Qayoom A, Anagha S, Almas SA, Naresh VK, Kumawat S, et al. Topical bilirubin-deferoxamine hastens excisional wound healing by modulating inflammation, oxidative stress, angiogenesis, and collagen deposition in diabetic rats. J Tissue Viability 2022;31(3):474–84. [CrossRef]
50. Assar DH, Elhabashi N, Mokhbatly AA, Ragab AE, Elbialy ZI, Rizk SA, et al. Wound healing potential of licorice extract in rat model: Antioxidants, histopathological, immunohistochemical and gene expression evidences. Biomed Pharmacother 2021;143:112151. [CrossRef]
51. Al-Abrash AS, Al-Quobaili FA, Al-Akhras GN. Catalase evaluation in different human diseases associated with oxidative stress. Saudi Med J 2000;21(9):826–30.
52. Fitzmaurice SD, Sivamani RK, Isseroff RR. Antioxidant therapies for wound healing: A clinical guide to currently commercially available products. Skin Pharmacol Physiol 2011;24(3):113–26. [CrossRef]
53. Afzali H, Khaksari M, Norouzirad R, Jeddi S, Kashfi K, Ghasemi A. Acidified nitrite improves wound healing in type 2 diabetic rats: Role of oxidative stress and inflammation. Nitric Oxide 2020;103:20–8. [CrossRef]
54. Shao HB, Chu LY, Lu ZH, Kang CM. Primary antioxidant free radical scavenging and redox signaling pathways in higher plant cells. Int J Biol Sci 2007;4(1):8–14. [CrossRef]
55. Venukumar MR, Latha MS. Antioxidant activity of Curculigo orchioides in carbon tetrachloride-induced hepatopathy in rats. Indian J Clin Biochem 2002;17:80–7. [CrossRef]
56. Hasanain B, Mooradian AD. Antioxidant vitamins and their influence in diabetes mellitus. Curr Diab Rep 2002;2(5):448–56. [CrossRef]
57. Mahmoud AM, Ashour MB, Abdel-Moneim A, Ahmed OM. Hesperidin and naringin attenuate hyperglycemia-mediated oxidative stress and proinflammatory cytokine production in high-fat fed/streptozotocin-induced type 2 diabetic rats. J Diabetes Complications 2012;26(6):483–90. [CrossRef]
58. Coskun O, Kanter M, Korkmaz A, Oter S. Quercetin, a flavonoid antioxidant, prevents and protects streptozotocin-induced oxidative stress and beta-cell damage in rat pancreas. Pharmacol Res 2005;51(2):117–23. [CrossRef]
59. Sun C, Wang L, Sun J, Wang Z, Tang Z. Hypoglycemic and hypolipidemic effects of rutin on hyperglycemic rats. J Tradit Chin Med 2020;40(4):640–5.
60. Ahmed Mobasher M, Galal El-Tantawi H, Samy El-Said K. Metformin ameliorates oxidative stress induced by diabetes mellitus and hepatocellular carcinoma in rats. Rep Biochem Mol Biol 2020;9(1):115–28. [CrossRef]
61. Erejuwa OO, Sulaiman SA, Wahab MS, Salam SK, Salleh MS, Gurtu S, et al. Comparison of antioxidant effects of honey, glibenclamide, metformin, and their combinations in the kidneys of streptozotocin-induced diabetic rats. Int J Mol Sci 2011;12(1):829–43. [CrossRef]
62. Chen C, Liu XC, Deng B. Protective effects of berberine on nonalcoholic fatty liver disease in db/db mice via AMPK/SIRT1 pathway activation. Curr Med Sci 2024;44(5):902–11. [CrossRef]

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Research Article

Immunological insights into recurrent spontaneous abortions: The role of GATA3 and cytokine expression in maternal and placental tissues

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Abstract

Objectives: Recurrent spontaneous abortion (RSA) is the successive loss of pregnancy experienced by 1-2% of women with clinically recognized pregnancies. The role of cytokines and their regulators in RSA has gained significant attention in recent years. The GATA3 transcription factor has significant implications for maternal-foetal health outcome by modulating the immune T-cell population to T helper cell subsets that produce the proinflammatory (IFNγ) and anti-inflammatory cytokines (IL4) required for pregnancy maintenance.

Methods: The study involved 65 case-women with RSA and 70 control-women without a history of RSA. IL-4, IFNγ, and GATA3 transcription factor levels were analysed in maternal serum and placental tissues. Correlation analysis was performed for GATA3 expression and cytokine levels, and the results were tested for statistical significance.

Results: The mRNA expression of the GATA3 transcription factor was significantly reduced in both the maternal blood and placental tissues of the RSA group compared to the control group undergoing medical termination ($p \leq 0.05$*). Additionally, compared to the control group, the levels of the Th1 cytokine IFN-γ were significantly elevated (11034 pg/ml & 87.4735 pg/g), while the levels of the Th2 cytokine IL4 were significantly decreased (48.9832 pg/ml & 6320 pg/g) in RSA mother and their placenta samples respectively. Moreover, cytokine levels in the RSA group showed a significant correlation with GATA3 expression.

Conclusion: The study suggests that altered GATA3 levels and an increased IFN-γ/IL-4 ratio may increase the risk of recurrent spontaneous abortions in Telangana women.

Keywords: GATA3 transcription factor, IFNγ, IL4, maternal-foetal immunology, recurrent spontaneous abortion (RSA), TH1/TH2

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Recurrent spontaneous abortion has been redefined as the loss of two or more successive clinically recognised pregnancies before the 24th week of gestation, which poses a significant challenge in reproductive medicine [1]. The prevalence of recurrent spontaneous abortion is estimated to be approximately 1–2% of all couples attempting to conceive [2]. The etiology of RSA is multifaceted, with several contributing factors which include chromosomal abnormalities in either parent, uterine anomalies, hormonal imbalances, immunological factors, thrombophilia, or infections. Genetic factors, environmental influences, and lifestyle choices further contribute to the complexity of RSA etiology. Nevertheless, 50% of the exact cause behind the pathogenesis is unclear.

Cytokines are signalling molecules that orchestrate the immune responses crucial for implantation, placental development, and foetal growth. A delicate equilibrium between

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proinflammatory and anti-inflammatory cytokines is necessary to support maternal–foetal immune tolerance while ensuring protection against inflammation and rejection of semi allografts. During implantation, a controlled inflammatory response is vital for the invasion of trophoblast cells into the maternal endometrium followed by implantation. Proinflammatory cytokines, such as interferon gamma (IFNγ), tumour necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β), promote this early phase of pregnancy, and as pregnancy progresses, a shift towards an anti-inflammatory environment (IL10, IL4, and TGF beta) becomes essential to establish maternal–foetal tolerance. Piccinni and colleagues (2000) suggested that there was a decrease in Th1 cytokines and an increase in Th2 cytokines for the maintenance of a successful pregnancy [3]. Moreover, studies have shown that an excessive inflammatory response can lead to adverse pregnancy outcomes, such as recurrent loss of pregnancy and preterm birth [4,5]. GATA3 has emerged as an important transcriptional regulator of naïve T-cell differentiation into Th1 and Th2 effector cells that produce proinflammatory and anti-inflammatory cytokines, respectively, which are essential for maintaining successful and healthy gestation.

The placenta serves a vital role in immune modulation along with managing the hormonal, nutritional, and oxygen requirements of the foetus. The microenvironment established by the placenta plays a crucial role in the differentiation and function of immune cells infiltrating the implantation site. Helper T (Th) cells, pivotal in regulating immune responses, exhibit distinct contributions from different Th cell subsets during various stages of human pregnancy. Conversely, dysregulation of Th responses has been associated with numerous obstetric complications [6,7]. However, understanding the dynamics of foetal-maternal immune system throughout pregnancy remains incomplete.

The GATA family member GATA3-binding protein is a zinc finger transcription factor that recognises G-A-T-A in the promoter of target genes. GATA3 is involved in the control of CD4+ effector T-cell differentiation into Th2 subtypes that produce the anti-inflammatory cytokines IL4 and TGF-beta by simultaneously inhibiting the production of Th1 cell lineages that promote proinflammatory cytokines. GATA3 facilitates the conversion of the Th2 gene locus into an open conformation and increases accessibility to transcription activators along with GATA3, which initiates the transcription of Th2 genes, such as interleukin 4 (IL4), interleukin 5 (IL5), and interleukin 13 (IL13), and simultaneously suppresses the transcription of the potent proinflammatory Th1 cytokine interferon gamma (IFNγ), thereby skewing naïve T cells to Th2 effector cells, which are crucial for foetal implantation and maintenance [8].

Interleukin-4 is a pleiotropic anti-inflammatory cytokine of 20 kDa that contributes to the maintenance of pregnancy by coordinating vascularisation, placental development, and immune response adaptations at the maternal–foetal interface. IL-4 binds to its receptor, IL-4Rα, and activates the signal transducer STAT 6 signalling pathway, which helps polarize antigen-stimulated naïve Th cells into Th2 effector cells and promote Th2 responses. By suppressing the production of IFNγ, STAT6 may directly inhibit the growth of Th1 cells by inducing the zinc-finger transcription factor GATA3 (GATA-binding protein 3). The interleukins IL4, IL10 and IL13 are reported to have homeostatic functions during pregnancy [9]. These anti-inflammatory cytokines promote spiral artery remodelling by inducing the expression of leptin receptors for the ligand leptin, which plays a significant role in lipid metabolism, angiogenesis, vascular function, placental development and intrauterine foetal development [10,11]. IL-4 enhances the expression of VCAM-1 and induces changes in the morphology of human umbilical vascular ECs during the implantation phase of pregnancy [12,13].

IFNγ is a potent cytokine that is essential for the differentiation of naïve T cells into Th1 cells. IFNγ, which helps in the propagation of the immune response, belongs to the type II interferon subfamily. It has been proposed that during pregnancy, IFNγ is essential for the initiation of endometrial vasculature remodelling and angiogenesis during implantation. Previous findings have shown that the rejection of allografts is supported by elevated levels of IL-2 and IFNγ [14,15]. Conversely, it was observed that there was an increased production of IFNγ by natural killer cells during the first week of pregnancy, and these levels dampened during the latter phase for maintenance of the semi-allogenic foetus, thereby establishing a reduced Th1 profile and increased Th2 cytokine profile. An imbalance in IFNγ levels has been demonstrated to lead to severe pregnancy outcomes, such as spontaneous abortion, preterm birth, preeclampsia, and gestational diabetes [4,16–18]. IFNγ, the major Th1 cell differentiation cytokine, is regulated by various transcription activators, such as RUNX3, Tbet and STAT. Studies have shown that GATA3 not only promotes Th2 cell differentiation but also inhibits Th1 cell differentiation [19] through its interaction with activators and repression of their action. Ectopic GATA3 expression in developing Th1 cells inhibits IFN-γ production by repressing the expression of STAT4, which is normally highly expressed in Th1 cells compared with Th2 cells [20], and by suppressing T-cell differentiation into Th1 cytokine-producing cells.

However, to date, no studies have been investigated whether the differential protein levels of IL4 and IFNγ are associated with altered levels of GATA3 factors in the etiology of RSA. Therefore, taking into account the immunomodulatory properties of GATA3 and the importance of IL4 and IFNγ during pregnancy, the present study aimed to evaluate the correlation between altered GATA3 expression and cytokine levels (IL4 and IFNγ) in RSA pathogenesis.

Materials and Methods

Study cohorts

A total of 135 women who attended the Department of Gynaecology and Obstetrics, Government Maternity Hospital, Petlaburz, Hyderabad, Telangana were enrolled in the study.

Table 1. Quantitative real-time PCR primer sequences

Gene	Primer sequence	Product size
GATA 3	Forward: GGCGCCGTCTTGATACTT Reverse: CCGTCTCTCTCTCTTCTTCTC	100 bp
Beta Actin	Forward: GGATCAGCAAGCAGGAGTATG Reverse: AGAAAGGGTGTAACGCAACTAA	96 bp

PCR: Polymerase chain reaction.

Sixty-five women with RSA along with aborted tissue and seventy healthy women who underwent medical termination of pregnancy were considered cases and control subjects, respectively. Demographic details were recorded with the help of a standard proforma, and prior consent was obtained from all the subjects. The study was approved by the institutional ethics committee (Ref. No. 24/EC/NEW/INST/2023/4032. dtd. 22/12/2023) of the Institute of Genetics and Hospital for Genetic Diseases, Hyderabad, Telangana. The study was designed in accordance with the Helsinki Declaration.

Inclusion criteria

Both the case and control subjects with a mean age of less than 40 years and who were gestational matched were selected. Women with two or more consecutive spontaneous abortions were considered cases, whereas participants with no previous history of spontaneous abortions and who had at least two live births were considered controls.

Exclusion criteria

The case-control study subjects who demonstrated physiological anomalies, chromosomal and hormonal abnormalities, diabetes, APA antibodies, hypothyroidism, blood pressure and who consumed contraceptive pills were excluded from the study.

Sample collection

A total of 4 ml of blood sample and 100 mg aborted placental tissues from women with RSA and who underwent medical termination of pregnancy (MTP) were collected for quantitative analysis of cytokines. The samples were collected in Hi media RNA later as per standard operating conditions and stored at -80°C for RNA isolation.

Cytokine quantification

The protein levels of IL-4 and IFN-γ in the case, control, and maternal–placental study groups were quantified via commercially available ELISA kits. The IL-4 GENLISA™ ELISA and IFN-γ GENLISA™ ELISA were procured from Kishgen Biosystems for the quantification of cytokines in serum and tissue samples.

Tissue homogenate processing

For the enzyme-linked immunosorbent assay of conceptus, the tissues weighing 100mg were initially washed with PBS at pH 7.4 to remove excess blood and homogenised with 5 ml volume of PBS by mere mixing with a glass homogeniser on ice as described by the Weel et al. [21]. This ensures a weight-based normalisation of cytokine levels. Finally, the supernatant for the analysis was collected by centrifuging the homogenised samples at 12,000 rpm for 15 minutes and was used to determine cytokine levels which expressed as pg/g of placental tissue. The intra-assay and inter-assay coefficients of variation for all measurements were <8% and 10%, respectively, and were recorded in pg/ml for serum samples.

RNA isolation and cDNA conversion

Total RNA was isolated from both case and control maternal blood as well as their respective placental tissues via the Macherey-Nagel NucleoSpin RNA isolation Kit according to the manufacturer's instructions, and any DNA contamination was removed via on-column DNase treatment (Qiagen, Inc.). The quality of the RNA was assessed by measuring the 260/280 absorbance. The amount of RNA per microliter was quantified via a NanoDrop, and each sample was normalised prior to cDNA conversion. One microgram of total RNA was used for first-strand cDNA synthesis in a 20 µL reaction mixture via an iScript cDNA synthesis kit.

Expression analysis of GATA3

Three replicates of real-time PCR experiments were performed for each sample in a 96-well plate via an ABI 7000 Sequence Detection System from Applied Biosystems (Applied Biosystems). The primers for the target genes GATA3 and beta-actin were designed with Primer Express software (Applied Biosystems), and the sequences used for PCR are presented in Table 1. A total volume of 20 µl was used with 10 µl of SYBR Select Master mix (Cat. no. 4472908; Thermo Fisher Scientific, Inc.), 1 µl of each primer (10 µM), and 4 µl of template cDNA. The amplification protocol consisted of an initial denaturation step at 95°C for 4 min, followed by two-step PCR for 40 cycles at 95°C for 30 sec and 60°C for 30 sec. A melting curve analysis was also performed to check that no primer dimers or false amplicons interfered with the result. The Ct value was extracted for both the reference gene and target gene with an auto baseline and manual threshold, and the fold change in expression was calculated via the ΔΔCt method.

Statistical analysis

The data obtained were statistically evaluated using statistical package IBM© SPSS statistics 24.0 software. The sam-

Table 2. Demographic characteristics of the case-control group study subjects

Demographic characters	Case subjects n=65		Control subjects n=70		p	OR CI (95%)
	n	%	n	%		
Age (mean age±SD) (n)						
≤30 years	37	52.3	63	90.0	0.014*	2.704
>30 years	28	47.7	17	10.0	χ^2=7.983	(1.303–5.612)
No of miscarriages						
Women with 2 pregnancy loss	16	24.6	Nil		<0.0001***	–
Women with ≥3 pregnancy loss	49	75.4				
Consanguinity						
Yes	18	27.5	27	38.5	0.247	1.64
No	47	72.3	43	61.4	χ^2=1.795	(0.7935–3.388)
Time of previous abortion (gestation weeks)						
>12 week	50	80	41	58.6	0.037*	2.358
≤12 week	15	20	29	41.4		(1.116–4.98)
Socio economic status						
Rural	49	75.4	42	60	0.883	2.042
Urban	16	24.6	28	40	χ^2=2.131	(0.974–4.278)
Education						
Primary level	39	60	48	68.6	0.390	0.6875
Secondary level	26	40	22	31.4	χ^2=1.08	(0.3388–1.395)
Smoking						
No	44	67.7	56	80	0.194	1.909
Yes	21	32.3	14	20	χ^2=2.658	(0.872–4.177)

*: p≤0.05; **: p≤0.001; ***: p≤0.0001. OR: Odds ratio; CI: 95% Confidence interval; SD: Standard deviation; BMI: Body mass index.

ple size was calculated using a free open access software OpenEpi (http://www.OpenEpi.com/). All the quantitative data obtained were represented in mean±standard deviation. The strength of risk for the variables and recurrent spontaneous abortion was evaluated from Odds ratio at 95% of their confidence interval. Pearson test of correlation was performed to find relationship between the independent and dependent variables. Logistic regression was carried out to evaluate any confounding effect of independent variables on the disease condition. A p- value less than 0.05 was considered statistically significant.

Results

The mean age of the participants enrolled in the study was under 40 years, and the participants were grouped into those with a mean age below 30 years and those with a mean age above 30 years, as depicted in Table 2. The cut off maternal age as 30 years was selected as a woman's fertility begins to decline gradually around age 30, with a more rapid decrease after age 35 and increased the risk of miscarriage and other adverse pregnancy outcomes with advancement of maternal age [22]. The data indicated a notable difference in the mean age between the case and control groups, with χ^2=7.985, p≤0.05*. Specifically, the study demonstrated a twofold increased susceptibility to recurrent spontaneous abortion (RSA) among case subjects in the older group (≤30 years) compared with control subjects in the same age group (OR=2.7, p=0.01*). A comparison of the basal metabolic rates between the case and control groups revealed a significant difference (p=0.005*), with the case study group having a higher BMI (25.13±3.54) than the control group. Further classification on the basis of the number of pregnancy losses revealed a significantly greater proportion (75.4%) of cases with more than three pregnancy losses than those with 2 pregnancies lost (p≤0.0001**). The inclusion of women with consanguineous marriages was not associated with disease susceptibility. Additionally, the evaluation of the gestational period and susceptibility to RSA revealed statistical significance, with case subjects demonstrating a twofold increased risk during early gestation (OR=2.3, p=0.037*). Furthermore, socioeconomic status, education, and smoking habits were not significantly associated with disease susceptibility.

As depicted in Table 3, the levels of IFNγ and IL4 varied among the respective case and control groups, and the difference was statistically significant. Compared with their respective controls, women with RSA and their placental tissues presented lower levels of IL4 (63±20 pg/ml & 48.98±32 pg/g; p≤0.05*) and elevated levels of IFNγ (110±34 pg/ml & 87.47±35 pg/g; p≤0.05*, respectively). Furthermore, the

Table 3. Analysis of the levels of the circulating cytokines IL4 and IFN-γ in placental tissue and maternal blood

	Placental tissue		Maternal blood serum	
	IFN-γ (pg/g) Mean±SD	Il4 (pg/g) Mean±SD	IFN-γ (pg/ml) Mean±SD	Il4 (pg/ml) Mean±SD
Case	87.47±35	48.98±32	110±34	63±20
Control	71±28	66.47±21	95±30.1	79±31
p	0.003**	0.0002***	0.007**	0.001**

P<0.001 **: Significant; p<0.0001 ***: Significant. IL4: Interleukin 4; IFN-γ: Interferon gamma; SD: Standard deviation.

Table 4. Binary logistic regression analysis for predictors associated with RSA

	Cytokines	Odds ratio	95% CI	p
Maternal serum	IFNγ	1.07	1.002–1.094	0.053
	IL4	0.87	0.631–0.979	0.61
Placental	IFNγ	1.73	1.806–3.041	0.027*
Tissue	IL4	0.66	0.422–0.837	0.001**

The variables in the binary logistic regression are adjusted for age, BMI, gestational period, consanguinity, socioeconomic status and smoking habits. *: p≤0.05; **: p≤0.001. RSA: Recurrent spontaneous abortion; CI: Confidence interval; IFN-γ: Interferon gamma; IL4: Interleukin 4.

above parameters were adjusted for the demographic variables studied to determine whether any confounding effect resulted in the disease condition. However, binary regression analysis revealed that placental tissue levels of IFNγ (OR=1.73, p value=0.027*) and IL-4 (OR= 0.66, p=0.001*) were significant single predictors of RSA, as shown in Table 4.

The relative expression of GATA3 was assessed in the case and control groups of women with recurrent spontaneous abortion and their respective placental tissues to determine whether altered levels of GATA3 contributed to the pathogenesis of RSA. Figure 1a, b depict the comparative case–control mRNA expression levels of GATA3 in placental tissues and maternal blood, respectively. The relative expression of the GATA3 transcription factor revealed that GATA3 mRNA levels were downregulated in women with RSA (p<0.043*) and in the respective placental tissues by one-fold (p<0.002**) compared with those in the control groups. Finally, the correlation between GATA3 expression and the levels of IL4 and IFNγ was assessed using a Pearson correlation test. This assessment was conducted in women from the RSA group and their corresponding tissues. The correlation was deemed statistically significant if the p value was below 0.05. The Pearson correlation test showed a strong positive correlation between IL4 levels and GATA3 expression in placental tissue (r=0.782, p=0.0001***) and in the corresponding maternal group (r=0.572, p= 0.003**), as depicted in Figure 2a, b. Additionally, a negative correlation was found between IFNγ levels and GATA3 expression in both placental tissues and the maternal group. However, the correlation was moderate in the maternal group (r=-0.6601, p=0.017*), whereas it was strong in the placental group (r=-0.7827, p=<0.00001**), indicating statistical significance, as illustrated in Figure 3a, b.

Discussion

The study aims to investigate the expression profile of the GATA3 transcription factor, which modulates T helper cell activity, along with their cytokines, particularly IL4 and IFNG, obtained from women with recurrent spontaneous abortion along with their respective placental tissues and those with no history of RSA who underwent medical termination of pregnancy and their placental tissues.

Previous studies have emphasized the importance of down-regulating TH1 cytokines and upregulating TH2 cytokines during pregnancy. TH1 immunity, characterized by immune-inflammatory responses, predominates during the pre-implantation period and shortly after placental implantation [4]. Subsequently, early inflammatory TH1 immunity transitions to TH2 anti-inflammatory responses. The prevailing TH2 immunity at the placental implantation site helps maintain a balance with TH1 immunity, thereby safeguarding foetal and placental development [23,24] .Various immunomodulatory gene products, including transcription factors such as GATA3 protein, play crucial roles in orchestrating the TH1/TH2 shift throughout pregnancy. GATA3 is a key regulator of CD4+ T cell differentiation into Th2 effector cells. It activates the expression of signature Th2 cytokines like IL4, IL5, and IL13, which are pivotal for trophoblast establishment, maintenance, and differentiation. GATA3 executes these diverse functions by directly activating target genes or binding to cofactors that regulate gene expression and epigenetic modifications. GATA3 directly binds to the promoters of IL5 and IL13, intragenic areas of IL4, and the CGRE region within the IL13 locus, thereby promoting Th2 differentiation [25]. Additionally, GATA3 inhib-

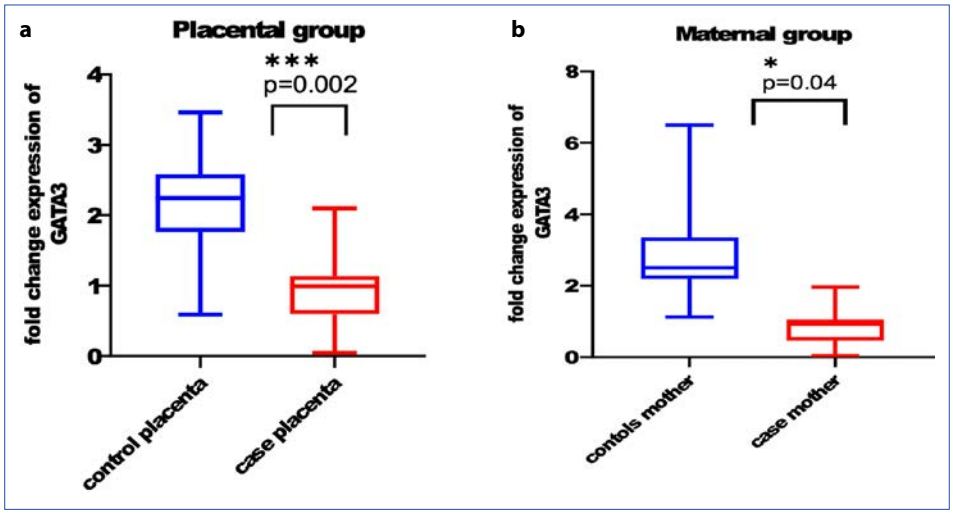

Figure 1. Graphical representation of the relative expression of GATA3 in case-control placental tissue and maternal blood.

GATA3: GATA binding protein 3.

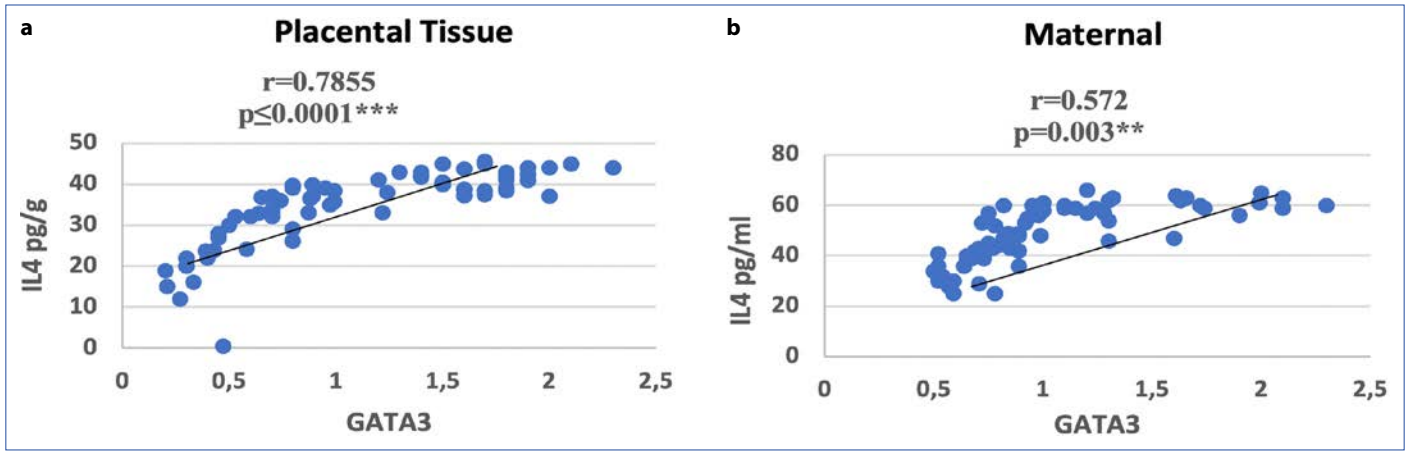

Figure 2. Correlation of GATA3 expression and IL4 levels, where r is the correlation coefficient and p is the statistical significance.

IL4: Interleukin 4.

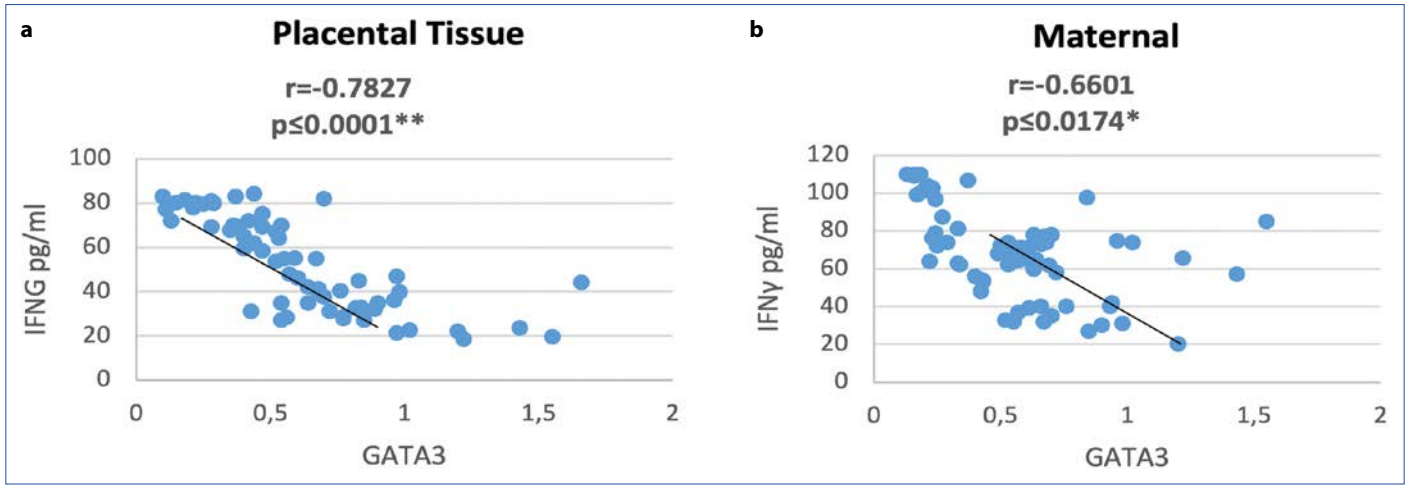

Figure 3. Correlation of GATA3 expression and IFNγ levels of placental and maternal blood, where r is the correlation coefficient and p is the statistical significance.

IFN-γ: Interferon gamma.

its Th1 differentiation by suppressing the production of STAT4 and IL12Rb2, both crucial for Th1 differentiation. Moreover, GATA3 interacts physically with Runt-related transcription factor 3 (Runx3), a regulator that stimulates Th1 differentiation, to suppress exomes expression and IFN-γ production.

Previous studies by Home et al. [26], and Saha et al. [27], demonstrated that the knockdown of GATA3 inhibited trophoectoderm maintenance, and also decreased expression of GATA3 in blastocysts of mouse models that further affected embryo hatching as well as the implantation respectively [26,27], underscoring the significance of GATA3 expression during pre-implantation. GATA3 expression promotes a TH2 response and modulates TH1 response, balancing TH2/TH1 levels crucial for maintaining successful pregnancy. Studies on tolerance induction to allografts have shown decreased levels of TH1 cytokines such as interleukin (IL)-2 and IFNγ, and increased levels of TH2 cytokines such as IL-4 and IL-10. Conversely, rejected allografts exhibited elevated levels of IL-2 and IFN [28].

GATA3 enhances IL4 production along with Stat5 by directly binding to the IL4 locus, promoting the opening of IL4 chromatin during the early stage of implantation while inhibiting the production of IFNγ, a potent cytokine in TH1 response and differentiation. Ectopic GATA3 expression in developing Th1 cells inhibits IFN-γ production through repressing the expression of STAT4, which is normally highly expressed in Th1 cells compared with Th2 cells and there by suppressing T cell differentiation to Th1 cytokine producing cells [8]. Further a study by Ribeiro et al. [29], reported that lower expression of GATA3 in preeclampsia women demonstrated altered levels of inflammatory cytokines, where there was increased levels of pro-inflammatory and lower anti-inflammatory cytokines. The present study results are in consistent with these findings, showing decreased levels of GATA3 protein expression in maternal blood and respective placental tissues from women with recurrent spontaneous abortions compared to those from healthy women who underwent medical termination of pregnancy. Furthermore, the study revealed differences in levels of potent Th1/Th2 cytokines IFNγ / IL4 crucial for pregnancy maintenance, indicating that altered levels of GATA3 and Th1/Th2 cytokines play a significant role in the etiology of recurrent spontaneous abortion.

Conclusion

The current study indicates a relationship between cytokine levels and GATA3 expression and suggests that immune pathway abnormalities may play a major role in the development of RSA. This highlights GATA3 as a potential target for reestablishing immunological homeostasis during pregnancy.

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Ethics Committee Approval: The study was approved by The Osmania University Institute of Genetics and Hospital for Genetic Diseases, Hyderabad, Telangana Ethics Committee (No: 24/EC/NEW/INST/2023/4032, Date: 22/12/2023).

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References

1. Ford HB, Schust DJ. Recurrent pregnancy loss: Etiology, diagnosis, and therapy. Rev Obstet Gynecol 2009;2(2):76.
2. Bender AR, Christiansen OB, Elson J, Kolte AM, Lewis S, Middeldorp S, et al; ESHRE guideline: Recurrent pregnancy loss: An update in 2022. Hum Reprod Open 2023;2023(1):hoad002. [CrossRef]
3. Piccinni MP, Scaletti C, Maggi E, Romagnani S. Role of hormone-controlled Th1- and Th2-type cytokines in successful pregnancy. J Neuroimmunol 2000;109(1):30–3. [CrossRef]
4. Saito S, Nakashima A, Shima T, Ito M. Th1/Th2/Th17 and regulatory T-Cell Paradigm in pregnancy. Am J Reproduct Immunol 2010;63(6):601–10. [CrossRef]
5. Moura E, Mattar R, Souza E de, Torloni MR, Gonçalves-Primo A, Daher S. Inflammatory cytokine gene polymorphisms and spontaneous preterm birth. J Reprod Immunol 2009;80(1–2):115–21. [CrossRef]
6. Raghupathy R. The immunology of unexplained recurrent spontaneous abortion: Cytokines as key mediators. Bull Kuwait Inst Med Spec 2003;2:32–8.
7. Raghupathy R. Cytokines and pregnancy complications: Modulation for prevention and treatment. Open Exploration 2019;2(3):414–27. [CrossRef]
8. Yagi R, Zhu J, Paul WE. An updated view on transcription factor GATA3-mediated regulation of Th1 and Th2 cell differentiation. Int Immunol 2011;23(7):415–20. [CrossRef]
9. Bryant AH, Spencer-Harty S, Owens SE, Jones RH, Thornton CA. Interleukin 4 and interleukin 13 downregulate the lipopolysaccharide-mediated inflammatory response by human gestation-associated tissues. Biol Reprod 2017;96(3):576–86. [CrossRef]
10. Childs GV, Odle AK, MacNicol MC, MacNicol AM. The Importance of Leptin to Reproduction. Endocrinology 2021;162(2):1–18. [CrossRef]
11. Pérez-Pérez A, Toro A, Vilariño-García T, Maymó J, Guadix P, Dueñas JL, et al. Leptin action in normal and pathological pregnancies. J Cell Mol Med 2018;22(2):716–27. [CrossRef]

12. Fukushi J, Ono M, Morikawa W, Iwamoto Y, Kuwano M. The activity of soluble VCAM-1 in angiogenesis stimulated by IL-4 and IL-13. J Immunol 2000;165(5):2818–23. [CrossRef]
13. Lee YW, Kühn H, Hennig B, Neish AS, Toborek M. IL-4-induced oxidative stress upregulates VCAM-1 gene expression in human endothelial cells. J Mol Cell Cardiol 2001;33(1):83–94. [CrossRef]
14. Strom TB, Roy-Chaudhury P, Manfro R. The Th1/Th2 paradigm and the allograft response. Curr Opin Immunol 1996;8(5):688–93. [CrossRef]
15. Piccinni MP. T cell tolerance towards the fetal allograft. J Reprod Immunol 2010;85(1):71–5. [CrossRef]
16. Al-Timimi FA, Darweesh MF, Hussein AR. The role of interferon-γ gene polymorphism in spontaneous abortion. Int J Sci Eng Res 2014;5(12):1295–302.
17. Pinheiro MB, Martins-Filho OA, Mota AP, Alpoim PN, Godoi LC, Silveira AC, et al. Severe preeclampsia goes along with a cytokine network disturbance towards a systemic inflammatory state. Cytokine 2013;62(1):165–73. [CrossRef]
18. Gomes CP, Torloni MR, Gueuvoghlanian-Silva BY, Alexandre SM, Mattar R, Daher S. Cytokine levels in gestational diabetes mellitus: A systematic review of the literature. Am J Reprod Immunol 2013;69(6):545–57. [CrossRef]
19. Usui T, Nishikomori R, Kitani A, Strober W. GATA-3 suppresses Th1 development by downregulation of Stat4 and not through effects on IL-12Rbeta2 chain or T-bet. Immunity 2003;18(3):415–28. [CrossRef]
20. Yagi R, Junttila IS, Wei G, Urban JF Jr, Zhao K, Paul WE, et al. The transcription factor GATA3 actively represses RUNX3 protein-regulated production of interferon-γ. Immunity 2010;32(4):507–17. [CrossRef]
21. Weel IC, Baergen RN, Romão-Veiga M, Borges VT, Ribeiro VR, Witkin SS, et al. Association between placental lesions, cytokines and angiogenic factors in pregnant women with preeclampsia. PLoS One 2016;11(6):e0157584. [CrossRef]
22. ACOG. Female age-related fertility decline. Available at: https://www.acog.org/-/media/project/acog/acogorg/clinical/files/committee-opinion/articles/2014/03/female-age-related-fertility-decline.pdf?rev=2eeed866997e400d8e43f5c183c53090&hash=036DE0E9244CF15474440DB9F16E8968. Accessed Feb 14, 2024.
23. Cyprian F, Lefkou E, Varoudi K, Girardi G. Immunomodulatory effects of vitamin D in pregnancy and beyond. Front Immunol 2019;10:475850. [CrossRef]
24. Kwak-Kim J, Skariah A, Wu L, Salazar D, Sung N, Ota K. Humoral and cellular autoimmunity in women with recurrent pregnancy losses and repeated implantation failures: A possible role of vitamin D. Autoimmun Rev 2016;15(10):943–47. [CrossRef]
25. Jenner RG, Townsend MJ, Jackson I, Sun K, Bouwman RD, Young RA, et al. The transcription factors T-bet and GATA-3 control alternative pathways of T-cell differentiation through a shared set of target genes. Proc Natl Acad Sci U S A 2009;106(42):17876–81. [CrossRef]
26. Home P, Ray S, Dutta D, Bronshteyn I, Larson M, Paul S. GATA3 is selectively expressed in the trophectoderm of peri-implantation embryo and directly regulates Cdx2 gene expression. J Biol Chem 2009;284(42):28729–37. [CrossRef]
27. Saha B, Home P, Ray S, Larson M, Paul A, Rajendran G, et al. EED and KDM6B coordinate the first mammalian cell lineage commitment to ensure embryo implantation. Mol Cell Biol 2013;33(14):2691–705. [CrossRef]
28. Chatterjee P, Chiasson VL, Bounds KR, Mitchell BM. Regulation of the anti-inflammatory cytokines interleukin-4 and interleukin-10 during pregnancy. Front Immunol 2014;5:1–1. [CrossRef]
29. Ribeiro VR, Romao-Veiga M, Nunes PR, de Oliveira LRC, Romagnoli GG, Peracoli JC, et al. Immunomodulatory effect of vitamin D on the STATs and transcription factors of CD4+ T cell subsets in pregnant women with preeclampsia. Clin Immunol 2022;234:108917. [CrossRef]

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Research Article

MicroRNAs and pro-inflammatory cytokines as candidate biomarkers for male infertility

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Abstract

Objectives: The effect of infertility does not exert a futuristic effect on society only but puts emotional and psychological stress on couples no matter who may show this problem. Thus, searching for obvious and hidden reasons to treat this problem took significant leaps to overcome it and provide couples with means of treatment. This article aims to investigate the role of specific microRNAs (miRNAs) as miR-429 and miR-425 and pro-inflammatory cytokines as interleukin-1 alpha (IL-1α) and tumor necrosis factor-alpha (TNF-α) on male fertility.

Methods: 100 semen samples were collected from healthy men with offspring and another 100 samples were collected from men suffering from fertility impairment for Semin fluid analysis (SFA). Cytokines levels in the serum were measured using sandwich ELISA technique, whereas DNA samples were obtained from both categories of participants from blood.

Results: Results showed that infertile patients showed high level of both tumor necrosis factor (TNF-α) and interleukin (IL-1A) which affected semen quality, motility, and to fertilize mature oocytes. In addition, high levels of miRNA 425, and 429 were detected in patients compared to control. We found specific type of single nucleotide polymorphism (SNPs) that reduced the ΔG in miRNAs within patients giving them the chance to be circulated for longer half time than the control which modified the RNA decay mechanism.

Conclusion: Immunological and epigenetic factors can play a crucial role in infertility manifestation in male. Since immunological factors are widely studied and been taken in concern in fertility clinics, epigenetic factors may be the key to overcome such clinical case and need to take in concern to provide a proper medical care.

Keywords: IL-1α, male infertility, miRNA-425, miRNA-429, TNF-α

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The case of infertility inflects a socially striking sensitivity than for other health conditions. Married couples mostly live in denial and lack the ability and medical knowledge to define themselves as infertile in the majority of cases regardless medical reports they are provided with by medical practitioners. Even more, medical models react with medical conditions as a phenomenon affecting the individual, while infertility is treated as condition affect the entire society in the

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industrial nations and could lead to functional impairment [1]. Developing of infertility is affected by various previously infected diseases and risk factors. Male factors occupy half of reasons in 13–18% infertility cases [2]. The failure to conceive a fetus after more than a year of regular unprotected sexual intercourse with the same partner is a feature of this condition [3]. Defective sperm function is the most known causes in male infertility. Abnormal semen parameters like low sperm concentration, impaired motility, and deformed morphology may play a crucial factor in infertility [4]. The infertility affects about 10% of women and 7% of men may produce a burden that may be announced high by WHO when subfertility / infertility reaches this percentage [5, 6]. Infertility may be categorized in in four reasons or factors: male factors, female factors, both male and female factors, in addition to unknown etiological factors [7]. A quantitative and signification idiopathic proportion of azoospermia, cryptozoospermia, and oligoasthenozoospermia may accompany male infertility and/or qualitative (asthenospermia, teratozoospermia, and necrospermia) abnormalities [8]. Male impairment or reduced fertility can be considered as a complex with multifactorial disease etiologies where genetic and epigenetic factors may play a key role and contribute to its manifestation [9]. Many genes were found to play a crucial role in controlling spermatogenesis, but the biological function of the majority of these genes in the control of the spermatogenesis process is still to be understood [10]. Among epigenetic factors affecting infertility, the single stranded miRNAs with length of 22 – 24 nucleotides were found to play a regulatory function in gene expression by forming semi – complementary structure in the untranslated 3' region related to mRNA [11]. About 1982 miRNAs playing a crucial role in cell cycle, cell differentiation, cell metabolism, and many biological processes, cell proliferation and apoptosis, male and female gametogenesis and embryo development were identified [12]. The body circulating extracellular miRNAs are categorized by stability and can be detected in body fluid samples of patients. The quantity of these miRNA may reflect the medical condition and disease status when compared to normal values [13]. The miR-429 is a member of miRNA-200 family, is highly expressed in in epithelial and mesenchymal tissues. Lack of regulation in these tissues due to lack of function of this type of miRNA may result in malignant tumors like oral squamous cell carcinoma [14]. The main function of miRNA-429 and its family as reported to inhibit inflammatory signaling and suppress multiple gene expression and production of IL8 [15]. They are an accumulative data regarding the importance and role of miRNA-429 in pathological and physiological events has and its possible role in oral inflammatory processes remains to be elucidated [16]. In addition, miRNA-425 was found to be associated with different diseases and biological processes like anti-angiogenesis [17], tumorigenesis [18] and inflammatory cytokine production [19]. In a separated report [20], miR-425 contributes to tumor development by inhibiting the tumor suppressor catenin α-3 in hepatocellular carcinoma. miR-425 is also reportedly upregulated in gastric cancer [21].

The purpose behind this study is to establish the relationship among miRNA-429 which considered as a tumor suppression factor and highly related to the function of immune system, and miRNA-425 which plays a crucial role in healthy proliferation of ovaries and fertility through immunological, genetic and epigenetic analysis.

Materials and Methods

Ethical approval

This study was approved by the Ethic Committee in Biotechnology Research Center under Ref. no. M. B 52 in 2/1/2022.

Consent of participation

All participants were asked to sign a participation form, and a questionnaire in which necessary information were recorded. No personal photos, videos, clinical tests, and names help to identify participants was published. The Helsinki declaration was followed during collecting personal data from patients.

Exclusion criteria

Men with chronic disease e.g., Cardiovascular, Diabetes Mellitus and Hypertension Varicocele (according to the clinical examination and ultrasonic waves), were excluded from this study.

Inclusion criteria

Males with fertility impairment showing no signs of chronic diseases, and previously had offspring were included in this study.

Sample collection

Semen samples were collected from 100 healthy men (normozoospermia) with a normal reproductive history and physical examination. All controls had children within 1 year, whereas 100 semen samples were collected from men with fertility impairment. Patients and control fall in the same age group 18 – 55 years.

Semen quality analysis

After two to five days of sexual abstinence, sperm samples were collected and incubated at 370C. After 30 minutes of incubation, liquefaction was observed and semen was analyzed according to World Health Organization recommendations, 2010, which included volume, sperm concentration, motility, and morphology.

Determination of IL-1A and TNF-α levels using Enzyme-Linked Immunosorbent Assay kit (ELISA)

The serum levels of human IL-1A and TNF- were determined using the human IL-1A and TNF-ELISA Kit (sandwich ELISA technique, Abcam, USA) according to manufacturer protocol. Resulting data were recorded and statistically analyzed.

Isolation of miRNAs

miRNAs were isolated from spermatozoa by using special EasyPure® miRNA Kit from TransGen biotech (China) as instructed by the manufacturer.

Isolation of total RNA

Trizol (TRIzol LS Reagent), was used to extract total RNA from sperm pellets, as directed by the manufacturer. Spectrophotometry was utilized to assess the purity and concentration of total RNA using the Nanodrop 2000 (Thermo Scientific). The isolated RNA samples were kept at -800C until use.

First-strand synthesis

The first strand was generated using EasyScript® One-Step cDNA synthesis kit (China). A volume of 5 µ of miRNA was mixed with kit components to final volume of 20 µl as instructed by the manufacturer. qPCR program was 940C for 5 sec, 600C for 15sec. in 40 cycles. The same protocol was used to generate cDNA from total RNA.

Primers used in Quantitative PCR (qRT–PCR)

Primers used during qRT – PCR amplification are listed below:

Genes	Forward primer (5´--->3´)	Reverse primer (3´ --->5´)	TM
rnub6B	AGAGAAGATTAGCATGGCCCCT	GCGAGCACAGAATTAATACGAC	62
mir-425	AATGACACGATCACTCCCGTTGA	GCGAGCACAGAATTAATACGAC	62
mir-429	TAATACTGTCTGGTAAAACCGT	GCGAGCACAGAATTAATACGAC	58
gapdh	GAAATCCCATCACCATCTTCCAGG	GAGCCCCAGCCTTCTCCATG	64
il-1A	TGTATGTGACTGCCCAAGATGAAG	AGAGGAGGTTGGTCTCACTACC	66
tnf-α	CTCCAGGCGGTGCTTGTTC	GGCTACAGGCTTGTCACTCG	58

Quantitative real-time PCR

Three microliters of cDNA were employed as templates in 20-mL reaction volume for quantitative real-time (qRT) PCR assessment of chosen genes. The primers utilized to evaluate gene expression are detailed in Table 1. including a thirty-second activation stage at 940C, followed by 40 cycles of five seconds at 940C (denaturation step), thirty seconds at 600C (annealing step), and ten seconds at 720C (extension step). The melting curves of the final dissociation curves were then generated. The Cepheid (smart cycler) Real-Time PCR Detection System was used for all of the tests, and the data (Ct values) were compared to internal controls RNU6B (U6) and GADPH. When housekeeping genes are employed in molecular studies, it is expected that the cells or tissue being analyzed will maintain constant levels of expression. These controls were found to be a very reliable method for qRT-PCR normalization when utilized in clinical research.

Analysis of gene expression

The levels of expression of the four chosen genes were normalized relative to endogenous controls. Using the comparative ΔCt technique, the relative miRNA and mRNA levels in the examined samples from fertile and infertile males were estimated individually. The threshold cycle (Ct) in a qRT-PCR is the number of cycles when a fluorescence curve initiated inside an interaction crosses the threshold.

The ΔCt has been computed by deducting the Ct amount of RNU6B and GAPDH from the ΔCt amount of target miRNA and mRNA, respectively: ΔCt (Ct of target miRNA-Ct RNU6B), ΔCt (Ct mRNA of interest-Ct GADPH). The ΔΔCt of the specimen from males with suspected infertility was then subtracted from the ΔCt of the healthy samples: ΔΔCt=(ΔCt1-ΔCt calibrator). This approach compares how a nucleic acid sequence is expressed in a test specimen to how it is expressed in a control sample containing the same sequence. The equation ΔΔCt was used to figure out where the fold-change cutoff was for each gene.

Statistical analysis

The Statistical Packages of Social Sciences (SPSS 2018) was used for statistical analysis. The parameters measured during the study were input to calculate the least significant difference (LSD) to signify the difference among tested groups.

Results

Basic seminal parameters distribution of the sterile and healthy men groups

Certain macroscopic and microscopic semen parameters showed some differences between the infertile and healthy control groups as displayed in Table 1.

Correlation between semen quality and TNFα

This current study investigated the correlation between TNFα and semen quality in infertile patient of males. Results obtained are illustrated in Table 2.

Relation between ILA1 and infertility

Levels of IL1A that produces interleukin 1 alpha was determined in serum of both patients and control. Results indicated that IL-1A were significantly higher compared to that

Table 1. Semen microscopical analysis for all groups

Parameters	Fertile healthy group (no=100)	Infertile group (no=100)	p‡
Volume (ml)	2.81±1.01[a]	3.22±1.21[a]	NS
Sperm concentration (million/ml)	77.64±59.22[a]	47.93±36.12[b]	**0.001**
Total motility (%)	71.36±8.38[a]	43.33±19.45[b]	**0.001**
Morphologically normal sperm (%)	37.43±15.66[a]	12.97±2.26[b]	**0.001**

Data were prepared as mean±SD; T-TEST was employed for statistical analysis. [a, b]: Indicates if there is a significant difference between compared data. NS: Not statistically significant; SD: Standard deviation.

Table 2. The correlation between TNF-α and semen quality in patients under study

Variable of semen analysis	n	% (n) of samples with elevated TNF-α conc. (≥20 pg/mL)[a]	p[b]	% (n) of samples with very high TNF-α conc. (≥40 pg/mL)[c]	p[b]	Median (range), pg/mL	p[d]
Sperm count							
<20×10⁶/ml	6	66.7 (4)	NS	33.3 (2)	NS	28.7 (9.2–74.5)	NS
≥20×10⁶/ml	94	46.5 (66)		25.4 (36)		17.7 (0.1–144.4)	
<40×10⁶/ml	26	57.7 (15)	NS	15.4 (4)	NS	25.0 (5.0–71.5)	NS
≥40×10⁶/ml	74	45.1 (55)		27.9 (34)		16.7 (0.1–144.4)	
Progressive motility							
<40%	88	44.7 (42)	NS	23.4 (22)	NS	16.9 (0.3–136.2)	NS
≥40%	12	51.9 (28)		29.6 (16)		20.9 (1.5–142.7)	
Total sperm count per ejaculate							
<96×10⁶[a]	24	50.0 (12)	NS	25.0 (6)	NS	19.9 (5.2–86.0)	NS
≥96×106	55	46.8 (58)		25 (32)		18.7 (0.1–144.4)	
<200×10⁶[c]	10	41.1 (23)	NS	17.9 (10)	NS	15.2 (1–144.4)	NS
≥200×10⁶	11	51.1 (47)		30.4 (28)		20.7 (0.1–142.7)	
Ejaculate volume							
<4.5 mL	92	42.4 (39)	NS	22.8 (21)	NS	15.3 (0.1–144.4)	NS
≥4.5 mL	8	55.4 (31)		30.4 (17)		25.3 (1.5–142.7)	

a, b, are not significant from each other in the statistical sense. c, and d are not significantly different. TNF-α: Tumor necrosis factor-α; NS: Not statistically significant.

in the control group (p=0.025, p<0.001). Table 3 illustrates the results obtained in this study.

Quantification of IL-A1 and TNFα expression using real-time PCR

The current study results revealed that there were significant differences in IL-A1 gene expression between patients and control. Results are shown in Table 4.

Measurement of miRNA – 429 and miRNA – 425 in study groups

The level of gene expression of miRNA – 429 and miRNA – 425 was measured in study groups. Results obtained are shown in Table 5.

Person correlation between varies study parameters

This current study investigated the Pearson correlation between some important factors related to Iraqi infertile patient groups males, correlation coefficient analysis was done including TNF alpha and IL-1 expression fold (2-ΔΔCt), TNF alpha and IL-1 serum level and finally (miR-429 and miR-425) expression fold, the results are shown in Table 6.

Molecular analysis of miRNA – 425 gene

DNA sequence of miRNA – 425 gene was performed for both control and patients and aligned to identify difference among them. The alignment data in shown in Appendix 1.

From alignment and sequence obtained, the similarity matrix was generated as shown in Figure 1.

Table 3. Comparison of IL-1A between patients and healthy group (mean±SD)

Item (pg/ml)	POI group (20)	Control group (22)	p
Serum IL-1α	1.07±1.61	0.48±0.53	0.025*
Follicular fluid IL-1α	3.65±4.16	1.67±1.03	<0.001*

*: p<0.05. IL-1A: Interleukin; SD: Standard deviation, POI: Patients of Interest.

Molecular analysis of miRNA – 429

An equal no. of patients and control DNA sequences were analyzed for DNA alignment, genomic location of the query, and percent identity matrix as shown in Appendix 2, and Figure 2.

Discussion

Socially, the literature on fertility is increasingly elaborating the cultural context of living this experience among individuals [22]. Accessing the medical record for survey and providing medical care and studying social attitudes may not provide a complete picture of who may or may not receive such care. Referencing a cross–sectional study population–based sample women showed that self – the definition of being infertile may provide the key to treatment [23]. However, the factors for infertility are a lot and some of them are either immunologically of genetically determined. Infection with microbes will trigger both Tumor necrosis factor alpha (TNF-α) and interleukin 1 (IL-1) as the key mediators of acute inflammatory reactions. TNF-α shows a wide variety of biologic actions which might interfere

Table 4. Levels of IL1A and TNF-α expression in patients and normal measured using qRT–PCR

IL1A						
Group	**Mean of Ct IL1 alpha**	**Mean of Ct gapdh**	**ΔCt (mean of Ct IL1 alpha-mean of Ct gapdh**	**2-ΔCt**	**Experimental group/ control group**	**Fold of gene expression**
Group 1 (patient)	20.755	22.11	-1.355	2.558	2.558/0.454	5.637
Group 2 (control)	23.25	22.11	1.140	0.454	0.454/0.454	1.000
TNF-α						
Group	**Mean of Ct TNF alpha**	**Mean of Ct gapdh**	**ΔCt (mean of Ct TNF alpha-mean of Ct gapdh**	**2-ΔCt**	**Experimental group/ control group**	**Fold of gene expression**
Group 1 (patient)	22.838	22.11	0.728	0.604	0.604/0.110	5.510
Group 2 (control)	25.3	22.11	3.190	0.110	0.110/0.110	1.000

IL-1A: Interleukin; TNF-α: Tumor necrosis factor-α; qRT–PCR: Quantitative real-time–PCR.

Table 5. Level of expression of miRNA 429 and miRNA – 425 compared to control group

miRNA 429						
Group	**Mean of Ct miRNA 425**	**Mean of Ct RNU6B mean of Ct RNU6B**	**ΔCt (mean of Ct miRNA 425-**	**2-ΔCt**	**Experimental group/ control group**	**Fold of gene expression**
Infertile group	23.23	22.18	1.05	0.482	0.482/0.151	3.19
miRNA – 425						
Group	**Mean of Ct miRNA 425**	**Mean of Ct RNU6B mean of Ct RNU6B**	**ΔCt (mean of Ct miRNA 425-**	**2-ΔCt**	**Experimental group/ control group**	**Fold of gene expression**
Infertile group	21.6	22.18	-0.58	1.494	1.494/0.503	2.96
Fertile (control) group 2	23.1	22.11	0.99	0.503	0.503/0.503	1
Fertile (control) group	24.83	22.11	2.72	0.151	0.151/0.151	1

Table 6. Person correlation among immunological and epigenetic factors measured in this study

	TNF alpha fold	**IL1 A fold**	**TNF alpha serum level**	**IL1 A serum level**	**miRNA429**	**miRNA425**
TNF alpha fold	1	0.032	0.033	0.071	-0.146	0.053
IL1 A fold	0.032	1	-0.088	0.022	-0.153	0.356
TNF alpha serum level	0.033	-0.088	1	-0.018	-0.263	0.103
IL1 A serum level	0.071	0.022	-0.018	1	-0.308	0.457
miRNA429	-0.146	-0.153	-0.263	-0.308	1	0.060
miRNA425	0.053	0.356	0.103	0.457	0.060	1

IL-1A: Interleukin; TNF-α: Tumor necrosis factor-α.

Figure 1. Percent identity matrix of patients and control. The letter C refers to control (healthy) subjects, whereas P refers to patients.

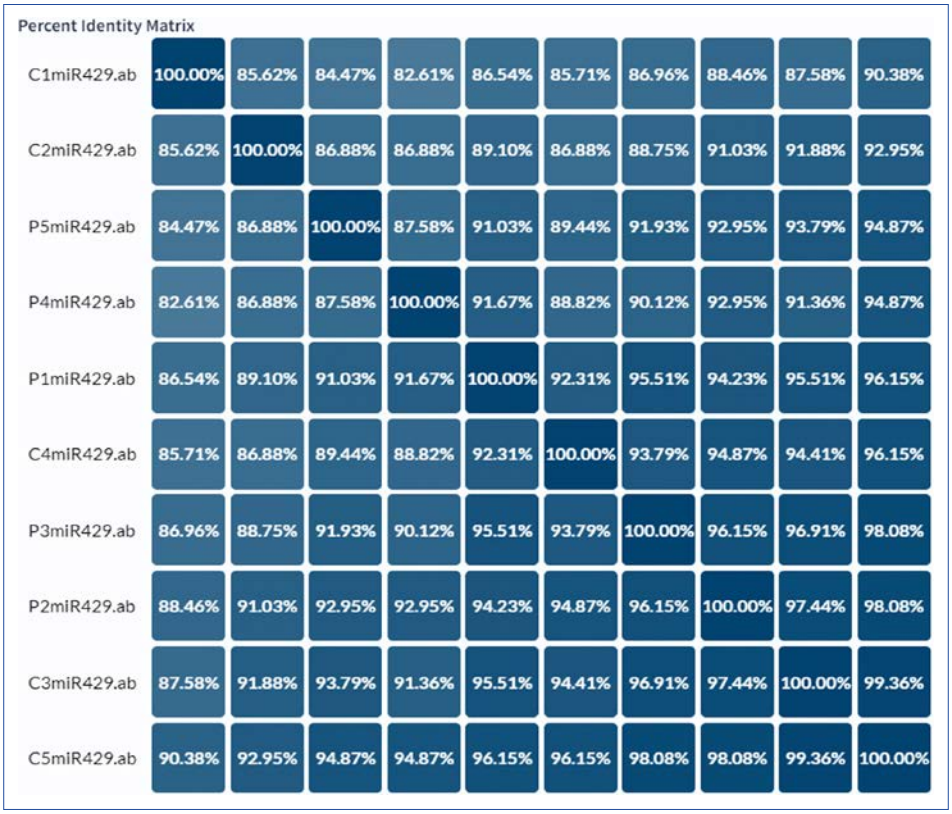

Figure 2. Percent identity matrix of patients and control for miRNA – 429. The letter C refers to control (healthy) subjects, whereas P refers to patients.

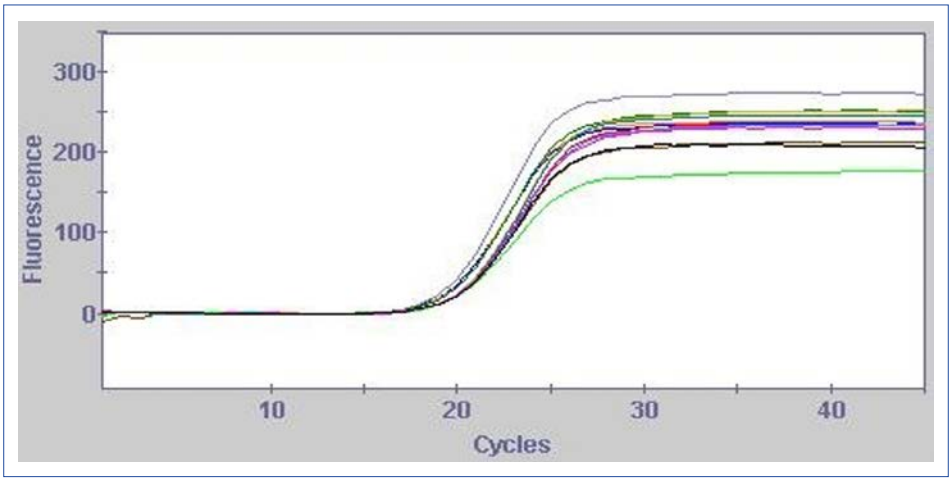

Figure 3. qRT–PCR of miRNA–425, and miRNA–429 in patient group. The figure shows amplification started at Ct 20 indicating high concentration of both miRNAs in blood.
qRT–PCR: Quantitative real-time–PCR.

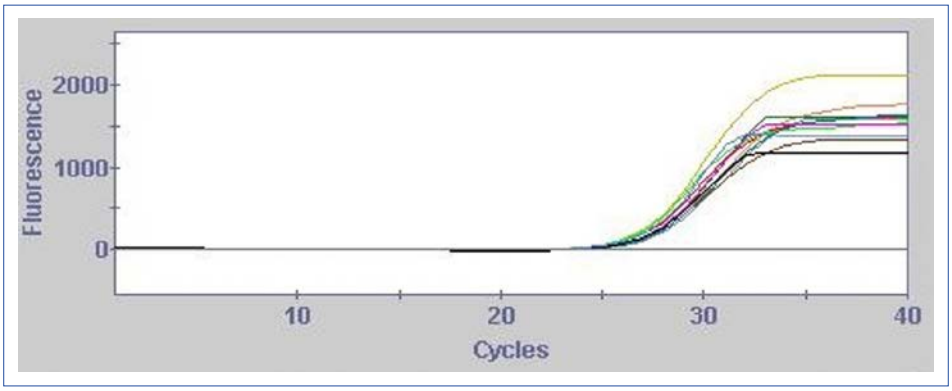

Figure 4. qRT–PCR of miRNA–425, and miRNA–429 in control group. The figure shows amplification started at Ct 30 indicating normal concentration of both miRNAs in blood.

with reproductive functions, like induction of the immune-cascade and chemotactic activity on neutrophils, cytolytic and cytostatic effects on tumor cells, induction of fibroblastic growth, stimulation of collagenase, prostaglandin synthesis, and potential influence on sperm motility and functional capacity. In Tables 2, 3, and 4 the correlation between TNFα and semen characteristics was determined. We found a decrease in semen criteria with elevation of TNFα concentration in blood that included semen quality, motility, and size of ejaculate. This may be attributed to that macrophages in testicles may produce cytokine-guided paracrine regulatory influence on Leydig cell function as an example of immune-endocrine interactions in the male reproductive system [24]. Interleukin 1 also is an important mediator of immunologic and pathologic responses to stress, infection, and antigenic challenge. In Table 6, it acts synergistically with other factors in the activation and differentiation of B- cells to immunoglobulin secreting cells, and it stimulates the activation and differentiation of natural killer (NK) cells, fibroblasts, and thymocytes. It acts anti-proliferatively, increases the cytotoxicity of macrophages. It has a variety of effects in the brain, such as induction of fever as an endogenous pyrogen, alteration of slow-wave sleep, and an important role in modulating reproductive functions through stimulation of corticotropin-releasing factor and ACTH secretion and further influence on the hypothalamic-pituitary-gonadal axis [25].

Since both miRNA- 425, and miRNA – 429 are both subject of interest and are major affecters in levels of TNFα and IL 1A, their expression rate was measured in both groups under study. An elevation was measured in patient group that reached 5 folds compared to control group as an indicator for increased need of the body for the effect of these miRNAs to take place in reduction of inflammation. Figure 3, we noticed that Ct value began at an early stage (Ct20) whereas the reaction began at Ct 25 in control group as shown in Figure 4.

The study extended further toward sequencing of both miRNA – 425 and miRNA – 429 genes form which we were able to identify variations that affected the role of the gene. In patients with infertility problem, the high level of both miRNAs may be attributed to epigenetic factors appeared in two points: first multiple SNPs were identified in patients that are listed in details in Table 7, and Table 8 respectively.

Table 7. DNA SNPs identified in miRNA – 425 gene

Variant	Class	Location	Alleles	Ambiguity code	Source	Consequence
rs769996937	SNP	3:49020120	C/G	S	dbSNP	Intron variant
rs1265172024	SNP	3:49020128	A/C	M	dbSNP	Intron variant
rs1250721195	SNP	3:49020144	C/G	S	dbSNP	Intron variant
rs759930741	indel	3:49020146-49020179	CCGAAAGAGCACTG	Non	dbSNP	Mature miRNA variant
rs759930741	indel	3:49020146-49020179	CCGAAAGAGCACTG	Non	dbSNP	Mature miRNA variant
rs368663793	SNP	3:49020225	A/G	R	dbSNP	Non coding transcript exon variant
rs1302210268	SNP	3:49020229	G/A	R	dbSNP	Non coding transcript exon variant
rs745809471	SNP	3:49020235	C/T	Y	dbSNP	Intron variant

SNPs: Single nucleotide polymorphism.

Table 8. DNA SNPs identified in miRNA – 429 gene

Variant	Class	Location	Alleles	Ambiguity code	Source	Consequence
rs375624023	SNP	1:1169013	G/A/C	V	dbSNP	non coding transcript exon variant
rs749785386	SNP	1:1169015	T/C	Y	dbSNP	non coding transcript exon variant
rs370935426	SNP	1:1169058	T/C	Y	dbSNP	mature miRNA variant
rs375039921	SNP	1:1169068	T/C	Y	dbSNP	mature miRNA variant
rs368678282	SNP	1:1169075	G/A	R	dbSNP	mature miRNA variant

Second, variation in folds of normal and patients miRNAs is associated with RNA decay mechanism that is mainly depend upon the free energy of RNAs and configuration. In this case the configuration and free energy of both miRNAs were measured (Fig. 5).

Results for thermodynamic ensemble prediction is that the free energy of the thermodynamic ensemble is -87.66 kcal/mol. The frequency of the MFE structure in the ensemble is 0.01%.

The ensemble diversity is 50.04 for healthy subjects. Whereas results for thermodynamic ensemble prediction for patients were that the free energy of the thermodynamic ensemble is - 84.29 kcal/mol and the frequency of the MFE structure in the ensemble is 0.04 %. The ensemble diversity is 55.55.

With such data it can be said that the mechanism for RNA decay is different between the studied group estimating a longer half-

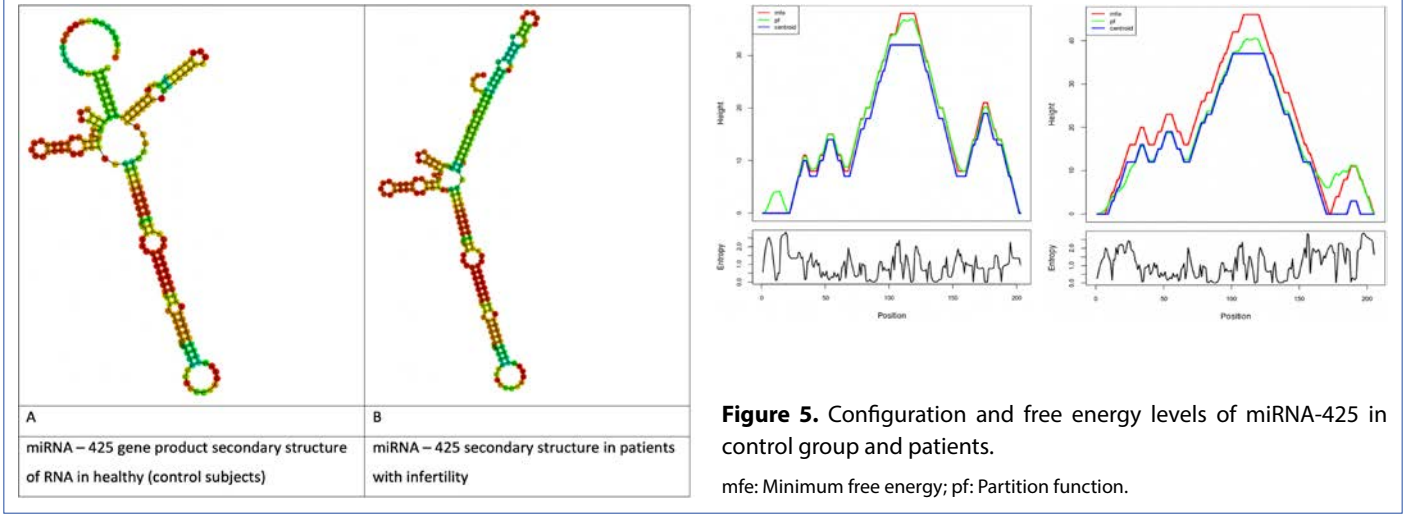

Figure 5. Configuration and free energy levels of miRNA-425 in control group and patients.

mfe: Minimum free energy; pf: Partition function.

life of miRNAs in patients than healthy which increases the concentration of these molecules in blood initiating a clinical case.

Conclusion

In patients with infertility problem, TNFα was found to in high levels accompanied with IL 1A indicating a case of inflammation affected semen quality, but with high levels of miRNA 425 and 529, symptoms may be mimicked showing no signs of infection, clinical case, and drive patients to seek other ways of treatment. Thus, it is highly recommended that such immunological factors and epigenetic factors should be measured to provide a proper way of treatment.

Appendix Files: https://jag.journalagent.com/ijmb/abs_files/IJMB-92668/IJMB-92668_(3)_Appendixes.pdf

Ethics Committee Approval: The study was approved by The Al-Nahrain University Biotechnology Research Center Ethics Committee (No: M. B 52, Date: 02/01/2022).

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References

1. Greil A, McQuillan J, Slauson-Blevins K. The social construction of infertility. Sociol Compass 2011;5(8):736–46. [CrossRef]
2. Öztekin Ü, Caniklioğlu M, Sarı S, Selmi V, Gürel A, Işıkay L. Evaluation of male infertility prevalence with clinical outcomes in Middle Anatolian Region. Cureus 2019;11(7):e5122. [CrossRef]
3. Marzec-Wróblewska U, Kamiński P, Lakota P, Szymański M, Wasilow K, Ludwikowski G, et al. Zinc and iron concentration and SOD activity in human semen and seminal plasma. Biol Trace Elem Res 2011;143(1):167–77. [CrossRef]
4. Chyra-Jach D, Kaletka Z, Dobrakowski M, Machoń-Grecka A, Kasperczyk S, Birkner E, Kasperczyk A. The associations between infertility and antioxidants, proinflammatory cytokines, and chemokines. Oxid Med Cell Longev 2018;2018:8354747. [CrossRef]
5. Sarkar S, Gupta P. Socio-demographic correlates of women's infertility and treatment seeking behavior in India. J Reprod Infertil 2016;17:123.
6. Krausz C, Riera-Escamilla A. Genetics of male infertility. Nat Rev Urol 2018;15:369–384. [CrossRef]
7. Hashem R, Al-Obaidi ZF, Samawi FT, Baher H. Analysis of anti-apoptotic protein (Bcl-xl) levels and mRNA expression in infertile patients. Afr J Reprod Health 2022;26(10):63–71.
8. Ferlin A, Arredi B, Speltra E, Cazzadore C, Selice R, Garolla A, et al. Molecular and clinical characterization of Y chromosome microdeletions in infertile men: A 10-year experience in Italy. J Clin Endocrinol Metab 2007;92:762–70. [CrossRef]
9. Krausz C, Giachini C. Genetic risk factors in male infertility. Arch Androl 2007;53(3):125–33. [CrossRef]
10. Gianotten J, Lombardi MP, Zwinderman AH, Lilford RJ, van der Veen F. Idiopathic impaired spermatogenesis: Genetic epidemiology is unlikely to provide a short-cut to better understanding. Hum Reprod Update 2004;10(6):533–9. [CrossRef]
11. Boyd SD. Everything you wanted to know about small RNA but were afraid to ask. Lab Investig 2008;88:569–78. [CrossRef]
12. Salas-Huetos A, James ER, Aston KI, Carrell DT, Jenkins TG, Yeste M. The role of miRNAs in male human reproduction: A systematic review. Andrology 2020;8:7–26. [CrossRef]
13. Saito A, Horie M, Ejiri K, Akira A, Katagiri S, Maekawa S, et al. MicroRNA profiling in gingival crevicular fluid of periodontitis-a pilot study. FEBS Open Bio 2017;7:981–94. [CrossRef]
14. Arunkumar G, Deva Magendhra Rao AK, Manikandan M, Prasanna Srinivasa Rao H, Subbiah S, Ilangovan R, et al. Dysregulation of miR-200 family microRNAs and epithelial-mesenchymal transition markers in oral squamous cell carcinoma. Oncol Lett 2018;15:649–57. [CrossRef]
15. Wang P, Cao J, Liu S, Pan H, Liu X, Sui A, et al. Upregulated microRNA-429 inhibits the migration of HCC cells by targeting TRAF6 through the NF-κB pathway. Oncol Rep 2017;37:2883–90. [CrossRef]
16. Kawasaki H, Amano H. Anti-inflammatory role of microRNA-429 in human gingival epithelial cells-inhibition of IL-8 production through direct binding to IKKβ mRNA. Mol Med Rep 2021;24(2):581. [CrossRef]
17. Gao Y, Yin Y, Xing X, Zhao Z, Lu Y, Sun Y, et al. Arsenic-induced anti-angiogenesis via miR-425-5p-regulated CCM3. Toxicol Lett 2016;254:22–31. [CrossRef]
18. Liu L, Zhao Z, Zhou W, Fan X, Zhan Q, Song Y. Enhanced expression of miR-425 promotes esophageal squamous cell carcinoma tumorigenesis by targeting SMAD2. J Genet Genomics 2015;42:601–11. [CrossRef]
19. Nakagawa R, Muroyama R, Koike K, Saeki C, Ito S, Morimoto S, et al. Decreased mir-425 induced inflammatory cytokine production via N-RAS upregulation in CD4+ T cells of primary biliar cholangitis. J Hepatol 2016;64(Suppl):S643–4. [CrossRef]
20. He B, Li T, Guan L, Liu FE, Chen XM, Zhao J, et al. CTNNA3 is a tumor suppressor in hepatocellular carcinomas and is inhibited by miR-425. Oncotarget 2016;7:8078–89. [CrossRef]
21. Demir M, Ozdem S. Evaluation of total antioxidant capacity in human seminal plasma. Int J Med Biochem 2022;5(1):54–9. [CrossRef]
22. Parry DC; Work, Leisure, and Support Groups. An examination of the ways women with infertility respond to pronatalist ideology. Sex Roles 2005;53(5–6):337–46. [CrossRef]
23. White L, McQuillan J, Greil AL. Explaining disparities in treatment seeking: The case of infertility. Fertil Steril 2006;85(4):853–7. [CrossRef]
24. Huleihel M, Lunenfeld E. Involvement of intratesticular IL-1 system in the regulation of Sertoli cell functions. Mol Cell Endocrinol 2002;187:125–32. [CrossRef]
25. Reichenberg A, Yirmiya R, Schuld A, Kraus T, Haack M, Morag A, et al. Cytokine-associated emotional and cognitive disturbances in humans. Arch Gen Psychiatry 2001;58:445–52. [CrossRef]

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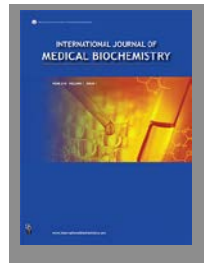

Research Article

Association of serum Maresin-1 levels with insulin-resistance indices in obese individuals

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Abstract

Objectives: This study aimed to investigate serum Maresin-1 (MaR1) levels among obese, overweight, and normal-weight groups, as well as to evaluate their association with various metabolic parameters, including insulin resistance-related indices and lipid profiles.

Methods: Ninety subjects were classified into three distinct groups in terms of body mass index (BMI). Using a median MaR1 value of 608 pg/mL as the threshold, the participants were also categorized into two distinct groups. Serum MaR1 levels were quantified via an ELISA. The study also evaluated several other indicators: metabolic score for insulin resistance (METS-IR), triglyceride glucose-body mass index (TyG-BMI), HbA1c, and various components of the lipid profile.

Results: MaR1 levels were significantly lower in the obese and overweight categories compared to the normal-weight categories. Nevertheless, no statistically significant difference was observed in the MaR1 levels between the obese and overweight groups. MaR1 levels were negatively linked to METS-IR ($r=-0.444$, $p<0.001$) and TyG-BMI ($r=-0.427$, $p<0.001$), whereas quantitative insulin sensitivity check index ($r=0.318$, $p=0.002$) levels were positively correlated. METS-IR had the highest AUC value (0.706), with 73.3% sensitivity and 57.8% specificity to identify high levels of MaR1 ($p<0.001$).

Conclusion: Ordinal logistic regression revealed a significant independent relationship between MaR1 levels and BMI categories. The close association between MaR1 and metabolic indices such as METS-IR and TyG-BMI suggests its role in insulin sensitivity and obesity-associated metabolic disorders.

Keywords: Insulin resistance, Maresin-1, METS-IR, obesity, specialized pro-resolving mediators, TyG-BMI

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Obesity is a global medical concern closely linked to chronic inflammation and dysfunctions of carbohydrate metabolism. In obesity, triglyceride accumulation occurs in the adipose tissue, as increased inflammation and insulin resistance promote fatty acid production in the liver. An increase in adiposity in adipose tissue reduces the responsiveness of insulin-sensitive cells to the physiological effects of insulin [1, 2]. In obese patients, adiposity allows the fat tissue to function as an endocrine organ. Adipokines secreted by adipocytes regulate metabolic pathway defects that arise from inflammation and insulin resistance. While numerous molecules have been recognized in this context, research has increasingly concentrated on the impact of new regulatory molecules, including specialized pro-resolving mediators (SPMs), on the obesity-induced metabolic dysfunction [3]. SPMs are bioactive compounds categorized into four primary categories: lipoxins, maresins, protectins, and resolvins [4]. Maresin 1 (MaR1), derived from docosahexaenoic acid (DHA), demonstrates anti-inflammatory in different tissue types, particularly in white adipose tissue. It is synthesized by macrophages through

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enzymatic conversion of DHA via the 14S-hydroxy-DHA and 12-lipoxygenase (12-LOX) pathways [5]. In obesity, elevated free fatty acids activate proinflammatory pathways by upregulating cyclooxygenase (COX) and LOX enzymes, resulting in increased production of mediators such as leukotriene B4 (LTB_4), which recruit neutrophils and exacerbate insulin resistance. This chronic inflammation is further amplified by neutrophil-released cytokines, which disrupt insulin signaling and sustain a proinflammatory state. The resolution of a self-limited acute inflammatory response involves a transition away from generating pro-inflammatory substances, like LTB_4, to the synthesis of counterregulatory substances known as SPMs. To counteract this, MaR1 is synthesized during the resolution phase of inflammation to stop further neutrophil recruitment and promote the clearance of apoptotic neutrophils and debris, thereby preventing chronic inflammation [6, 7].

Although numerous animal studies have explored the relationship between MaR1 and lipid and glucose metabolism, research in humans remains limited. Understanding the role of MaR1 in metabolic regulation is essential for developing effective therapeutic strategies to prevent and manage obesity-related metabolic disorders. This study purposed analyze serum MaR1 levels in patient cohorts stratified into three groups depending on body weight (normal weight, overweight, and obese), and to assess the correlation between MaR1 levels and metabolic indicators.

Materials and Methods

This study was conducted with authorization the Firat University Ethics Committee for Non-interventional Research (Number: 2023/10-23, Date: 27/07/2023). Ninety individuals who attended the Internal Medicine outpatient department at Yerkoy State Hospital between August 2023 and November 2023 were included after obtaining informed consent forms. All stages of the study were carried out conformity the Helsinki Declaration.

This study excluded participants based on the following criteria: age under 18 years, type 2 diabetes mellitus (T2DM), liver diseases, chronic kidney disease, history of bariatric or metabolic surgery, hematological disorders or malignancies, systemic inflammatory or infectious diseases, and use of anti-inflammatory or steroid therapy. Physical examinations were conducted during outpatient assessment, and height and weight measurements were recorded. The participants were grouped into three categories according to their body mass index (BMI): normal weight (18.5–24.9 kg/m^2), overweight (25–29.9 kg/m^2), and obese (≥30 kg/m^2) [8]. Additionally, based on the median MaR1 level (608 pg/mL), the study participants were divided into two categories.

Following an 8-hour fasting period, blood specimens have been collected from the participants using serum separator tubes. The samples were allowed to clot for at least 30 minutes before being centrifuged at 2000×g for 10 minutes. The resulting sera were separated, transferred to Eppendorf tubes, and stored at −20°C until analysis. Glucose, alanine aminotransferase (ALT), hemoglobin A1c (HbA1c), aspartate aminotransferase (AST), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), triglyceride (TG), and low-density lipoprotein cholesterol (LDL-C) levels were measured via a Beckman Coulter DxC 700 AU (Beckman Coulter Inc., Brea, CA, USA) clinical chemistry analyzer. Insulin levels were analyzed using a chemiluminescent immunoassay method on a Snibe Maglumi X3 analyzer (Snibe Diagnostics, Shenzhen, China). Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) was computed via the equation: [glucose (mg/dL) × insulin (mU/L)] / 405 [9]. Metabolic Score for Insulin Resistance (METS-IR) was computed via the equation: Ln [(2 × glucose) + TG] × BMI / Ln [HDL-C] [10]. Body fat percentage (BF%) was estimated using the Deurenberg equation: BF%= 1.2 × BMI (kg/m^2) + 0.23 × age (years) – 10.8 × gender (female= 0, male= 1) – 5.4 [11]. The triglyceride glucose index (TyG), which is considered an effective surrogate marker for insulin resistance, was calculated using the formula: ln [(fasting glucose × TG) / 2]. TyG-BMI was determined by multiplying TyG index by BMI [12]. Quantitative Insulin Sensitivity Check Index (QUICKI) was calculated as follows: 1/(log insulin + log glucose) [13]. The McAuley index was calculated as follows: $=e^{[2.63-0.28\times \ln\ (\text{Insulin})-0.31\times \ln(\text{TG})]}$ [14]. The Castelli risk index (CRI-I) was determined by dividing TC by HDL-C, whereas the CRI-II was derived by dividing LDL-C by HDL-C [15]. The atherosclerotic index (AI) was determined analytically using the formula (TC−HDL-C) / HDL-C [16].

A Human MaR1 enzyme-linked immunosorbent assay (ELISA) (Catalog No: 201–12–7339; Sunred Biotechnology Company, Shanghai, CHINA) was carried out adherence the procedures described in the procedures specified by the manufacturer. The optical density at 450 nm was quantified spectrophotometrically with a CLARIOstar PLUS device (BMG Labtech, Germany). Test results are reported in pg/mL. The measurement range of the MaR1 kit was 7.5 pg/mL to 2000 pg/mL, with a sensitivity of 7.247 pg/mL. The MaR1 kit has an intra-assay coefficient of variation (CV) of less than 10% and an inter-assay CV of less than 12%.

Statistical evaluation

To determine whether the dataset conformed to a normality, Shapiro–Wilk was applied for statistical evaluation. Categorical variables were examined using Pearson's Chi-square test, Continuity Correction, or Fisher's Exact test, depending on the minimum expected count value. When the data did not follow a normal distribution, the Mann-Whitney U was utilized, whereas the Student's t was conducted for data exhibiting normal distribution. To compare three independent groups, ANOVA was performed for normality assumptions, and the Kruskal–Wallis was performed for non-normality assumptions. Post-hoc Tukey or Tamhane's T2 tests were employed for pairwise comparisons among groups following the one-way ANOVA. The correlations between the parameters were assessed using Spearman correlation analysis. ROC analysis was employed to distinguish individuals with high Maresin 1

Table 1. Comparison of demographic and laboratory parameters across the three groups

Parameter	Normal weight (n= 30)	Overweight (n= 30)	Obese (n=30)	p*
Age (years)	39 (31–46)	39 (34–43)	34 (27–43)	0.570
Gender (n, %)				
Male	8 (26.7)	6 (20.0)	7 (23.3)	0.830
Female	22 (73.3)	24 (80.0)	23 (76.7)	
Body mass index (kg/m^2)	23.3 (21.1–24.3)	27.7 (26.4–28.6)^{a1}	31.3 (30.6–34.0)$^{a1, b1}$	**<0.001**
Body fat (%)	29.1 (24.4–31.9)	35.4 (32.3–38.1)^{a2}	39.3 (37.2–43.5)$^{a1, b2}$	**<0.001**
Maresin 1 (pg/mL)	987 (550–2204)	462 (290–1177)^{a2}	480 (380–990)^{a2}	**0.003**
Glucose (mg/dL)	83.2±8.01	88.1±6.88	94.3±8.87$^{a1, b2}$	**<0.001**
Insulin (mIU/L)	8.65 (5.70–11.7)	12.9 (10.3–17.4)^{a2}	19.8 (13.5–32.0)$^{a1, b2}$	**<0.001**
HOMA-IR	1.70 (1.23–2.24)	2.80 (2.40–3.82)^{a1}	4.58 (3.06–7.74)$^{a1, b2}$	**<0.001**
METS-IR	32.4 (29.9–34.2)	41.3 (37.7–44.5)^{a1}	49.4 (45.9–52.9)$^{a1, b1}$	**<0.001**
Triglyceride-glucose index	8.35±0.47	8.57±0.52	8.96±0.51$^{a1, b2}$	**<0.001**
TyG-BMI	192 (176–208)	237 (223–248)^{a1}	284 (275–306)$^{a1, b1}$	**<0.001**
QUICKI	0.36±0.02	0.33 ±0.02^{a1}	0.31±0.02$^{a1, b1}$	**<0.001**
Mcauley index	7.46 ±1.12	6.29±1.23^{a2}	5.02±1.16$^{a1, b1}$	**<0.001**
Hemoglobin A1c (%)	5.23 (5.14–5.50)	5.71 (5.40–5.90)^{a2}	5.90 (5.60–6.20)^{a1}	**<0.001**
Alanine transaminase (U/L)	19.2±6.45	19.5±7.59	18.9±7.33	0.953
Aspartate transaminase (U/L)	20.0 (17.0–23.0)	19.0 (17.2–25.0)	20.5 (17.2–24.0)	0.875
Cholesterol (mg/dL)	187±30.1	179±41.5	203±48.6	0.070
Triglyceride (mg/dL)	101 (79–141)	111 (89–189)	179 (126–224)$^{a1, b2}$	**0.001**
LDL-C (mg/dL)	112 (89–132)	102 (88–127)	132 (98–156)	0.083
HDL-C (mg/dL)	53.0 (47.0–61.0)	45.0 (40.0–60.0)	46.0 (39.0–54.0)^{a2}	**0.031**
TG/HDL-C	2.13 (1.41–2.57)	2.43 (1.53–4.30)	3.51 (2.39–5.39)^{a1}	**<0.001**
Castelli risk index I	3.55±0.77	3.83±1.14	4.56±1.40^{a2}	**0.003**
Castelli risk index II	2.18 (1.55–2.69)	2.16 (1.76–2.76)	2.82 (2.07–3.64)$^{a2, b2}$	**0.006**
Atherosclerotic index	2.55±0.77	2.83±1.14	3.56±1.40$^{a2, b2}$	**0.004**

*: p<0.05: Statistically significant. For pairwise comparisons between the three groups, Bonferroni correction was applied, setting the statistical significance threshold at p<0.017. ^{a}: Comparison with normal weight group; ^{a1}: <0.001; ^{a2}: <0.017; ^{b}: Comparison with overweight group. ^{b1}: <0.001; ^{b2}: <0.017. HOMA-IR: Homeostatic model assessment of insulin resistance; METS-IR: Metabolic score for insulin resistance; TyG-BMI: Triglyceride-glucose index and body mass index; QUICKI: Quantitative insulin sensitivity check index; LDL-C: Low-density lipoprotein cholesterol; HDL-C: High-density lipoprotein cholesterol; TG/HDL-C: Triglycerides to high-density lipoprotein cholesterol ratio.

levels. P-value below 0.05 was regarded as statistically significant. However, for pairwise comparisons between the three groups, Bonferroni correction was applied, setting the statistical significance threshold at p<0.017. Statistical analyses were performed and graphs were generated using SPSS v. 26 (IBM Corp., Armonk, NY, US) and GraphPad Prism v. 8.3.0 (GraphPad Software, San Diego, California, US). Post-hoc power analyses were performed using G*Power version 3.1.9.7 (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany).

Results

Glucose, insulin, HOMA-IR, METS-IR, TyG, TyG-BMI, HbA1c, TG, TG/HDL-C, CRI-I, CRI-II, and AI were higher in obese individuals than in normal-weight individuals; QUICKI, Mcauley index, and HDL-C were lower in obese individuals than in normal-weights. Insulin, HOMA-IR, METS-IR, TyG-BMI, and HbA1c levels were higher in overweight patients than in normal-weight patients. QUICKI, and McAuley index levels were lower in overweights than in normal-weights. Glucose, insulin, HOMA-IR, METS-IR, TyG, TyG-BMI, TG, CR-II, and AI were higher in obese individuals than in overweight individuals. QUICKI and Mcauley indices were lower in obese subjects than in overweight subjects. MaR1 levels were lower in both the obese [480 (380–990)] and overweight [462 (290–1177)] groups than in the normal-weight group [987 (550–2204)]. The analysis revealed that MaR1 levels did not differ between individuals classified as obese and those classified as overweight (Table 1 and Fig. 1a). A post-hoc power analysis was conducted for Maresin 1 among the BMI groups. The mean and standard deviation values were used to determine the effect size, which was determined to be Cohen's f=0.34, with an alpha of 0.05, a total sample size of 90, and three groups, yielding a power of 0.82. Given that the power exceeded the ideal value of 0.80, it could be concluded that the power was sufficient.

There were no differences in age, sex, glucose, TyG, Mcauley index, ALT, AST, cholesterol, TG, LDL-C, HDL-C, TG/HDL-C, CRI-I, CR-II, and AI parameters between the groups with MaR1≥608 levels and MaR1<608 levels. BMI, BF%, insulin,

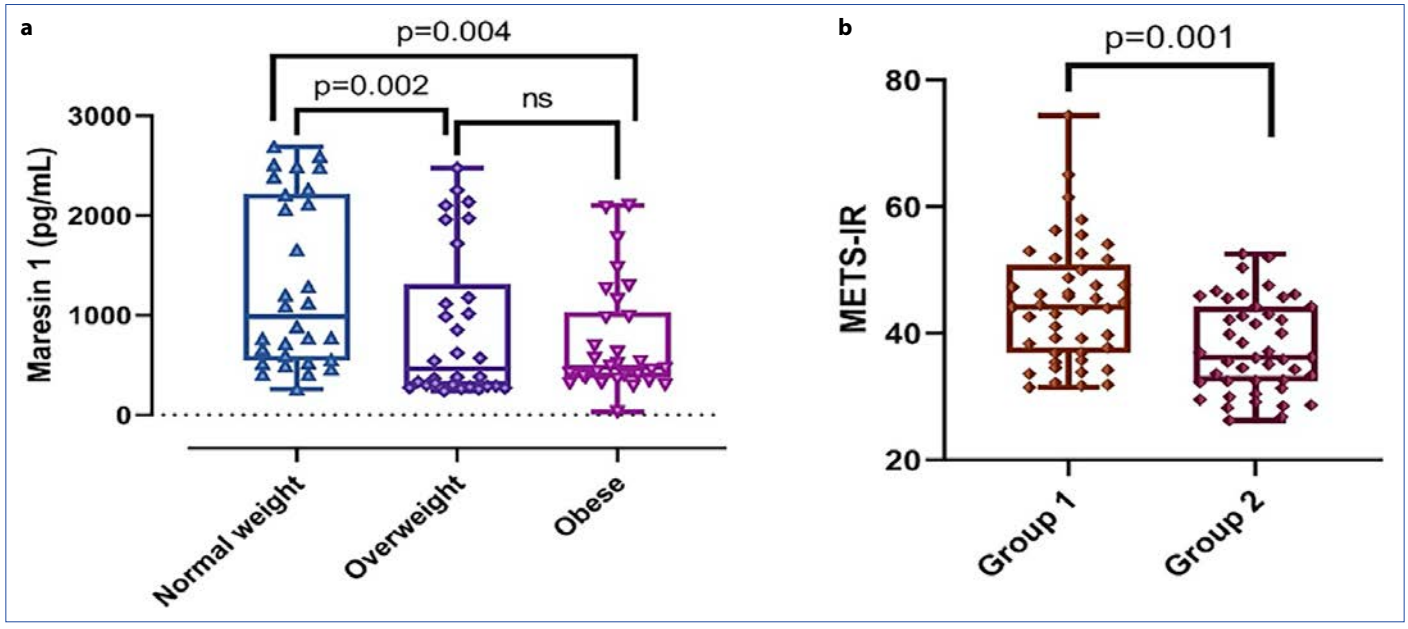

Figure 1. (a) Comparison of Maresin-1 levels among the three groups. (b) Comparison of METS-IR between the two groups based on the median Maresin-1 levels.

ns: Non-significant; Group 1: Maresin-1 <608 pg/mL; Group 2: Maresin-1≥ 608 pg/mL. METS-IR: Metabolic score for insulin resistance.

HOMA-IR, METS-IR, TyG-BMI, and HbA1c were higher in the MaR1<608 group than in the MaR1≥608 group, whereas only QUICKI was lower in the MaR1<608 group than in the MaR1≥608 group (Table 2 and Fig. 1b). A post-hoc power analysis was conducted for METS-IR between the two groups. The mean and standard deviation values were used to determine the effect size, which was determined to be Cohen's d=0.80, with an alpha of 0.05, the sample size for each group of 45, and two groups, yielding a power of 0.97. Given that the power output exceeded the ideal value of 0.80, it can be concluded that the power was sufficient.

MaR1 levels were negatively related to BMI (r=-0.495, p<0.001), BF% (r=-0.366, p<0.001), glucose (r=-0.294, p=0.005), insulin (r=-0.285, p=0.006), HOMA-IR (r=-0.318, p=0.002), METS-IR (r=-0.444, p<0.001), TyG-BMI (r=-0.427, p<0.001), and HbA1c (r=-0.247, p=0.019), whereas QUCKI (r=0.318, p=0.002) levels were positively correlated. The metabolic index with the highest correlation coefficient with MaR1 level was METS-IR (Table 3 and Fig. 2).

In the ROC analysis of the metabolic indicators for the identification of high levels of MaR1, METS-IR had the highest AUC value of 0.706 (95% CI=0.600–0.797), presenting 73.3% sensitivity and 57.8% specificity, p<0.001. TyG-BMI had an AUC value of 0.701 (95% CI=0.596–0.793), with 68.9% sensitivity and 71.1% specificity, p<0.001. HbA1c had an AUC value of 0.637 (95% CI=0.528–0.735), with 68.9% sensitivity and 57.8% specificity, p=0.021. HOMA-IR showed an AUC value of 0.635 (95% CI=0.527–0.734), with 60.0% sensitivity and 64.4% specificity, p=0.022. QUICKI performed the AUC value was 0.634 (95% CI=0.525–0.733), presenting 46.7% of sensitivity and 75.6% of specificity, p=0.022 (Table 4 and Fig. 3).

The findings of ordinal logistic regression exhibited an independent inverse association between MaR1 levels and BMI categories. In Model 1 (unadjusted), MaR1 was significantly associated with BMI categories, showing an odds ratio (OR) value of 0.9992 (95% CI:0.9987–0.9998, p=0.005). Adjusted for age, sex, and AI, this association remained significant, with an OR value of 0.9992 (95% CI:0.9986–0.9997, p=0.004), in Model 2. In Model 3, which included adjustments for age, sex, TyG and ALT, the association persisted, with an OR value of 0.9991 (95% CI:0.9985–0.9997, p=0.003) (Table 5).

Discussion

Lower serum MaR1 levels were found in obese and overweight subjects compared to normal-weight individuals, along with their correlation with BMI and BF%, indicating an association between MaR1 and obesity. This study found negative correlations between MaR1 levels and several metabolic parameters, including HOMA-IR, METS-IR, TyG-BMI, and HbA1c. Conversely, MaR1 levels were positively linked to QUICKI, suggesting an association between higher MaR1 levels and improved insulin sensitivity. Among the metabolic indicators examined, METS-IR demonstrated the strongest correlation with MaR1 levels (r=-0.444). ROC analysis revealed that METS-IR and TyG-BMI were the most effective parameters for identifying high MaR1 levels, with AUC of 0.706 and 0.701, respectively. METS-IR and TyG-BMI are useful surrogate markers for insulin resistance with significant associations with various cardiovascular conditions [17, 18]. These findings indicate that MaR1 is strongly associated with various insulin-resistance indices, including METS-IR and TyG-BMI, which are linked to high cardiovascular risk.

Table 2. Comparison of demographic and laboratory parameters between the two groups based on median Maresin 1 levels

Parameter	Maresin 1<608 pg/mL (n= 45)	Maresin 1≥608 pg/mL (n= 45)	p*
Age (years)	37.2±9.88	38.8±9.94	0.459
Gender (n, %)			
Male	10 (22.2)	11 (24.4)	0.803
Female	35 (77.8)	34 (75.6)	
Body mass index (kg/m^2)	29.4 (27.2–32.5)	25.6 (23.1–29.1)	**<0.001**
Body fat (%)	36.3±7.34	31.6±6.86	**0.002**
Glucose (mg/dL)	90.3±9.68	86.8±8.20	0.068
Insulin (mIU/L)	13.5 (10.1–22.3)	11.7 (8.16–14.8)	**0.036**
HOMA-IR	2.96 (2.30–5.58)	2.58 (1.62–3.19)	**0.028**
METS-IR	44.2 (36.9–50.4)	36.2 (32.4–44.3)	**0.001**
Triglyceride-glucose index	8.62±0.61	8.64±0.50	0.896
TyG-BMI	250 (217–291)	218 (190–263)	**0.001**
QUICKI	0.32±0.03	0.34±0.03	**0.019**
Mcauley index	6.09±1.60	6.42±1.45	0.314
Hemoglobin A1c (%)	5.69±0.48	5.50±0.39	**0.035**
Alanine transaminase (U/L)	19.0 (15.8–24.3)	18.0 (13.0–23.0)	0.175
Aspartate transaminase (U/L)	20.0 (18.0–24.3)	19.0 (16.8–24.3)	0.411
Cholesterol (mg/dL)	187 (157–214)	186 (163–222)	0.614
Triglyceride (mg/dL)	113 (91.3–185)	135 (93.8–187)	0.548
LDL-C (mg/dL)	109 (92.5–138)	109 (89.0–145)	0.981
HDL-C (mg/dL)	49.0 (40.8–58.0)	49.0 (41.8–56.5)	0.657
TG/HDL-C	2.60 (1.55–4.64)	2.37 (1.83–3.56)	0.971
Castelli risk index I	3.88 (3.06–4.72)	3.76 (3.10–4.27)	0.625
Castelli risk index II	2.44 (1.88–3.17)	2.22 (1.81–2.82)	0.503
Atherosclerotic index	2.88 (2.06–3.72)	2.76 (2.10–3.27)	0.625

*: p<0.05: Statistically significant. HOMA-IR: Homeostatic model assessment of insulin resistance; METS-IR: Metabolic score for insulin resistance; TyG-BMI: Triglyceride-glucose index and body mass index; QUICKI: Quantitative insulin sensitivity check index; LDL-C: Low-density lipoprotein cholesterol; HDL-C: High-density lipoprotein cholesterol; TG/HDL-C: Triglycerides to high-density lipoprotein cholesterol ratio.

Insulin resistance serves as a pivotal factor in the emergence of numerous metabolic disorders such as obesity, cardiovascular diseases, non-alcoholic fatty liver disease (NAFLD) [19]. Studies investigating the relationship between MaR1, obesity, and insulin resistance in the human population are notably limited in the current literature. T2DM patients, particularly those with diabetic foot ulcers, exhibited lower plasma MaR1 concentrations compared to individuals with normal glucose tolerance. Reduced MaR1 levels were closely linked to obesity, reduced insulin secretion, and elevated insulin resistance (HOMA-IR). MaR1 levels were positively related to beta-cell function (HOMA-β), acute insulin response, and HDL-C [20]. In a study, no significant differences in MaR1 levels were observed between individuals with mild and morbid obesity. However, differences in diabetes remission and the capacity for inflammation resolution following surgery were identified as significant factors influencing MaR1 levels. Specifically, diabetic patients who failed to achieve remission experienced a substantial impairment in MaR1 production. Sufficient MaR1 production was linked to the control of inflammation and improved insulin sensitivity. Therefore, therapies aimed at enhancing MaR1 biosynthesis might represent potential strategies against insulin resistance [6]. MaR1 activates brown adipose tissue while causing browning of white adipose tissue, suggesting that this molecule might

Table 3. Significant correlations between Maresin 1 and the other variables in all groups

Parameter	Maresin 1 (pg/mL)	
	r	p
Body mass index (kg/m^2)	-0.495	<0.001
Body fat (%)	-0.366	<0.001
Glucose (mg/dL)	-0.294	0.005
Insulin (mIU/L)	-0.285	0.006
HOMA-IR	-0.318	0.002
METS-IR	-0.444	<0.001
TyG-BMI	-0.427	<0.001
QUICKI	0.318	0.002
Hemoglobin A1c (%)	-0.247	0.019

r: Spearman correlation. HOMA-IR: Homeostatic model assessment of insulin resistance; METS-IR: Metabolic score for insulin resistance; TyG-BMI: Triglyceride-glucose index and body mass index; QUICKI: Quantitative insulin sensitivity check index.

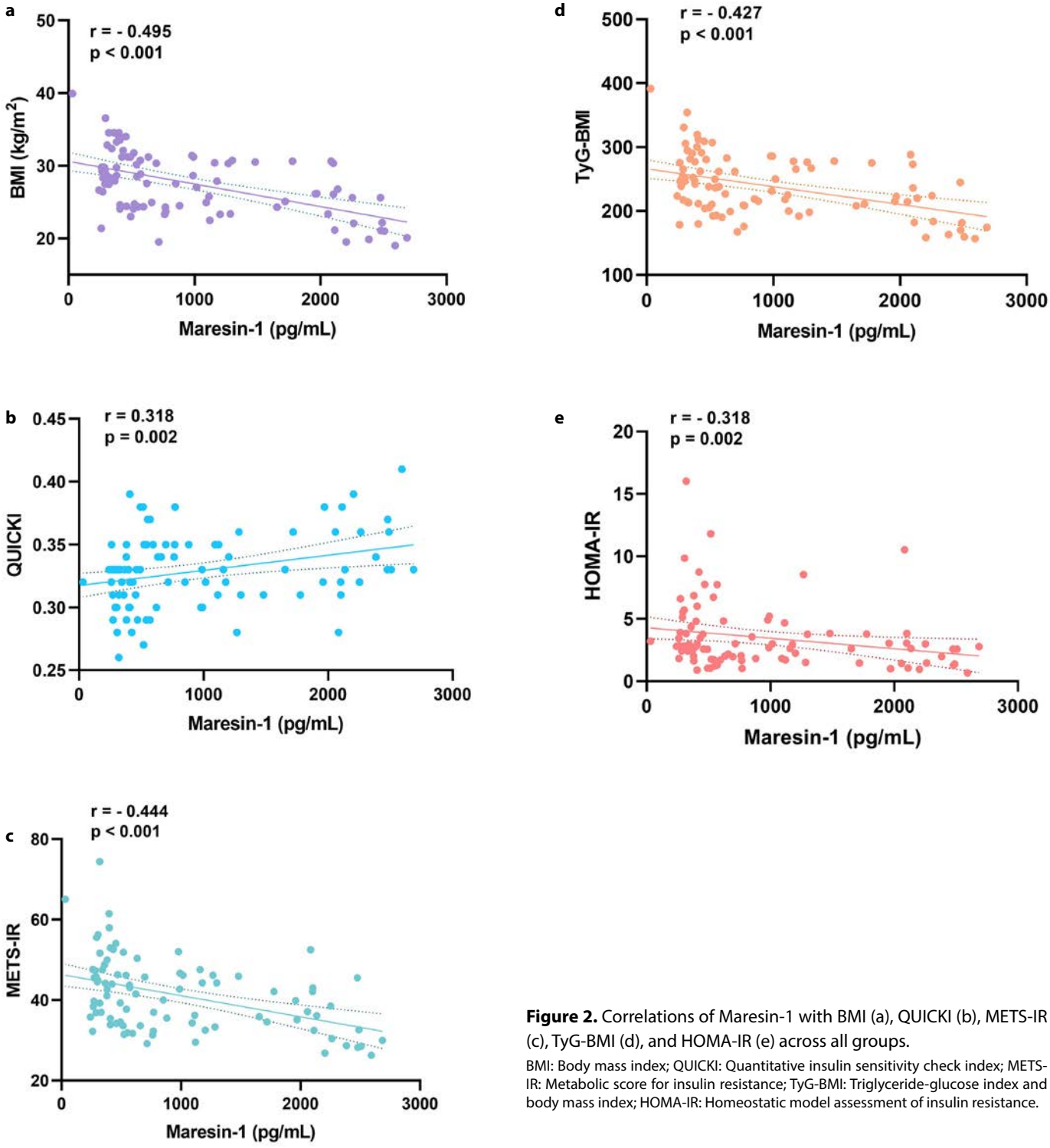

Figure 2. Correlations of Maresin-1 with BMI (a), QUICKI (b), METS-IR (c), TyG-BMI (d), and HOMA-IR (e) across all groups.

BMI: Body mass index; QUICKI: Quantitative insulin sensitivity check index; METS-IR: Metabolic score for insulin resistance; TyG-BMI: Triglyceride-glucose index and body mass index; HOMA-IR: Homeostatic model assessment of insulin resistance.

contribute to the control of adipokine synthesis and release in obese individuals [21, 22]. Circulating MaR1 concentrations were found to be markedly reduced in individuals with NAFLD. MaR1 levels were inversely correlated with BMI, glucose, ALT, GGT, and TG levels. Conversely, MaR1 levels were positively and independently associated with AST/ALT ratio, albumin level, albumin-globulin ratio, and HDL-C level. The proportion of patients diagnosed with NAFLD showed a progressive decline across ascending MaR1 quartiles. This relationship points to MaR1's potential role in metabolic health and inflammation, as decreased MaR1 might contribute to metabolic dysfunctions associated with higher BMI [23]. Our research revealed no association between MaR1 concentrations and the analyzed lipid measurements and parameters.

Table 4. ROC analysis of metabolic indicators for identifying high Maresin 1 levels

Parameter	AUC (95 CI%)	Cut-off	Sensitivity	Specificity	LR (+)	LR (–)	p
METS-IR	0.706 (0.600–0.797)	≤43.0	73.3%	57.8%	1.74	0.46	<0.001
TyG-BMI	0.701 (0.596–0.793)	≤236	68.9%	71.1%	2.38	0.44	<0.001
Hemoglobin A1c (%)	0.637 (0.528–0.735)	≤5.70	68.9%	57.8%	1.63	0.54	0.021
HOMA-IR	0.635 (0.527–0.734)	≤2.69	60.0%	64.4%	1.69	0.62	0.022
QUICKI	0.634 (0.525–0.733)	>0.33	46.7%	75.6%	1.91	0.71	0.022

ROC: Receiver operating characteristic; AUC: Area under the curve; CI: Confidence interval; LR: Likelihood ratio; METS-IR: Metabolic score for insulin resistance; TyG-BMI: Triglyceride-glucose index and body mass index; HOMA-IR: Homeostatic model assessment of insulin resistance; QUICKI: Quantitative insulin sensitivity check index.

Table 5. Ordinal logistic regression analysis results

Regression model	Maresin-1 (pg/mL)				
	Estimate	SE	Wald	OR (95%CI)	p
Model 1	-0.001	0.000	7.974	0.9992 (0.9987–0.9998)	**0.005**
Model 2	-0.001	0.000	8.341	0.9992 (0.9986–0.9997)	**0.004**
Model 3	-0.001	0.000	8.672	0.9991 (0.9985–0.9997)	**0.003**

Model 1: Unadjusted; Model 2: Age, sex, AI; Model 3: Age, sex, TyG, ALT. SE: Standard error; OR: Odds Ratio; CI: Confidence interval; TyG: triglyceride glucose index; ALT: alanine aminotransferase.

MaR1 enhanced insulin sensitivity and reduces inflammation in the white adipose tissue of obese mice by modulating inflammatory markers and activating insulin signaling pathways, such as Akt and AMPK. MaR1 treatment has the potential to serve as an effective therapeutic approach aimed at improving insulin sensitivity in obese mouse models, thereby addressing the key metabolic dysfunctions associated with obesity and potentially mitigating the risk of related complications [22, 24]. MaR1 has been demonstrated to modulate the expression of adipokines in obese models. In cultured human adipocytes, MaR1 increased the basal expression of adiponectin, leptin, dipeptidylpeptidase 4, cardiotrophin 1, and irisin while effectively counteracting the effects of TNF-α. This regulatory mechanism could counteract inflammation and improve insulin sensitivity. The study suggested that MaR1 tissue-specific actions could be harnessed to improve metabolic profiles by reducing inflammation and restoring healthy adipokine levels in obesity [25]. Additionally, MaR1 suppresses lipid accumulation and endoplasmic reticulum stress in hepatocytes, resulting in reduced hepatic steatosis and improved lipid metabolism in high-fat diet-fed mice [26]. Mitochondrial damage in liver cells is frequently observed in fatty liver disease associated with metabolic dysfunction. MaR1 enhanced liver mitochondrial and metabolic performance, protecting liver cells from mitochondrial impairment that was induced by factors promoting obesity and fibrosis [27]. Preclinical studies have suggested that SPMs could be effective in preventing and managing cardiovascular disease by enhancing endogenous SPM production through polyunsaturated fatty acids or by administering synthetic SPM analogues [4]. Generally,

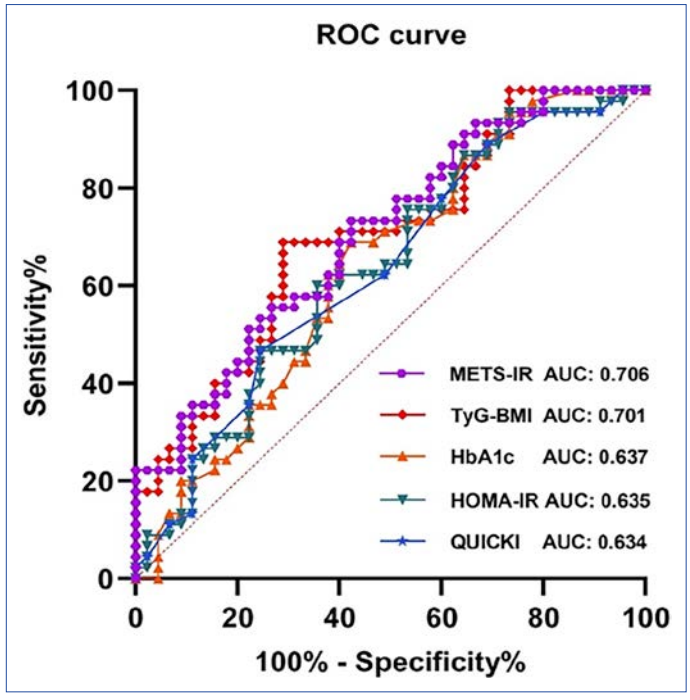

Figure 3. ROC analysis of metabolic indicators for identifying high Maresin 1 levels.

ROC: Receiver operating characteristic; METS-IR: Metabolic score for insulin resistance; TyG-BMI: Triglyceride-glucose index and body mass index; HbA1c: Hemoglobin A1c; HOMA-IR: Homeostatic model assessment of insulin resistance; QUICKI: Quantitative insulin sensitivity check index.

MaR1 and its related metabolites have cardiovascular protective functions and/or inhibit the progression of cardiovascular

diseases [28]. Since most of the studies on MaR1 and obesity are experimental, conclusive results can be achieved through data gathered from more comprehensive human studies.

Although the relationship between MaR1 and lipid and glucose indicators has been demonstrated on a molecular basis in mouse models, research exploring this connection in humans remains limited. The current literature does not directly investigate the association between MaR1 and METS-IR, TyG-BMI, or QUICKI indices. METS-IR index, which combines fasting triglyceride, glucose, BMI and HDL-C, is now recognized as a more accurate tool for evaluating insulin sensitivity. METS-IR is associated with various cardiovascular events, and its ability to predict inflammatory activity and endothelial dysfunction has been emphasized [29, 30]. In non-diabetic Korean individuals, a high METS-IR score demonstrated a significant prognostic value for future occurrences of ischemic heart disease [31]. The TyG-BMI index combines the TyG index, which reflects glucose and triglyceride levels, with BMI, which is a measure of adiposity. This combination seems to offer a more extensive evaluation of metabolic status than the TyG index or BMI alone [32]. According to a cohort investigation, TyG-BMI emerged as the most effective indicator for predicting metabolic syndrome in male subjects [33]. TyG-BMI and METS-IR were shown to be strongly associated with NAFLD and were identified as the most valuable IR-related indicators with high discriminatory ability for NAFLD screening [34].

This study has some limitations, including its single-center cross-sectional design. Inflammatory parameters, including cytokines, which could have provided insights into the relationship between MaR1 and inflammation in obesity, have not been evaluated. Although liquid chromatography–mass spectrometry (LC-MS) is widely used for the analysis of SPMs such as Maresin 1 due to its high specificity, precision, and accuracy in lipid quantification, ELISA was chosen for this study because of its sensitivity, methodological simplicity, and feasibility, as LC-MS was not available in our laboratory [35]. Prospective follow-up to predict obesity-related vascular events or morbidity could not be performed.

Conclusion

Our study demonstrated significant associations between MaR1 levels and insulin resistance indices, such as METS-IR and TyG-BMI, which are also indicative of cardiovascular risk in obese patients. Furthermore, ordinal regression analysis revealed an independent negative relationship between the MaR1 levels and obesity. This investigation addresses a crucial void in current research by offering novel perspectives on the connection between MaR1 and these key insulin resistance indices. Research into the role of lipid mediators like MaR1 in resolving inflammation and improving insulin sensitivity could provide new therapeutic avenues. To fully elucidate the metabolic mechanisms associated with obesity and to explore the therapeutic efficacy of MaR1 in humans, more extensive clinical investigations are essential.

Ethics Committee Approval: The study was approved by The Firat University Non-interventional Research Ethics Committee (No: 2023/10-23, Date: 27/07/2023).

Authorship Contributions: Concept – L.D., M.Y.; Design – L.D., M.Y.; Supervision – L.D.; Materials – M.Y., M.A.S.; Data collection &/or processing – M.Y., M.A.S.; Analysis and/or interpretation – L.D., R.F.A.; Literature search – L.D., M.Y., M.A.S., R.F.A.; Writing – L.D., M.Y., R.F.A.; Critical review – L.D., M.Y., M.A.S., R.F.A.

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References

1. Czech MP. Insulin action and resistance in obesity and type 2 diabetes. Nat Med 2017;23(7):804–14. [CrossRef]
2. Chen G. The Interactions of Insulin and Vitamin A Signaling Systems for the Regulation of Hepatic Glucose and Lipid Metabolism. Cells. 2021;10(8):2160. [CrossRef]
3. Goossens GH. The metabolic phenotype in obesity: Fat mass, body fat distribution, and adipose tissue function. Obes Facts 2017;10(3):207–15. [CrossRef]
4. Salazar J, Pirela D, Nava M, Castro A, Angarita L, Parra H, et al. Specialized proresolving lipid mediators: A potential therapeutic target for atherosclerosis. Int J Mol Sci 2022;23(6):3133. [CrossRef]
5. Laiglesia LM, Escote X, Sainz N, Felix-Soriano E, Santamaria E, Collantes M, et al. Maresin 1 activates brown adipose tissue and promotes browning of white adipose tissue in mice. Mol Metab 2023;74:101749. [CrossRef]
6. Schulte F, Asbeutah AA, Benotti PN, Wood GC, Still C, Bistrian BR, et al. The relationship between specialized pro-resolving lipid mediators, morbid obesity and weight loss after bariatric surgery. Sci Rep 2020;10(1):20128. [CrossRef]
7. Serhan CN, Chiang N, Dalli J, Levy BD. Lipid mediators in the resolution of inflammation. Cold Spring Harb Perspect Biol 2014;7(2):a016311. [CrossRef]
8. Seidell JC, Flegal KM. Assessing obesity: Classification and epidemiology. Br Med Bull 1997;53(2):238–52. [CrossRef]
9. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: Insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28(7):412–9. [CrossRef]
10. Hou Y, Wu X, Shi Y, Xu X, Zhang Y, Jiang L, et al. METS-IR as an important predictor of neurological impairment severity in patients with severe cerebral infarction: A multicenter study based on the Chinese population. Front Neurol 2024;15:1450825. [CrossRef]
11. Romero-Saldana M, Tauler P, Vaquero-Abellan M, Lopez-Gonzalez AA, Fuentes-Jimenez FJ, Aguilo A, et al. Validation of a

non-invasive method for the early detection of metabolic syndrome: A diagnostic accuracy test in a working population. BMJ Open 2018;8(10):e020476. [CrossRef]
12. Er LK, Wu S, Chou HH, Hsu LA, Teng MS, Sun YC, et al. Triglyceride glucose-body mass index is a simple and clinically useful surrogate marker for insulin resistance in nondiabetic individuals. PLoS One 2016;11(3):e0149731. [CrossRef]
13. Katz A, Nambi SS, Mather K, Baron AD, Follmann DA, Sullivan G, et al. Quantitative insulin sensitivity check index: A simple, accurate method for assessing insulin sensitivity in humans. J Clin Endocrinol Metab 2000;85(7):2402–10. [CrossRef]
14. McAuley KA, Williams SM, Mann JI, Walker RJ, Lewis-Barned NJ, Temple LA, et al. Diagnosing insulin resistance in the general population. Diabetes Care 2001;24(3):460–4. [CrossRef]
15. Andrades CDS, Poletti VV, Closs VE, Gustavo ADS, Oliveira MDS, Donadio MVF, et al. Prevalence of dyslipidemia, atherogenic and cardiovascular risk in overweight and obese adolescents. Rev Paul Pediatr 2023;41:e2021312. [CrossRef]
16. Zhang D, Hu RH, Cui XM, Jiang XH, Zhang S. Lipid levels and insulin resistance markers in gastric cancer patients: Diagnostic and prognostic significance. BMC Gastroenterol 2024;24(1):373. [CrossRef]
17. Wang S, Zhang X, Keerman M, Guo H, He J, Maimaitijiang R, et al. Impact of the baseline insulin resistance surrogates and their longitudinal trajectories on cardiovascular disease (coronary heart disease and stroke): A prospective cohort study in rural China. Front Endocrinol Lausanne 2023;14:1259062. [CrossRef]
18. Qian T, Sheng X, Shen P, Fang Y, Deng Y, Zou G. Mets-IR as a predictor of cardiovascular events in the middle-aged and elderly population and mediator role of blood lipids. Front Endocrinol Lausanne 2023;14:1224967. [CrossRef]
19. Drabczyk M, Kosyra K, Magda I, Marczyńska Z, Zyśk A. Insulin resistance and metabolic diseases - a review. J Educ Health Sport 2024;61:186–99. [CrossRef]
20. Miao T, Huang B, He N, Sun L, Du G, Gong X, et al. Decreased plasma Maresin 1 concentration is associated with diabetic foot ulcer. Mediators Inflamm 2020;2020:4539035. [CrossRef]
21. Spite M, Claria J, Serhan CN. Resolvins, specialized proresolving lipid mediators, and their potential roles in metabolic diseases. Cell Metab 2014;19(1):21–36. [CrossRef]
22. Martinez-Fernandez L, Gonzalez-Muniesa P, Laiglesia LM, Sainz N, Prieto-Hontoria PL, Escote X, et al. Maresin 1 improves insulin sensitivity and attenuates adipose tissue inflammation in ob/ob and diet-induced obese mice. FASEB J 2017;31(5):2135–45. [CrossRef]
23. Fang X, Wang H, Ye T, Fu X, Tan X, Zeng Y, et al. Low serum Maresin-1 levels are associated with non-alcoholic fatty liver disease: A cross-sectional study. Lipids Health Dis 2021;20(1):96. [CrossRef]
24. Martinez-Fernandez L, Gonzalez-Muniesa P, Sainz N, Escote X, Martinez JA, Arbones-Mainar JM, et al. Maresin 1 regulates insulin signaling in human adipocytes as well as in adipose tissue and muscle of lean and obese mice. J Physiol Biochem 2021;77(1):167–73. [CrossRef]
25. Martinez-Fernandez L, Burgos M, Sainz N, Laiglesia LM, Arbones-Mainar JM, Gonzalez-Muniesa P, et al. Maresin 1 exerts a tissue-specific regulation of adipo-hepato-myokines in diet-induced obese mice and modulates adipokine expression in cultured human adipocytes in basal and inflammatory conditions. Biomolecules 2023;13(6):919. [CrossRef]
26. Jung TW, Kim HC, Abd El-Aty AM, Jeong JH. Maresin 1 attenuates NAFLD by suppression of endoplasmic reticulum stress via AMPK-SERCA2b pathway. J Biol Chem 2018;293(11):3981–8. [CrossRef]
27. Lopez-Vicario C, Sebastian D, Casulleras M, Duran-Guell M, Flores-Costa R, Aguilar F, et al. Essential lipid autacoids rewire mitochondrial energy efficiency in metabolic dysfunction-associated fatty liver disease. Hepatology 2023;77(4):1303–18. [CrossRef]
28. Liu WC, Yang YH, Wang YC, Chang WM, Wang CW. Maresin: Macrophage mediator for resolving inflammation and bridging tissue regeneration-a system-based preclinical systematic review. Int J Mol Sci 2023;24(13):11012. [CrossRef]
29. Bello-Chavolla OY, Almeda-Valdes P, Gomez-Velasco D, Viveros-Ruiz T, Cruz-Bautista I, Romo-Romo A, et al. METS-IR, a novel score to evaluate insulin sensitivity, is predictive of visceral adiposity and incident type 2 diabetes. Eur J Endocrinol 2018;178(5):533–44. [CrossRef]
30. Wang Z, Li W, Li J, Liu N. The nonlinear correlation between a novel metabolic score for insulin resistance and subclinical myocardial injury in the general population. Front Endocrinol Lausanne 2022;13:889379. [CrossRef]
31. Yoon J, Jung D, Lee Y, Park B. The Metabolic Score for Insulin Resistance (METS-IR) as a predictor of incident ischemic heart disease: A longitudinal study among Korean without diabetes. J Pers Med 2021;11(8):742. [CrossRef]
32. Song K, Park G, Lee HS, Choi Y, Oh JS, Choi HS, et al. Prediction of insulin resistance by modified triglyceride glucose indices in youth. Life Basel 2021;11(4):286. [CrossRef]
33. Gui J, Li Y, Liu H, Guo LL, Li J, Lei Y, et al. Obesity- and lipid-related indices as a predictor of obesity metabolic syndrome in a national cohort study. Front Public Health 2023;11:1073824. [CrossRef]
34. Li H, Shi Z, Chen X, Wang J, Ding J, Geng S, et al. Relationship between six insulin resistance surrogates and nonalcoholic fatty liver disease among older adults: A cross-sectional study. Diabetes Metab Syndr Obes 2023;16:1685–96. [CrossRef]
35. Cross TG, Hornshaw MP. Can LC and LC-MS ever replace immunoassays? J Appl Bioanal 2016;2(4):108–16. [CrossRef]

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Research Article

Unnecessary serum protein electrophoresis test requests in the follow-up of multiple myeloma patients can be prevented

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Abstract

Objectives: Appropriate testing is a part of good laboratory practices. "Requesting the right test with the right method, at the right time, to the right patient, to produce the right result at the right cost" has been defined as an appropriate test request. This study was intended to measure the impact of an attend to regulate requests for serum protein electrophoresis tests before and after applying rejection rules and clinical management.

Methods: In a meeting in December 2022, hematologists declared to be more careful about proper testing in electrophoresis. In addition, the laboratory was decided to be involved in test request management through test rejection rules. Multiple myeloma patients with measurable M protein spikes in the gamma regions of serum protein electrophoresis tests were chosen due to relatively well-defined follow-up protocols. Number of hospital visits of the patients and electrophoresis test requests were compared with the year before (2022) and the year after (2023) the meeting.

Results: Selected 92 patients visited our hospital 493 times in 2022 and 583 times in 2023 (number of visits). A total of 423 serum protein electrophoresis (SPE) and 416 serum immunofixation electrophoresis (SIFE) tests were requested in 2022 while 427 SPE and 470 SIFE tests were requested in 2023. In 2023, 51 SPE and 36 SIFE test requests were rejected according to the defined test rejection rules.

Conclusion: From 2022 to 2023 total patient visits increased by 18%, while SPE test requests increased by less than 1% and SIFE test requests increased by 13%. The common will by the Hematology Clinic and the Clinical Biochemistry Laboratory to reduce unnecessary electrophoresis test requests achieved their goal as the rise in test requests were under the rise in hospital visits. After a year of experience, we could confidently propose that our test rejection rules can be adopted by laboratories and used for electrophoresis test management.

Keywords: Continuous quality management, electrophoresis, good laboratory practices, medical laboratory, multiple myeloma

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Appropriate testing is a part of good laboratory practices. "Requesting the right test with the right method, at the right time, to the right patient, to produce the right result at the right (reasonable) cost" has been defined as an appropriate test request [1]. Particularly considering serum protein electrophoresis (SPE) and serum protein immunofixation electrophoresis (SIFE), in addition to the high cost of the tests, a major concern is the cost of time and effort of a very specialized technician and laboratory specialist. Gel electrophoresis is one of the last conventional tests in the clinical laboratory. Semi-automated gel electrophoresis requires plenty of handwork of a specialized and experienced technician while reporting the tests requires extra time and effort of a specialized and experienced laboratory specialist [2].

SPE and/or SIFE tests maintain their importance in the diagnosis and follow-up of multiple myeloma (MM) patients. The effort in harmonization of reporting electrophoresis test results is guided by the fact that the test report must provide the clinician with sufficient data to observe the response of the patient under treatment [3]. While the changes in the amount of M

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(myeloma) protein detected in SPE are evaluated as an indicator of the tumor's response to treatment; appearance or disappearance of a monoclonal band defines the clinical condition of the MM patient as relapse or remission. There are international and national guidelines (Multiple Myeloma Diagnosis and Treatment Guidelines) which update at regular intervals [4, 5].

This project of utilization of SPE and SIFE test requests was limited to patients with an M protein spike in the gamma region of SPE due to relatively clear-cut directions for the follow-up electrophoresis test requesting in the guidelines. It started with a meeting attended by laboratory specialists and hematologists. In the meeting, a joint decision was taken so that the hematologists would pay extra care in test requesting while the laboratory specialists would contribute by rejecting the unnecessary tests that escaped the clinician's attention. This retrospective study aimed to measure the effect of the project by comparing electrophoresis test requests one the year before (2022) and one year after (2023) the meeting.

Materials and Methods

Good laboratory practices

In December 2022, we conducted a presentation at The Hematology Clinic with the participation of all hematologists, where we repeated the basics of follow-up of MM patients with SPE and SIFE in line with the national clinical guideline [4]. Any request made outside the agreed national guideline was defined as inappropriateness. While the hematologists owed to be more careful about their test requests, some test rejection rules to be performed by the laboratory were defined:

Rejection rule 1: When SPE and SIFE were requested together and SPE showed a measurable M protein in the same location with the previous SPE, SIFE was rejected as an inappropriate test request, if the patient had a previous positive SIFE.

Rejection rule 2: When SPE and SIFE were requested together and SIFE was negative, SPE was rejected as an inappropriate test request.

All electrophoresis test requests were checked for rejection rules prior to performing the test. Previous electrophoresis test results were checked in detail by the laboratory specialist. The clinicians requesting the tests, which were decided to be rejected, were informed by text messages. The requested tests were rejected only after the approval of the requesting hematologist. Patient samples of the rejected tests were stored at 2–8°C for 48 hours as a caution.

Patients

The study was conducted at a 1270-bed tertiary care, medical school–affiliated medical center. Laboratory information system was searched retrospectively between 01/01/2022 and 31/12/2023. Patients who admitted to the Hematology inpatient or out-patient clinics were filtered. Within the 2 years, patient visits with at least 1 electrophoresis test request and type of electrophoresis tests requests in a visit were recorded (Visit: Visits with at least 1 electrophoresis test request). Test data were included from both in-patient and out-patient clinical encounters where any of the following were ordered: SPE and/or SIFE. Number of patients was high enough to select group patients with more than 1 year follow-up. Patients with a measurable M spike in the gamma region of a positive SPE were selected. Only patients with MM diagnosis were included (Fig. 1). The diagnosis of the patients was identified by the ICD code and confirmed by the hematologist.

This study was performed in accordance with the ethical standards set by the Declaration of Helsinki and was approved by the local ethics committee (2024-165).

Microsoft Excel 2013 was used to calculate results and create graphics.

Results

The results of 470 patients who admitted to The Hematology inpatient and out-patient clinic and with at least one SPE and/or SIFE were evaluated retrospectively. Patients without any positive SPE and/or SIFE, patients with less than 5 visits were excluded. 390 patients were with at least one SPE and/or SIFE positive test result. Of these 390 patients, 256 (65.64%) were male and 134 (34.35%) were female, with a mean age of 65,98 (±10.78) in men and 66,22 (±10.37) in women. When patients were examined in

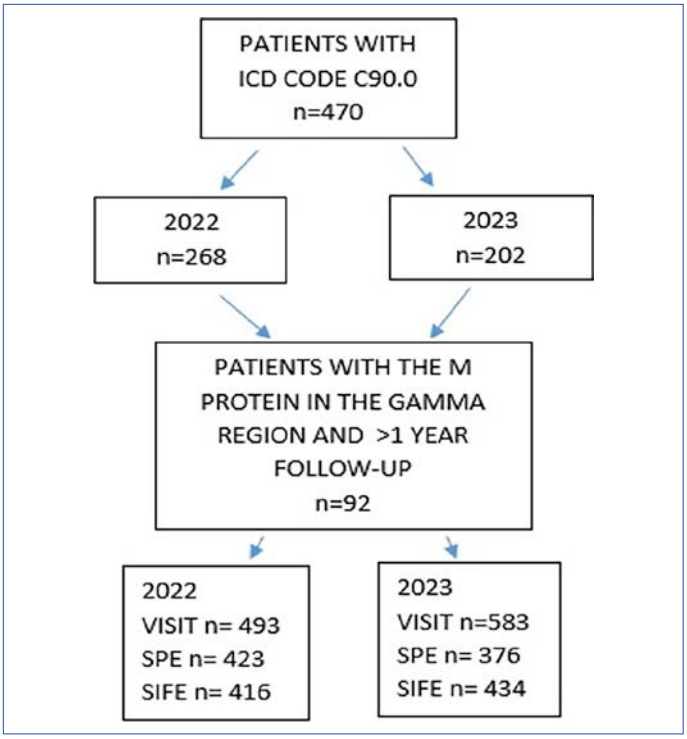

Figure 1. A total of 470 patients admitted to hematology outpatient and inpatient clinics and with the ICD code used to follow-up of monoclonal gammopathy patients in 2022 and 2023. Among the 470 patients, 390 had at least one positive SPE and/or SIFE. 92 of these 390 patients had a measurable M protein in the gamma region of SPE and had more than one year follow-up.

SPE: Serum protein electrophoresis; SIFE: Serum protein immunofixation electrophoresis.

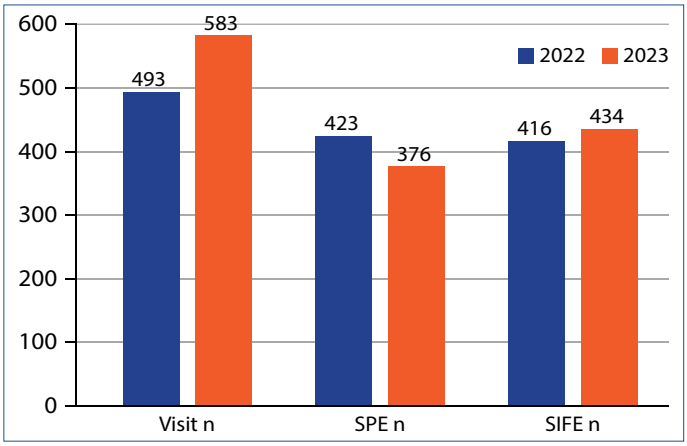

Figure 2. From 2022 to 2023 total patient visits increased by 18%, while SPE test requests increased by less than 1% and SIFE test requests increased by 13% which showed positive effort of the clinicians. The figure shows the number of tests after 51 SPE and 36 SIFE test requests were rejected by the laboratory according to test rejection rules in 2023.
SPE: Serum protein electrophoresis; SIFE: Serum protein immunofixation electrophoresis.

types of paraproteinemia, in order of frequency, we detected IgG-Kappa in 158 patients (40.51%), IgG-Lambda in 81 patients (20.76%), IgA-Kappa in 48 patients (12.30%), IgA-Lambda in 20 patients (5.12%), IgM- Kappa in 15 patients (3.84%), IgM Lambda in 7 patients (1.79%), monoclonal Kappa in 34 patients (8.71%) and monoclonal Lambda in 27 patients (6.92%).

92 of 470 patients were diagnosed and followed with MM diagnosis for more than one year period including 2022 and 2023. Of these 92 patients, 45 were male (48.91%) and 47 were female (51.08%); with a mean age of 65.76 (±8.97) in men and 66.09 (±11.49) in women. When patients were examined in types of paraproteinemia, in order of frequency, we detected IgG-Kappa in 55 patients (59.78%), IgG-Lambda in 25 patients (27.17%), IgA-Kappa in 5 patients (5.43%), IgA-Lambda in 4 patients (4.34%), IgM-Kappa in 1 patient and IgM-Lambda in 2 patients.

These 92 patients visited our hospital 493 times in 2022 and 583 times in 2023 (number of visits). A total of 423 SPE and 416 SIFE tests were requested in 2022 while 427 SPE and 470 SIFE tests were requested in 2023 (Fig. 2). In 2023 51 SPE and 36 SIFE test requests were rejected by the laboratory according to test rejection rules.

Discussion

In MM patients with a measurable M spike in the gamma region of SPE, all characteristics of the M protein are followed in the SPE test [4, 5]. The International Myeloma Working Group has determined the limit of 1 g/dL for SPE and concentrations above this limit mean a measurable M protein [3]. At concentrations below these levels, detection of M protein changes is considered clinically unreliable. Methodically, MM patients with an M protein level above 1 g/dL can be followed by protein electrophoresis until the measured M protein level drops below 1 g/dL during the treatment process. After the M protein level drops below 1 g/dL, the quantitative follow-up of the M protein by SPE will continue qualitatively by SIFE. Patients whose M protein disappears in the SPE test and without a detectable M protein in subsequent SIFE tests will now be followed up with the diagnosis of "Complete Remission" [1, 6, 7]. Unfortunately, MM is still an incurable disease and every patient will inevitably face a relapse in which the disappeared M protein will re-appear in follow-up electrophoresis tests. The Turkish Hematology Association Multiple Myeloma Diagnosis and Treatment Guideline recommends that the tests for the assessment of response to MM treatment be repeated once a month or every two months until a response is achieved, and once a response plateau is achieved, the intervals should be increased and repeated every 3–6 months [4]. In case of possible biochemical progression, the follow-up intervals are reduced to 1–2 months again [4].

MM is a very heterogeneous disease and the follow-up of each patient will include differences in its own way [4]. For example, in about 2% of patients, tumor cells do not synthesize M protein (non-secretory MM). Electrophoresis applications are naturally useless in the follow-up of these patients. In approximately 15% of cases, the M protein consists only of light chain immunoglobulins. This type of M protein may not be detected in the SPE test due to its low molecular weight and rapid clearance from the serum. M protein peaks located in the alpha and beta areas in serum protein electrophoresis may not be measured accurately due to the natural protein loads of these areas. Nevertheless, MM cases outside these groups, which show measurable M protein located in the gamma area in SPE, constitute 66% of all cases (54% in the gamma area, 12% in the gamma-beta border) [6, 7]. In the follow-up of these cases, a standard method agreed between laboratory and clinical branches should consider cost-effectiveness balances as well as proper follow-up of patients. During the treatment process, as long as detectable M protein can be observed in the gamma field, SPE alone will be sufficient to evaluate the patient's response to treatment [4]. If the patient continues to respond well to treatment, M protein will gradually decrease, and when M protein finally falls below the detectable threshold in SPE, follow-up with more sensitive tests, such as serum and urine immunofixation tests, will be appropriate. The sensitivity of serum immunofixation electrophoresis to detect M protein is approximately 10 times higher than that of the SPE test. Therefore, it is a mathematical reality that as long as measurable M protein is present in the SPE test, the SIFE test will be positive, and as long as the SIFE test is negative, the SPE test will not be positive. In national and international guidelines, MM patients with measurable amounts of M protein in the gamma field are followed up quantitatively with the SPE test [1, 6]. At this stage, the SIFE test, which gives qualitative results, has no clinical benefit and is considered an unnecessary test request [7].

Although not intended to be measured at the beginning of the study, a surprising finding of the study was the 'more than expected' decrease in MM patient hospital visits that we observed during the early post-pandemic period. It was evidenced by the 18% increase in patient visits from 2022 to 2023 which we call to be turning to the normal. This finding will contribute to the difficulty of the pandemic conditions in

this patient group, which we know to be extremely adherent and meticulous about treatment protocols. The impacts of COVID-19 pandemic on the therapy delivery in MM were significant. Utilizing access to electronic patient reports from health care organizations, Martinez-Lopez and colleagues were able to highlight the decrease in the survival of newly diagnosed MM patients [8]. Interrogating multi-national datasets, the authors found that MM patients have been more severely impacted by COVID-19 pandemic than non-MM patients.

From 2022 to 2023 total patient visits increased by 18%, while SPE test requests increased by less than 1% and SIFE test requests increased by 13% (Fig. 2). In 2023 51 SPE and 36 SIFE test requests were rejected according to the rejection rules. Both SPE and SIFE test requests were lower than the % visit rise. We were more successful in SPE test requests than SIFE test requests. After all laboratory tests of the patients were studied and verified by the laboratory and the patients were evaluated by the hematologists none of the rejected tests were re-requested confirming the feasibility of the application. A main concern of false rejection did not happen.

Pressure on hospitals to restrain health-care expenditure has resulted in cost-cutting strategies. In this regard, Turkish Ministry of Health released 'Good Laboratory Practices Project' in 2018 [9]. A practical guide for adequate test requesting was part of this project. Practically, attempts to reduce unnecessary laboratory test requests include two major approaches: education of the clinicians and designing the test requests [10–12]. Our experience showed that the educational approach is short-lived, with effects disappearing shortly after cessation of the educational effort. On the other hand, efforts to design the test ordering practice of clinicians promise a longer-lived effect. Designing test ordering practices necessitate close co-operation of the clinicians and the laboratory [13]. In our project, we were lucky to have a national clinical guide to compose a consensus between the clinicians and laboratory specialists. We did not set a target goal in reducing unnecessary electrophoresis test requests when we started the project. We are confident to call our effort 'successful' just because it works. Our rejection rules are evidence based, in line with the national clinical guide on multiple myeloma; so they can safely be used by all hospitals and laboratories for this purpose. Most importantly, we managed to decrease unnecessary test requests.

Conclusion

Considering the proportion of patients in this group who have M protein in the gamma field in the electrophoresis test, standardizing the treatment follow-up of this limited group in accordance with the guidelines will be quite efficient in terms of labor and cost. Laboratories should play an active role in the diagnosis and treatment of monoclonal gammopathies, including multiple myeloma.

Urine protein electrophoresis and urine immunofixation electrophoresis were not included in this project due to different applications of clinicians on these tests like using serum free light chain assays instead.

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Ethics Committee Approval: The study was approved by The Antalya Training and Research Hospital Clinical Research Ethics Committee (No: 2024-165, Date: 13/06/2024).

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References

1. Plebani M, Zaninotto M, Faggian D. Utilization management: A European perspective. Clin Chim Acta 2014;427:137–41. [CrossRef]
2. Aydin O, Erturk I. Serum protein electrophoresis in diagnosing monoclonal gammopathies. Turk Clin Biochem J [Article in Turkish] 2022;20(1):106–14.
3. Keren DF, Bocsi G, Billman BL, Etzell J, Faix JD, et al. Laboratory detection and initial diagnosis of monoclonal gammopathies: Guideline from the College of American pathologists in collaboration with the American Association for Clinical Chemistry and the American Society for Clinical Pathology. Arch Path Lab Med 2022;146:575–90. [CrossRef]
4. Turkish Society of Hematology. Multiple Myeloma Diagnosis and Treatment Guidelines. Available at: https://www.thd.org.tr/thdData/Books/77/kilavuzu-tek-parca-halinde-goruntulemek-icin-tiklayiniz.pdf. Accessed Feb 28, 2025.
5. Dimopoulos MA, Moreau P, Terpos E, Mateos MV, Zweegman S, Cook G, et al. Multiple myeloma: EHA-ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Ann Oncol 2021;32(3):309–22. [CrossRef]
6. Migkou M, Avivi I, Gavriatopoulou M, Cohen YC, Fotiou D, Kanellias N, et al. Clinical characteristics and outcomes of oligosecretory and non-secretory multiple myeloma. Ann Hematol 2020;99:1251–5. [CrossRef]
7. Kyle RA, Gertz MA, Witzig TE, Lust JA, Lacy MQ, Dispenzieri A, et al. Review of 1027 patients with newly diagnosed multiple myeloma. Mayo Clin Proc 2003;78:21–33. [CrossRef]
8. Martinez-Lopez J, Hernandez-Ibarburu G, Alonso R, Sanchez-Pina JM, Zamanillo I, Lopez-Muñoz N, et al. Impact of COVID-19 in patients with multiple myeloma based on a global data network. Blood Cancer J 2021;11:198. [CrossRef]
9. Türkiye Health Ministry. Good laboratory practices project. Doc No: 95966346-040.99-95966346-040.99-E.272.
10. Aykal G, Keşapli M, Aydin Ö, Esen H, Yeğin A, Güngör F, et al. Pre-test and post-test applications to shape the education of phle-

botomists in a quality management program: An experience in a training hospital. J Med Biochem 2016;35(3):347–53. [CrossRef]
11. Aydin O, Erturk I. Stepwise testing in the diagnosis of monoclonal gammopathies. LLM Derg [Article in Turkish] 2023;7(1):40–2. [CrossRef]
12. Aydin O, Erturk I. Electrophoresis testing in the follow-up of multiple myeloma patients with a measurable m protein in gamma region: Good laboratory practice for the laboratory specialist. LLM Derg [Article in Turkish] 2023;7(2):82–5. [CrossRef]
13. Aydin O, Ellidag HY, Eren E, Yilmaz N.The laboratory should actively be ınvolved in the Therapeutic Drug Monitoring (TDM) Process. In J Phar Pract 2016;9(1):9–13. [CrossRef]

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Research Article

Association of hematological inflammatory indices and monocyte/HDL ratio with plaque formation in patients with atherosclerotic heart disease

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Abstract

Objectives: There are insufficient studies on the combined effect of neutrophil-lymphocyte ratio (NLR), systemic immune-inflammation (SII) index and monocyte-to-high-density lipoprotein ratio (MHR) on plaque status and risk of cardiovascular disease (CVD) occurrence. The aim of this study was to demonstrate the feasibility of using NLR, SII index and MHR, which are preferable markers in terms of favorable cost/benefit ratio and easy measurement, to monitor and evaluate the severity of the disease, considering that CVD is an inflammatory disease.

Methods: Two thousand two hundred seventy-three patients presenting with complaints of shortness of breath or chest pain who were followed up in the Cardiovascular Surgery outpatient clinic of Gaziosmanpaşa Training and Research Hospital between January 2024 and October 2024 were retrospectively included in the study.

Results: Lymphocyte levels were significantly higher in the deceased patients (p=0.02). Conversely, the NLR and the SII were higher in the surviving patients compared to the deceased patients (p<0.001; p<0.001). LDL levels and plaque status were statistically significantly different between the groups. Patients in the moderate-risk group had significantly lower LDL levels compared to those in the mild-risk group (p<0.001).

Conclusion: These results suggest that MHR, a novel biomarker derived from the inflammatory marker monocyte and the antiatherogenic HDL, may be associated with CAD. Given that CVD is an inflammatory disease, NLR, SII and MHR may be preferable in terms of favorable cost/benefit ratio and easy measurement. These markers can also be calculated practically and inexpensively from whole blood and HDL values, which are routine tests that can be performed in primary health care centers. It also demonstrates NLR and MHR are associated with plaque formation in patients with atherosclerotic heart disease.

Keywords: Monocyte-to-high-density lipoprotein ratio, neutrophil-lymphocyte ratio, plaque formation, systemic immune-inflammation index

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Cardiovascular diseases (CVD) encompass a range of conditions affecting the heart and blood vessels, with coronary heart disease (CHD) and stroke being the most prevalent and fatal forms. In 2019, CVD accounted for approximately 18.6 million deaths worldwide, underscoring their significant global health burden [1]. At the core of

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many CVDs, particularly CHD, is atherosclerosis, a chronic inflammatory process characterized by the development of plaques within arterial walls. Epidemiology studies show that among many genetic and environmental factors, elevated serum cholesterol levels alone are sufficient for the development of atherosclerosis, even in the absence of other known risk factors. Oxidation of low-density lipoprotein cholesterol (LDL-C) in the endothelial is the initiating factor in the development of atherosclerosis [2].

Atherosclerosis begins with impaired endothelial function. In the final stage of plaque rupture, endothelial dysfunction plays an important role. Lymphocytes and neutrophils, as a subgroup of leukocytes, play a role in the formation of plaque rupture as well as in infarct healing and reperfusion injury [3, 4]. The recently widely used neutrophil to lymphocyte ratio (NLR) is a reliable, accessible, and clinically relevant marker of acute coronary syndrome (ACS). Increased NLR has been proven to be associated with the severity and prevalence of atherosclerosis in ST-elevation heart attack patients [5–8]. Although, the association between NLR and the morbidity of CVD with vulnerable plaque characteristics is not fully understood to date.

The systemic immune-inflammation index (SII), new index is an emerging composite biomarker that reflects the balance between the immune system and inflammatory processes. It combines three key blood parameters: neutrophil count, platelet count, and lymphocyte count. The index is designed to provide a more comprehensive view of systemic inflammation and immune activation, which plays a crucial role in many diseases, including CVD [9].

Recently, the monocyte/ high-density lipoprotein (HDL) ratio (MHR) has been shown to be a predictor of cardiovascular events in patients with chronic renal failure and to be associated with coronary slow flow and stent thrombosis [10]. The association of high monocyte count, and low HDL has emerged as a new prognostic marker of inflammation and oxidative stress associated with adverse outcomes in various cardiovascular diseases [11, 12]. Considering the contribution of monocytes and macrophages to inflammation and oxidative stress and the inhibitory effect of HDL-C on them, the idea that the MHR can be used as a prognostic indicator in inflammation-related diseases has yielded significant results in studies conducted in renal failure and various heart diseases [6, 13–18].

Since CVD is largely driven by inflammation, markers such as NLR, SII and MHR could offer valuable insights into immune system activation and chronic inflammation, which are known to play a key role in the disease's progression. The aim of this study was to demonstrate the feasibility of using NLR, SII and MHR, which are preferable markers in terms of favorable cost/benefit ratio and easy measurement, to monitor and evaluate the correlation of these markers with traditional clinical outcomes (e.g. plaque burden, major adverse cardiovascular events).

Materials and Methods

Study design and population

The study protocol adhered to ethical guidelines and was approved by the Istanbul Atlas University Clinical Research Ethics Committee (Approval Date: 14/05/2022, Approval Number: E-22686390-050.99-42823). All procedures were conducted in compliance with the principles defined in the Declaration of Helsinki. Informed consent was obtained from participant or their relative.

Two thousand two hundred seventy-three patients presenting with complaints of shortness of breath or chest pain who were followed up in the Cardiovascular Surgery outpatient clinic of Gaziosmanpaşa Training and Research Hospital between January 2024 and October 2024 were retrospectively included in the study. All patients were questioned about cardiac risk factors such as smoking, hypertension, diabetes mellitus, hyperlipidaemia, family history of coronary heart disease and medications. All this information was obtained from the patients' medical record.

Inclusion criteria

Patients who underwent previous coronary angiography (CAG) and were found to have ≥50% stenosis in their major coronary arteries, who underwent percutaneous coronary procedure or bypass surgery for CAD, or who were newly diagnosed with CAD by myocardial perfusion sintigraphy (MPS) at outpatient clinic presentation were included.

Exclusion criteria

Active infection, inflammatory disease, acute coronary syndrome clinic, advanced heart failure, history of cancer, GFR<15 mL/minute, chronic obstructive pulmonary disease and liver failure, patients receiving antibiotics, immunosuppressive therapy, and non-steroidal anti-inflammatory drugs were excluded from the study. Patients with known significant valvular heart disease, malignancy, acute or chronic infections, those receiving systemic anti-inflammatory treatment, antihyperlipidemic therapy, or those with severe liver disease and renal failure were excluded from the study.

Electronic health records of patients hospitalized for CAD were retrospectively reviewed using the hospital's electronic database system. The demographic and clinical characteristics, systolic and diastolic blood pressure, complete blood count parameters, total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides, troponin, creatinine kinase-MB (CK-MB) levels were recorded from the hospital automation system and patient files.

Patients were evaluated according to survival and deceased status and according to plaque status detected at angiography.

The information you've provided is a description of how intra-plaque angiogenesis (IPN) is assessed using contrast-enhanced ultrasound (CEUS) imaging in the context of evaluating plaque stability [19]. The process of scoring involves evaluating the presence and distribution of microbubbles

within the plaque, which is used to infer the degree of angiogenesis (new blood vessel formation) within the plaque. Here's a breakdown of the IPN scoring system:

1. **IPN Score 0**: No microbubbles detected within the plaque, suggesting no angiogenesis is present within the plaque. This is considered a stable plaque.
2. **IPN Score 1:** Microbubbles are confined to the shoulder or adventitial side of the plaque, indicating some degree of angiogenesis, but the plaque remains relatively stable.
3. **IPN Score 2:** Microbubbles are seen throughout the entire plaque, suggesting significant intra-plaque angiogenesis, which is associated with plaque instability.

An IPN score of ≥2 (i.e., microbubbles throughout the plaque) is considered indicative of an unstable plaque, while an IPN score of <2 (i.e., microbubbles are confined to the shoulder or absent) indicates a stable plaque. This scoring system is important for evaluating the risk of plaque rupture or other complications, with unstable plaques generally being at a higher risk for rupture or causing adverse cardiovascular events [20].

Diagnoses of hypertension (HT), type 2 diabetes mellitus (T2DM) were made according to current guidelines [21]. T2DM was defined as fasting blood glucose levels ≥126 mg/dL and/or a previous diagnosis and treatment of diabetes. HT was defined as systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg, the use of antihypertensive therapy, and/or a known history of HT.

Height and weight measurements were obtained, and body mass index (BMI) was calculated according to the formula body weight (kg)/height (m^2). Obesity was defined as BMI >30 kg/m^2.

Laboratory parameters

Blood samples were collected in tubes, both plain (without anticoagulant) and containing EDTA. The CBC analyses were conducted using an automated hematology analyzer (Sysmex XN-1000, Norderstedt, Germany), ensuring precision and reliability in data acquisition. NLR were calculated from neutrophil/ lymphocyte count. The SII was calculated as (platelet count × neutrophil count) /lymphocyte count. MHR was calculated by taking the ratio of monocytes to HDL in peripheral blood count.

The high-sensitive cTnI (hs-cTnI) levels were assessed by the immunofluorescent method using fluorescent antibody conjugates (ARCHITECTR Abbott assay, USA).

Glucose, albumin, total cholesterol, HDL, LDL, CK-MB, CRP were assessed with an autoanalyzer (COBAS 8000, ROCHE-2007, Tokyo, Japan).

Statistical analysis

Statistical analyses were performed using SPSS v.26 (IBM Corp., Armonk, NY, USA) software. Descriptive data were expressed as frequency (n) and percent (%) for categorical variables, and as mean ± standard deviation or median (25^{th} percentile–75^{th} percentile) for numerical variables. Normality of the distribution was assessed using the Kolmogorov–Smirnov test, as well as Q–Q plots and histograms. Pearson chi-square test or Fisher's exact test was used to compare categorical variables. Mann-Whitney U test was used for comparisons between two independent groups, while Kruskal-Wallis test was used for comparisons between more than two independent groups. Numerical variables were also evaluated by Spearman Correlation test. Laboratory parameters were categorized using Receiver Operating Characteristic (ROC) curve analysis, and the cutoff point where sensitivity and specificity were maximized was selected. Youden index values were used to determine the optimal cutoff points. The area under the curve (AUC) was reported.

Multivariate logistic regression (Enter and Forward: LR) analysis was performed to identify risk factors associated with survival, and plaque formation. The goodness-of-fit of the model was evaluated using the Hosmer–Lemeshow test. The regression model does not include highly correlated variables. The significance level for statistical tests was set at $p<0.05$.

Results

The mean age of the patients was 63.5±13.2 years, and 49.8% were female (Table 1). The age distribution between the survival groups was similar. However, the sex distribution showed a significant difference, with a higher survival rate in males ($p=0.012$). The body mass index (BMI) was higher in the deceased group compared to the survival group ($p=0.001$). Lymphocyte levels were significantly higher in the deceased patients ($p=0.02$). Conversely, the NLR and the SII were higher in the surviving patients compared to the deceased patients ($p<0.001$; $p<0.001$). A statistically significant difference was observed between survival and plaque progression ($p<0.001$). The frequency of no plaque was higher in the survival group.

There was no statistically significant difference in age, sexes, and BMI between the plaque groups (Table 2). However, LDL levels were statistically significantly different between the groups. In pairwise comparisons, significant differences were observed across all groups ($p<0.001$). LDL levels were lower in the stable plaque group compared to the other groups, with the highest LDL levels observed in the without plaque group ($p<0.001$). Mild risk was higher in without plaque group, while moderate risk was higher in patients with stable plaque. Furthermore, all patients with stable plaque were categorized into the moderate-risk group, while all patients without plaque were classified into the mild-risk group.

Clinical and sociodemographic characteristics of the patients were compared across different risk groups (Table 3). Glucose, LDL, Troponin, CK-MB levels and plaque status were statistically significantly different between the groups. Patients in the moderate-risk group had significantly lower LDL levels compared to those in the mild-risk group ($p<0.001$). Additionally, Glocose, Troponin and CK-MB levels were significantly higher in the moderate-risk group compared to the mild-risk group ($p<0.001$).

Table 1. Comparison of demographic and clinical characteristics of the patients

Characteristic	All patients (n=2273)	Deceased group (n=673)	Survival group (n=1600)	p
Age (years)	63.5±13.2	64.0±13.2	63.2±13.2	0.244
	64.0(54.0–73.0)	65.0 (55.0–74.0)	64.0 (54.0–73.0)	
Sex (female)	1133 (49.8)	308 (27.2)	825 (72.8)	**0.012**
BMI (kg/m^2)	28.1 (24.6–32.5)	29.3 (25.4–32.7)	27.7 (24.0–32.4)	**0.001**
Systolic blood pressure (mmHg)	153.0 (143.0–163.0)	153.0 (143.0–163.0)	153.0 (144.0–163.0)	0.295
Diastolic blood pressure (mmHg)	85.0 (79.0–88.0)	84.0 (79.0–88.0)	85.0 (79.0–88.0)	**0.046**
LYMPH (10^{3}cell/µL)	1710.0 (1439.5–2008.5)	1730.0 (1414.0–2360.0)	1700.0 (1447.0–1983.0)	**0.020**
NEU (10^{3}cell/µL)	7812.0 (6687.5–8236.0)	7803.0 (6210.0–8315.0)	7812.0 (7260.0–8205.0)	0.941
MONO (10^{3}cell/µL)	398.0 (386.0–416.0)	399.0 (386.0–416.0)	398.0 (386.0–416.0)	0.880
WBC (10^{3}cell/µL)	9.0 (7.6–10.9)	8.9 (7.7–11.0)	9.0 (7.6–10.9)	0.813
PLT (10^3/uL)	207.2 (164.8–242.4)	208.2161.8–242.1)	206.4 (165.4–242.7)	0.757
Glucose (mg/dL)	120.0 (102.0–145.0)	125.0 (102.0–148.0)	120.0 (102.0–145.0)	**0.020**
Albumin (g/dL)	3.6 (2.7–4.1)	3.8 (3.3–4.1)	3.6 (2.6–4.0)	**<0.001**
HDL (mg/dL)	36.0 (26.0–44.0)	36.0 (26.0–42.0)	36.0 (26.0–46.0)	0.742
LDL (mg/dL)	159.0 (146.0–264.5)	153.0 (141.0–164.0)	186.0 (150.0–297.0)	**<0.001**
CRP (mg/L)	66.4 (33.3–108.8)	82.8 (46.5–134.7)	56.2 (33.3–100.8)	**<0.001**
NLR	4.4 (3.6–5.2)	4.1 (2.5–5.2)	4.5 (3.7–5.2)	**<0.001**
SII	852.0 (586.2–1113.8)	780.0 (449.4–1123.9)	876.0 (622.7–1112.2)	**<0.001**
MHR	11.0 (9.0–15.1)	11.0 (9.0–14.9)	10.9 (8.9–15.2)	0.726
Troponin I (ng/ml)	0.6 (0.1–18.8)	0.1 (0.05–5.4)	1.8 (0.1–21.9)	**<0.001**
CK-MB (ng/ml)	32.4 (15.0–144.0)	20.7 (12.5–100.5)	82.3 (17.2–154.0)	**<0.001**
Hypertension (+)	1758 (77.3)	503 (28.6)	1255 (71.4)	0.055
Diabetes (+)	1072 (47.2)	319 (29.8)	753 (70.2)	0.883
Dyslipidemia (+)	1844 (81.1)	570 (30.9)	1274 (69.1)	**0.005**
Smoking (+)	1310 (57.6)	349 (26.6)	961 (73.4)	**<0.001**
Alcoholism (+)	574 (25.3)	158 (27.5)	416 (72.5)	0.206
Plaque status				
Without plaque	463 (20.4)	80 (17.3)[a]	383 (82.7)[b]	**<0.001**
Unstabil plaque	912 (40.5)	265 (28.8)[a]	656 (71.2)[b]	
Stabil plaque	889 (39.1)	328 (36.9)[a]	561 (63.1)[b]	

Mann Whitney U Testi, Fisher Exact Test, Pearson Chi-Square Test. Comparisons were made between survival and deceased groups. Each superscript letter denotes a subset of survival categories whose column proportions do not differ significantly from each other at the 0.05 level. BMI: Body mass index; LYMPH: Lymphocyte; NEU: Neutrophil; MONO: Monocyte; WBC: White blood count; PLT: Platelet count; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; CRP: C-reactive protein; NLR: Neutrophil-lymphocyte ratio; SII: Systemic immune-inflammation; MHR: Monocyte-to-high-density lipoprotein ratio; CK-MB: Creatinine kinase-MB.

Correlation between inflammatory indices was evaluated. A statistically significant very high correlation was observed between NRI and BMI (p<0.01; r=0.999). There was a very weak but statistically significant correlation between CRP levels and LDL (p<0.01; r=-0.064). A highly significant correlation was observed between NLR and SII (p<0.01; r=0.784) (Table 4).

When evaluating the risk factors associated with survival, PNI (OR:1.018; 95% CI:1.006–1.031), CRP (OR:1.004; 95% CI:1.003–1.005), Albumin (OR:1.444; 95% CI:1.252–1.665) were considered as risk factors that increased the likelihood of death. Conversely, an increased Troponin (OR:0.992; 95% CI:0.986–0.998) and LDL levels (OR:0.996; 95% CI:0.995–0.997) were found to be a preventive factor, positively associated with survival. NRI and NLR were not independent risk factors for survival (p>0.05) (Table 5).

In Figure 1, the predictive performance of the MHR parameter for plaque formation is illustrated by the ROC curve. MHR parameter was found to be non-predictive for plaque formation (p=0.279).

Discussion

Inflammatory molecules and lipids are two key elements in CVD [22]. Studies with animal models have demonstrated the invasion of neutrophils into atherosclerotic plaques [23]. In the current study, lymphocyte levels were significantly higher in the deceased patients. Conversely, the NLR and the SII were higher in the surviving patients compared to the deceased patients. HDL levels were lower in the stable plaque group compared to the other groups, with the highest HDL levels observed in the non-plaque group. The MHR was higher in the stable plaque

Table 2. Comparison of demographic and clinical characteristics of patients according to plaque status detected at angiography

Characteristic	Without plaque (n=463)	Unstabil plaque (n=921)	Stabil plaque (n=889)	p
Age (years)	63.8±13.7	63.5±13.3	63.5±13.7	0.473
	65.0 (55.0–74.0)	64.0 (55.0–74.0)	64.0 (54.0–72.0)	
Sex (female)	222 (19.6)	455 (40.2)	456 (40.2)	0.476
BMI (kg/m^2)	28.3 (24.7–33.2)	28.1 (24.5–32.4)	28.0 (24.5–32.0)	0.241
Systolic blood pressure (mmHg)	153 (144–163)	153 (144–163)	153 (141–163)	0.487
Diastolic blood pressure (mmHg)	85.0 (79.0–88.0)	85.0 (79.0–88.0)	85.0 (79.0–88.0)	0.673
LYMPH (10^{3}cell/µL)	1698 (6439–8256)	1710 (1444–2016)	1722 (1431–2013)	0.855
NEU (10^{3}cell/µL)	7795 (6439–8256)	7817 (6495–8225)	7817 (7260–8225)	0.944
MONO (10^{3}cell/µL)	396 (386–416)	398 (386–416)	398 (386–416)	0.977
WBC (10^{3}cell/µL)	9.0 (7.7–11.0)	9.0 (7.6–11.0)	8.9 (7.6–10.8)	0.482
PLT (10^3/uL)	211.3 (165.4–247.2)	206.3 (164.6–242.4)	206.3 (164.8–240.8)	0.338
Glucose (mg/dL)	120.0 (100.0–145.0)	120.0 (102.0–145.0)	120.0 (102.0–145.0)	0.159
Albumin (g/dL)	3.6 (2.6–4.0)	3.6 (2.7–4.1)	3.6 (2.7–4.1)	0.076
HDL (mg/dL)	36.0 (26.0–46.0)	36.0 (26.0–44.0)	36.0 (26.0–44.0)	0.528
LDL (mg/dL)	193.0 (149–342)[a]	161.0 (146–243)[b]	156.0 (144–194)c	**<0.001**
CRP (mg/L)	66.4 (33.3–108.8)	66.4 (33.3–108.8)	66.4 (33.3–108.8)	0.682
NLR	4.4 (3.3–5.2)	4.3 (3.6–5.3)	4.4 (3.7–5.2)	0.839
SII	855.1 (575–1120)	837.5 (586–1114)	862.6 (591–1108)	0.862
MHR	10.8 (8.7–15.1)	11.0 (9.1–15.2)	10.9 (9.0–14.8)	0.520
Troponin I (ng/ml)	0.1 (0.04–4.8)	0.7 (0.05–18.6)	1.8 (0.05–23.6)	0.738
CK-MB (ng/ml)	26.6 (14.7–101.2)	28.0 (14.4–142.2)	53.4 (15.2–154.6)	0.640
Hypertension (+)	364 (20.6)	707 (39.6)	687 (39.8)	0.738
Diabetes (+)	221 (20.6)	424 (39.6)	427 (39.8)	0.671
Dyslipidemia (+)	377 (20.4)	740 (40.1)	727 (39.4)	0.727
Smoking (+)	277 (21.1)	529 (40.4)	504 (38.5)	0.536
Alcoholism (+)	112 (19.5)	225 (39.2)	237 (41.3)	0.463
Survival patients	383 (23.9)[a]	656 (41.0)[b]	561 (35.1)[c]	**<0.001**
Risk score				
Mild risk	463 (100.0)[a]	259 (28.1)[b]	0 (0.0)[c]	**<0.001**
Moderate risk	0 (0.0)[a]	662 (71.9)[b]	889 (100.0)[c]	

Kruskall -Wallis Test, Pearson Chi-Square Test. Each superscript letter denotes a subset of plaque categories whose column proportions do not differ significantly from each other at the 0.05 level. BMI: Body mass index; LYMPH: Lymphocyte; NEU: Neutrophil; MONO: Monocyte; WBC: White blood count; PLT: Platelet count; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; CRP: C-reactive protein; NLR: Neutrophil-lymphocyte ratio; SII: Systemic immune-inflammation; MHR: Monocyte-to-high-density lipoprotein ratio; CK-MB: Creatinine kinase-MB.

group compared to the others, and the without plaque group had the lowest MHR. Additionally, the moderate risk ratio was higher in the stable plaque group than in the other groups. Furthermore, all patients with stable plaque were classified in the moderate-risk group, whereas all patients without plaque were classified in the mild-risk group. Although monocytes and macrophages are the most common white blood cell seen in atherosclerotic plaque, increased MHR may have accelerated monocyte infiltration of neutrophils into atherosclerotic plaques. NLR and MHR are associated with plaque formation in patients with atherosclerotic heart disease.

Chronic inflammation is a key factor in the pathogenesis of atherosclerosis and the development of subsequent CVD. It contributes to the initiation, progression, and destabilization of atherosclerotic plaques. Recent studies have shown that CRP, platelet/lymphocyte and neutrophil/lymphocyte ratios used as inflammatory markers both increased in atherosclerotic CVD and that these markers are independently associated with CAD [24–27]. On the other hand, it has long been known that HDL levels are inversely associated with atherosclerosis [28]. This relationship is largely explained by the reverse cholesterol transport of HDL in the vessel wall. It has been demonstrated that HDL exerts a protective role in atherosclerosis by inhibiting the expression of endothelial adhesion molecules. This action reduces the recruitment and accumulation of monocytes at sites of vascular injury. By preventing monocyte adhesion to the endothelial cells, HDL helps to mitigate the inflammatory processes that contribute to the formation and progression of atherosclerotic plaques [28]. Kahraman et al. [8] found that the ratio of MHR consisting of monocytes, an inflammatory marker, and HDL, an antiatherogenic lipid parameter, was associated with high

Table 3. Evaluation of risk factors in patients

Characteristic	Mild risk (n=722)	Modereta risk (n=1551)	p
Age (years)	65.5 (54.0–74.0)	64.0 (54.0–73.0)	0.593
Sex (female)	340 (30.0)	793 (70.0)	0.073
BMI (kg/m^2)	28.0 (24.7–33.0)	28.1 (24.5–32.2)	0.210
Systolic blood pressure (mmHg)	153.0 (144–163)	153.0 (141–163)	0.199
Diastolic blood pressure (mmHg)	85.0 (79.0–88.0)	85.0 (79.0–88.0)	0.958
LYMPH (10^{3}cell/µL)	1700 (1432–2004)	1710 (1440–2011)	0.707
NEU (10^{3}cell/µL)	7795 (6418–8236)	7818 (7130–8236)	0.236
MONO (10^{3}cell/µL)	398 (386–416)	398 (386–416)	0.541
WBC (10^{3}cell/µL)	9.0 (7.6–11.0)	8.9 (7.6–10.9)	0.492
PLT (10^3/uL)	207.9 (165.5–247.1)	206.9 (164.5–240.9)	0.234
Glucose (mg/dL)	120 (100–145)	125 (102–145)	**0.01**
Albumin (g/dL)	3.6 (2.6–4.0)	3.6 (2.7–4.1)	0.08
HDL (mg/dL)	36.0 (26–46)	36.0 (26–43)	0.173
LDL (mg/dL)	186.0 (149–297)	159.0 (145–195)	**<0.001**
CRP (mg/L)	66.4 (33.3–107.8)	66.4 (33.3–108.8)	0.252
NLR	4.3 (3.4–5.3)	4.4 (3.6–5.2)	0.621
SII	854.5 (578–1119)	849.2 (590–1108)	0.778
MHR	10.9 (8.7–15.1)	11.0 (9.0–15.1)	0.229
Troponin I (ng/ml)	0.2 (0.05–5.1)	11.0 (9.0–15.1)	**<0.001**
CK-MB (ng/ml)	27.9 (14.7–105.3)	38.8 (15.0–154.0)	**<0.001**
Hypertension (+)	564 (32.1)	1194 (67.9)	0.548
Diabetes (+)	344 (32.1)	728 (67.9)	0.753
Dyslipidemia (+)	587 (31.8)	1257 (68.2)	0.884
Smoking (+)	422 (32.2)	888 (67.8)	0.591
Alcoholism (+)	171 (29.8)	403 (70.2)	0.240
Survival patients	570 (35.6)	1030 (64.4)	**<0.001**

Mann Whitney U Test, Fisher Exact Test, Pearson Chi-Square Test. BMI: Body mass index; LYMPH: Lymphocyte; NEU: Neutrophil; MONO: Monocyte; WBC: White Blood Count; PLT: Platelet Count; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; CRP: C-Reactive Protein; NLR: Neutrophil-lymphocyte ratio; SII: Systemic immune-inflammation; MHR: Monocyte-to-high-density lipoprotein ratio; CK-MB: Creatinine kinase-MB.

Table 4. Evaluation of correlation between parameters

	MHR	Troponin I	CK-MB	CRP	NLR	SII	PNI	NRI	Age	BMI	HDL
MHR	.										
Troponin I	0.038	.									
CK-MB	-0.056**	0.798**	.								
CRP	0.013	0.075**	-0.095**	.							
NLR	-0.045*	0.006	0.035	-0.012	.						
SII	-0.036	0.022	0.032	-0.016	0.784**	.					
PNI	0.015	-0.030	0.012	-0.065**	-0.608**	-0.495**	.				
NRI	0.013	-0.060**	-0.074**	0.193**	0.022	0.025	-0.096**	.			
Age	0.001	-0.006	0.006	0.006	-0.014	-0.025	0.029	0.017	.		
BMI	0.013	-0.059**	-0.073**	0.189**	0.022	0.026	-0.095**	0.999**	0.018	.	
HDL	-0.984**	0.031	0.056**	-0.010	0.045*	0.035	-0.015	-0.009	-0.002	-0.010	.
LDL	0.006	0.021	0.028	-0.064**	0.050*	0.072**	0.051*	-0.035	0.029	-0.034	-0.012

<0.25 very weak; 0.26–0.49 weak; 0.50–0.69 moderate; 0.70–0.89 high; 0.90–1.0 very high correlation. *: p<0.05; **: p<0.01. MHR: Monocyte-to-high-density lipoprotein ratio; CK-MB: Creatinine kinase-MB; CRP: C-Reactive Protein; NLR: Neutrophil-lymphocyte ratio; SII; Systemic immune-inflammation; PNI: Prognostic nutritional index; NRI: Net reclassification index; BMI: Body mass index; HDL: High-density lipoprotein; LDL: Low-density lipoprotein.

Table 5. Evaluation of risk factors associated with survival in patients

Characteristic	Multivariate logistic regression (Multivariate-Enter method)		Multivariate logistic regression (Multivariate-Forward:LR method)	
	OR (95% CI)	p	OR (95% CI)	p
NRI	1.009 (0.999–1.019)	0.091	–	–
NLR	0.974 (0.906–1.048)	0.484	–	–
PNI	1.015 (0.998–1.032)	**0.004**	1.018 (1.006–1.031)	**0.004**
CRP	1.003 (1.002–1.005)	**<0.001**	1.004 (1.003–1.005)	**<0.001**
Troponin I (ng/ml)	0.992 (0.986–0.998)	**0.009**	0.992 (0.986–0.998)	**0.007**
Albumin	1.446 (1.253–1.668)	**<0.001**	1.444 (1.252–1.665)	**<0.001**
LDL	0.996 (0.995–0.997)	**<0.001**	0.996 (0.995–0.997)	**<0.001**

Forward LR and Enter methods were used for logistic regression analysis. Enter Model: Hosmer Lemeshow test p=0.002, Cox & Snell R^2= 0.101, Nagelkerke R^2= 0.143, -2 Log Likelihood= 2520.2. Forward LR Model: Hosmer Lemeshow test p=0.001, Cox & Snell R^2= 0.100 Nagelkerke R^2=0.142, -2 Log Likelihood= 2523.4. OR: Odds ratio; CI: Confidence interval; NRI: Net reclassification index; NLR: Neutrophil-lymphocyte ratio; PNI: Prognostic nutritional index; CRP: C-reactive protein; LDL: Low-density lipoprotein.

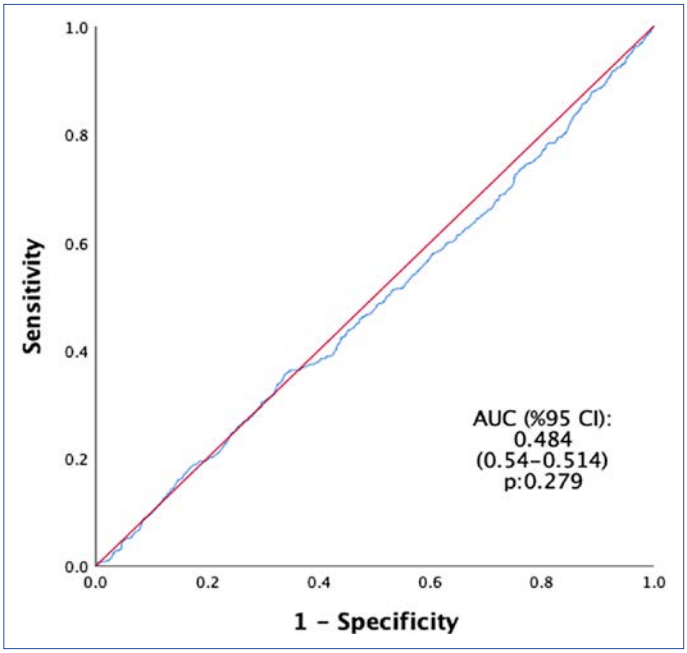

Figure 1. Predictive performance of the MHR parameter for plaque formation.

AUC: Under the curve; CI: Confidence interval.

SYNTAX score in patients with stable CAD. In a study conducted with patients with ACS, high NHR ratio consisting of neutrophils and HDL was found to be associated with long-term mortality [22]. In the same study, it was also shown that the prognostic value of NHR was superior to MHR and LDL/HDL. In another recent study, it was emphasized that high NHR ratio was associated with CAD severity in stable CAD [29]. In our study, like these studies, the NLR and the SII were higher in the surviving patients compared to the deceased patients. But no statistical significance was observed between survival and plaque progression. HDL levels and the MHR were statistically significantly different between the groups. In pairwise comparisons, significant differences were observed across all groups. HDL levels were lower in the stable plaque group compared to the other groups, with the highest HDL levels observed in the without plaque group. Previous studies have shown that HDL also has anti-inflammatory, antithrombotic and antioxidant effects. HDL exerts antiatherogenic effects by preventing macrophages from transporting lipid loads and lipids to the arterial wall. Furthermore, HDL-C inhibits endothelial expression of adhesion molecules through inhibition of CD11b activation and thus prevents monocyte adhesion to the arterial wall [30].

HDL is thought to prevent atherosclerosis primarily through reverse cholesterol transport in the vessel wall. In recent studies, mice with impaired reverse cholesterol transport pathway showed an increase in hematopoietic stem cells, monocytosis, neutrophilia and systemic foam cells and infiltration of many organs with myeloid cell lines. When these mice were infused with reconstituted HDL, the proliferation of hematopoietic stem cells and myeloid cell lines was reversed [31]. In the study by Qin et al. [32], it was shown that monocyte increase was a predictor of plaque development in previously plaque-free arteries. Excess lipids and inflammatory reactions (cellular and humoral) are considered the major contributors to plaque development, and the loss of smooth vascular muscle cells (VSMCs) and increased intraplaque hemorrhage are critical steps in necrotic core destabilization and enlargement [33].

There are also studies showing that HDL is effective in monocyte activation and inflammation in the development of atherosclerosis [34, 35]. Our results confirm this study. The fact that neutrophils, an inflammatory marker, and HDL, which have antiatherogenic properties, are both associated with atherosclerosis and with each other suggests that NHR may be a successful indicator for many diseases with an atherosclerotic background in the clinic. Indeed, in our study, we found that male gender, diabetes, and CAD were independent predictors of high MHR rate. We think that larger randomized prospective studies are needed in this regard.

Study Limitations

There were several limitations in our study. One of them is the retrospective design of the study. Another limitation is that the coronary artery calcium (CAC) test was not performed. Future studies investigating the association between MHR and plaque and cardiovascular events should be prospectively designed in larger patient series.

Conclusion

These results suggest that MHR, a novel biomarker derived from the hematological inflammatory marker monocyte and the antiatherogenic HDL, may be associated with CAD. Given that CVD is an inflammatory disease, NLR, SII and MHR may be preferable in terms of favorable cost/benefit ratio and easy measurement. These markers can also be calculated practically and inexpensively from whole blood and HDL values, which are routine tests that can be performed in primary health care centers. It also demonstrates the usability of NLR, SII and MHR for monitoring and assessing disease severity. Although the predictive performance of the MHR parameter for plaque formation (sensitivity and selectivity 0.59 and 0.53, respectively) was low, it was found to be higher in the stable plaque group compared to the others, while it had the lowest value in the plaque-free group. NLR and MHR are associated with plaque formation in patients with atherosclerotic heart disease.

Ethics Committee Approval: The study was approved by The Istanbul Atlas University Non-interventional Scientific Research Ethics Committee (No: E-22686390-050.99-42823, Date: 14/05/2022).

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References

1. Timmis A, Vardas P, Townsend N, Tobica A, Katus H, De Smedt D, et al; Atlas Writing Group, European Society of Cardiology. European Society of Cardiology: Cardiovascular disease statistics 2021. Eur Heart J 2022;43:716–99.
2. Jung E, Kong SY, Ro YS, Ryu HH, Shin SD. Serum cholesterol levels and risk of cardiovascular death: A systematic review and a dose-response meta-analysis of prospective cohort studies. Int J Environ Res Public Health 2022;19(14):8272. [CrossRef]
3. Tudurachi BS, Anghel L, Tudurachi A, Sascău RA, Stătescu C. Assessment of inflammatory hematological ratios (NLR, PLR, MLR, LMR and Monocyte/HDL-Cholesterol Ratio) in acute myocardial infarction and particularities in young patients. Int J Mol Sci 2023;24(18):14378. [CrossRef]
4. Cosarca MC, Hălmaciu I, Muresan AV, Suciu BA, Molnar C, Russu E, et al. Neutrophil to lymphocyte, platelet to lymphocyte and lymphocyte to monocyte ratios are associated with amputation rates in patients with peripheral arterial disease and diabetes mellitus who underwent revascularization: A Romanian regional center study. Exp Ther Med 2022;24(5):703. [CrossRef]
5. Li X, Li J, Wu G. Relationship of neutrophil-to-lymphocyte ratio with carotid plaque vulnerability and occurrence of vulnerable carotid plaque in patients with acute ischemic stroke. Biomed Res Int 2021;2021:6894623. [CrossRef]
6. Sharma DJ Sr, Nath HJ, Batta A, Goala AK. Neutrophil-to-lymphocyte Ratio (NLR) useful as a cost-effective preliminary prognostic marker in ST-Elevation Myocardial Infarction (STEMI): An observational study from a tertiary care hospital in Northeast India. Cureus 2023;15(3):e36885. [CrossRef]
7. Maleki M, Tajlil A, Separham A, Sohrabi B, Pourafkari L, Roshanravan N, et al. Association of neutrophil to lymphocyte ratio (NLR) with angiographic SYNTAX score in patients with non-ST-Segment elevation acute coronary syndrome (NSTE-ACS). J Cardiovasc Thorac Res 2021;13(3):216–21. [CrossRef]
8. Kahraman S, Agus HZ, Avci Y, Serbest NG, Guner A, Erturk M. The neutrophil to lymphocyte Ratio (NLR) is associated with residual syntax score in patients with ST-Segment Elevation Myocardial Infarction. Angiology 2021;72(2):166–73. [CrossRef]
9. Ye Z, Hu T, Wang J, Xiao R, Liao X, Liu M, et al. Systemic immune-inflammation index as a potential biomarker of cardiovascular diseases: A systematic review and meta-analysis. Front Cardiovasc Med 2022;9:933913. [CrossRef]
10. Acar B, Yayla C, Gul M, Karanfil M, Unal S, Uçar F, et al. Monocyte-to-HDL-cholesterol ratio is associated with ascending aorta dilatation in patients with bicuspid aortic valve. Afr Health Sci 2021;21(1):96–104. [CrossRef]
11. Caimi G, Lo Presti R, Urso C, Brucculeri S, Carlisi M. Neutrophil/HDL-C, Lymphocyte/HDL-C and Monocyte/HDL-C in subjects with asymptomatic carotid atherosclerosis. Clin Hemorheol Microcirc 2024;88(1):1–11. [CrossRef]
12. Sucato V, Comparato F, Ortello A, Galassi AR, Novo G. Residual cardiovascular risk: Role of remnants cholesterol, Monocyte/HDL Ratio and lipoprotein ratios on personalized cardiovascular prevention. J Pers Med 2024;14(5):460. [CrossRef]
13. Gembillo G, Siligato R, Cernaro V, Satta E, Conti G, Salvo A, et al. Monocyte to HDL ratio: A novel marker of resistant hypertension in CKD patients. Int Urol Nephrol 2022;54(2):395–403. [CrossRef]
14. Villanueva DLE, Tiongson MD, Ramos JD, Llanes EJ. Monocyte to High-Density Lipoprotein Ratio (MHR) as a predictor of mortality and Major Adverse Cardiovascular Events (MACE) among ST Elevation Myocardial Infarction (STEMI) patients undergoing primary percutaneous coronary intervention: A meta-analysis. Lipids Health Dis 2020;19(1):55. [CrossRef]
15. Yakar HI, Kanbay A. Could monocyte level/HDL cholesterol ratio predict cardiovascular diseases in patients with COPD? Niger J Clin Pract 2020;23(4):450–5. [CrossRef]

16. Song Y, Zhao Y, Shu Y, Zhang L, Cheng W, Wang L, et al. Combination model of neutrophil to high-density lipoprotein ratio and system inflammation response index is more valuable for predicting peripheral arterial disease in type 2 diabetic patients: A cross-sectional study. Front Endocrinol Lausanne 2023;14:1100453. [CrossRef]
17. Ustundag Y, Demir C, Demir M, Huysal K, Yesil MR, Karaca MS. The relationship between serum vitamin D levels and hematological inflammatory indices in patients with heart failure. Int J Med Biochem 2024;7(1):1–5. [CrossRef]
18. Zeki D, Uzun H. Can additional information be obtained in the diagnosis of heart failure in type 2 diabetics by evaluating the hematological indices. Int J Med Biochem 2023;6(1):15–20.
19. Sun C, Xi N, Sun Z, Zhang X, Wang X, Cao H, et al. The relationship between intracarotid plaque neovascularization and Lp (a) and Lp-PLA2 in elderly patients with carotid plaque stenosis. Dis Markers 2022;2022:6154675. [CrossRef]
20. Arányi Z, Csillik A, Dévay K, Rosero M. Ultrasonographic demonstration of intraneural neovascularization after penetrating nerve injury. Muscle Nerve 2018;57(6):994–9. [CrossRef]
21. Cosentino F, Grant PJ, Aboyans V, Bailey CJ, Ceriello A, Delgado V, et al. 2019 ESC Guidelines on diabetes, pre-diabetes, and cardiovascular diseases developed in collaboration with the EASD. Eur Heart J 2020;41:255–323.
22. Kou T, Luo H, Yin L. Relationship between neutrophils to HDL-C ratio and severity of coronary stenosis. BMC Cardiovasc Disord 2021;21:127. [CrossRef]
23. Şaylık F, Çınar T, Selçuk M, Tanboğa İH. The relationship between uric acid/albumin ratio and carotid intima-media thickness in patients with hypertension. Arq Bras Cardiol 2023;120(5):e20220819.
24. Serhatlioglu F, Cetinkaya Z, Yilmaz Y. The role of glucose-lymphocyte ratio in evaluating the severity of coronary artery disease. J Clin Med 2024;13(22):6711. [CrossRef]
25. Zhu M, Lin J, Wang C, Yang M, Lv H, Yang M, et al. The relationship among angiotensinogen genes polymorphisms and hs-CRP and coronary artery disease. J Clin Lab Anal 2019;33(5):e22881. [CrossRef]
26. Groenen AG, Halmos B, Tall AR, Westerterp M. Cholesterol efflux pathways, inflammation, and atherosclerosis. Crit Rev Biochem Mol Biol 2021;56(4):426–39. [CrossRef]
27. Pedro-Botet J, Climent E, Benaiges D. Atherosclerosis and inflammation. New therapeutic approaches. Med Clin Barc 2020;155(6):256–62. [CrossRef]
28. Huang JB, Chen YS, Ji HY, Huang JB, Chen YS, Ji HY, et al. Neutrophil to high-density lipoprotein ratio has a superior prognostic value in elderly patients with acute myocardial infarction: A comparison study. Lipids Health Dis 2020;19:59. [CrossRef]
29. Lamichhane P, Agrawal A, Abouainain Y, Abousahle S, Regmi PR. Utility of neutrophil-to-high-density lipoprotein-cholesterol ratio in patients with coronary artery disease: A narrative review. J Int Med Res 2023;51(4):3000605231166518. [CrossRef]
30. Zhang YL, Bai J, Yu WJ, Lin QY, Li HH. CD11b mediates hypertensive cardiac remodeling by regulating macrophage infiltration and polarization. J Adv Res 2024;55:17–31. [CrossRef]
31. Barrett TJ, Distel E, Murphy AJ, Hu J, Garshick MS, Ogando Y, et al. Apolipoprotein AI) promotes atherosclerosis regression in diabetic mice by suppressing myelopoiesis and plaque inflammation. Circulation 2019;140(14):1170–84. [CrossRef]
32. Qin W, Gan F, Liang R, Li J, Lai X, Dai Y, et al. Identification of monocyte-associated genes related to the instability of atherosclerosis plaque. Oxid Med Cell Longev 2022;2022:3972272. [CrossRef]
33. Mebrat Y. Cellular and molecular mechanisms that underlies the formation of atherosclerotic plaque and plaque rupture-review. Int J Med Biochem 2023; 6(3):210–22. [CrossRef]
34. Guo X, Ma L. Inflammation in coronary artery disease-clinical implications of novel HDL-cholesterol-related inflammatory parameters as predictors. Coron Artery Dis 2023;34(1):66–77. [CrossRef]
35. Jiang M, Yang J, Zou H, Li M, Sun W, Kong X. Monocyte-to-high-density lipoprotein-cholesterol ratio (MHR) and the risk of all-cause and cardiovascular mortality: A nationwide cohort study in the United States. Lipids Health Dis 2022;21(1):30. [CrossRef]

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Review

Branched-chain amino acids in obesity and diabetes: Implications and insights

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Abstract

Branched-chain amino acids (BCAAs), such as leucine, isoleucine, and valine, are vital in metabolic processes and in regulating energy equilibrium. In obesity and diabetes, BCAAs have been implicated in various metabolic dysfunctions, such as insulin resistance and altered glucose metabolism. Elevated BCAA levels are often observed in individuals with these conditions, suggesting a potential link between BCAA metabolism and the etiology of obesity and diabetes. Understanding the implications of BCAAs in these disorders could provide insights into novel therapeutic strategies to improve metabolic health and manage these chronic diseases. Previous reviews on BCAAs in the context of obesity and diabetes have often lacked a comprehensive analysis of their dual role in metabolic pathways. These reviews have focused solely on their positive effects, such as muscle protein synthesis, or potential negative impacts, like insulin resistance. Considering recent research findings and clinical studies, a thorough evaluation of the nuanced effects of BCAAs is necessary. This review seeks to fill these gaps by offering an impartial viewpoint on the metabolic consequences of BCAAs in individuals with obesity and diabetes, highlighting areas for future research and covering the metabolic role of BCAAs, their impact on feed intake patterns, and biochemical insights into BCAA metabolism. The review also delves into leucine's role in diabetes, examining its therapeutic potential and clinical implications. It also investigates mechanisms linking BCAAs to insulin resistance and BCAAs' relationship to mitochondrial dysfunction in obesity, providing a comprehensive understanding of BCAAs' metabolic effects. Given the rising prevalence of obesity and diabetes, this review is crucial for informing therapeutic strategies and identifying areas for future research.

Keywords: Branched-chain amino acids (BCAAs), diabetes, insulin resistance, metabolic dysfunction, obesity, therapeutic strategies

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Although branched-chain amino acids (BCAAs) were initially identified and thoroughly examined in the late 1800s, it is only within the last two decades that their significance in several areas of health and illnesses has become evident. Scientists have begun to reveal the functions of BCAAs that go beyond the processes of protein synthesis and breakdown. Leucine, isoleucine, and valine are examples of BCAAs. Mammals cannot synthesize BCAAs endogenously and rely on obtaining them from their

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nutritional intake. Compelling evidence indicates that bacteria found in the human microbiome can produce BCAAs [1]. These amino acids act as biomarkers for several disorders and conditions, including type 2 diabetes (T2D), obesity (OB), cardiovascular diseases (CVD), and some forms of cancer. Their metabolites can regulate gene expression and the epigenome [2].

BCAAs are essential to a comprehensive and balanced diet. Leucine is the main component of protein. Isoleucine and valine provide carbon atoms for the process of glucose synthesis. The catabolism of BCAAs provides the necessary fuel for the tricarboxylic acid cycle, thereby supplying cells with energy. Leucine, an intracellular amino acid, stimulates the rapamycin complex 1 (mTORC1) activation [3]. This intricate structure performs a pivotal function in regulating cellular growth and metabolism. The organism detects signals from nutrient and energy sources and stimulates cellular development when sufficient resources are present. However, it also stimulates catabolism in the absence of food.

Primarily, the liver is responsible for the breakdown of amino acids via a process known as degradation [4]. However, the liver cannot synthesize the branched-chain aminotransferase (BCAT) enzyme, which is essential for the transamination process of BCAAs [5]. As a result, BCAAs are absorbed directly from the gastrointestinal tract into the bloodstream. This partially explains the rapid fluctuation in blood levels of BCAAs, which depends on diet and the pace of protein synthesis or breakdown. BCAA concentration significantly impacts several physiological processes, including the creation of new mitochondria, energy production, inflammation, and glycolysis [6].

BCAAs, particularly leucine, stimulate protein synthesis initiation and rely on the activation of the mTOR signaling pathway [7]. Consequently, there has been an increase in the use of BCAAs as supplementary substances to augment performance and stimulate muscle development among bodybuilders and those striving to boost their physical fitness. The precise impacts of BCAAs when consumed in isolation or in the presence of other crucial amino acids or hormones still need to be understood [8]. BCAAs are essential for preserving the structural integrity and functionality of proteins and muscles. The favorable effects of BCAA supplementation on protein turnover and the prevention of muscle loss can be demonstrated in individuals with cirrhosis, renal failure, hepatic malignancies, and sepsis. Moreover, BCAAs are crucial as dietary cues and metabolic regulators alongside their anabolic response (Fig. 1).

BCAA

BCAAs enhance insulin production by modulating proteins and transcription in pancreatic β-cells [9]. In addition, they can regulate fatty tissue metabolism [10] and maintain glucose homeostasis [11]. They also enhance the growth and well-being of the intestines, boost milk supply from the mammary gland, support immunological function, and alter the composition and activities of gut microorganisms. The comprehensive examination of the metabolic and health benefits of consuming BCAAs has been explored in earlier research articles [12].

Preliminary research has shown that, unlike other amino acids, BCAAs can circumvent the liver and reach the bloodstream directly. The liver lacks the enzyme BCAT (branched-chain aminotransferase), which is responsible for breaking down BCAAs [13]. Conversely, the enzyme BCAT converts amino acids into branched-chain keto acids (BCKAs) in organs such as skeletal muscle, kidneys, heart, and adipose tissue. These BCKAs are then released into the circulation and mostly absorbed by the liver for oxidation. The enzyme complex known as branched-chain α-keto acid dehydrogenase (BCKDH) is essential for facilitating the oxidation process. Following synthesis, acyl-coenzyme A (acyl-CoA) undergoes oxidation via a sequence of enzymes, forming succinyl-CoA or acyl-CoA. These molecules enter the tricarboxylic acid cycle to produce adenosine triphosphate (ATP) [14].

McGarrah [15] recently reported research employing *in vivo* isotopic tracing to demonstrate that several peripheral or-

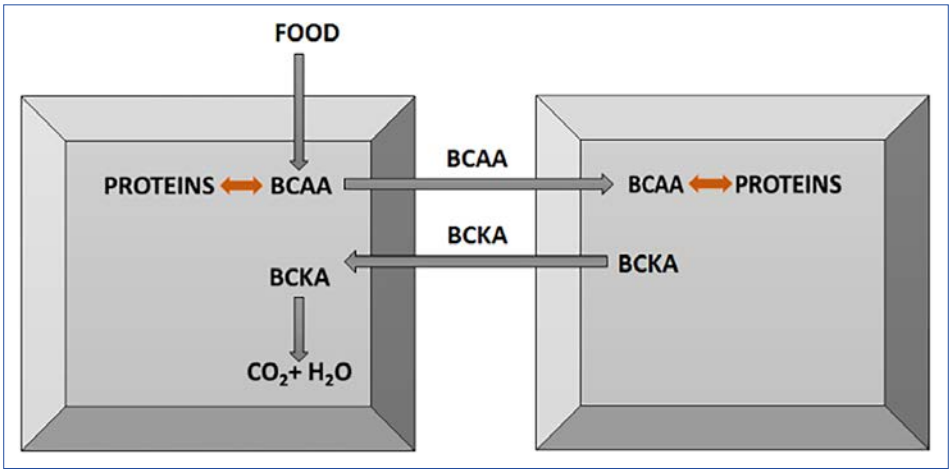

Figure 1. Pathways of BCAA catabolism.

BCAA: Branched-chain amino acid; BCKA: Branched-chain keto acid.

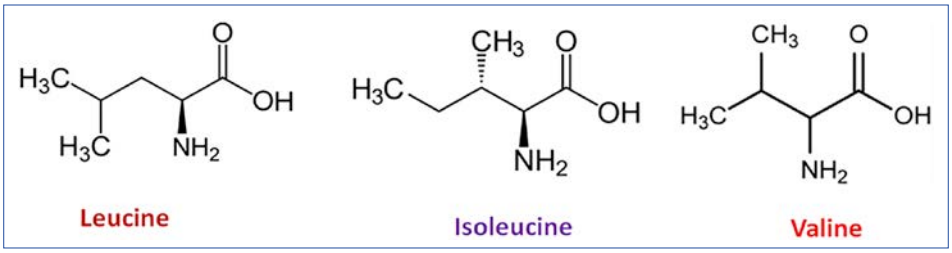

Figure 2. Branched chain amino acids.

gans have the potential to metabolize BCAAs. These findings suggest that organs other than the liver may significantly influence BCAA metabolism.

The liver and muscles primarily store glucose and fatty acids, which are used in substantial amounts as glycogen. Conversely, white adipose tissue stores them in the form of triglycerides. Amino acids, including BCAAs, are not converted into protein for subsequent use. Therefore, the only method of controlling excessive BCAAs is through their catabolic route [16]. The importance of BCAA catabolism is evident in people with inherited metabolic deficiencies, such as those with maple syrup urine disease [17]. This rare genetic disorder occurs when mutations in the BCKDH enzyme complex are inherited in an autosomal recessive manner. The human body cannot digest BCAAs, resulting in abnormally high amounts of BCAAs and BCKAs in the bloodstream. Consequently, these individuals suffer from hypotonia and ketoacidosis, along with severe neurological problems such as developmental delay, hallucinations, seizures, and coma [18].

Type 2 diabetes (T2D) is a very common global condition that is closely linked to the widespread problem of obesity. Obesity may lead to T2D, which occurs when pancreatic β-cells do not produce enough insulin to compensate for the decreased insulin sensitivity often associated with obesity.

In recent years, there has been a growing recognition of the significance of BCAA breakdown in the development of insulin resistance in individuals with obesity and T2D [19]. BCAA concentrations are markedly elevated in the plasma and tissues of these individuals. Furthermore, a significant association exists between elevated levels of BCAAs in the circulatory system and the development of insulin resistance in people with obesity and T2D [20]. The reasons for these high levels of BCAAs and their association with insulin resistance remain unclear. However, a dysfunctional breakdown of BCAAs might be a contributing factor.

Multiple studies have investigated the circulation levels of BCAAs in persons with obesity and T2D. However, more research is needed on the importance of dietary BCAAs for metabolic diseases and their impact on circulating BCAA levels [21]. Circulating BCAAs may increase negative feedback signaling by activating mammalian target of rapamycin (mTOR), which leads to insulin resistance and poor glucose metabolism [22].

Furthermore, epidemiological studies conducted on several demographic groups have shown a negative correlation between increased BCAA consumption and the risk of obesity. This review seeks to understand the processes behind elevated BCAA levels in obese individuals with T2D. It is important to consider their role in the development of insulin resistance. Moreover, this review examines pharmacological and alternative lifestyle intervention options to reduce plasma BCAA levels and their impact on metabolic health.

Metabolic Significance of BCAAs

BCAAs, the amino acids leucine, isoleucine, and valine, are important in several metabolic processes. The human body cannot produce these crucial amino acids, so they must be acquired via diet. BCAAs are distinct from other amino acids as they are mostly broken down in the muscle rather than the liver [23]. This highlights their important function in muscle protein synthesis and energy generation. Leucine has a vital role in regulating the mechanistic target of the rapamycin (mTOR) pathway, which is essential for the synthesis of muscle proteins. Leucine is vital in promoting muscle tissue development and repair by activating mTOR [24], making it essential for athletes and individuals involved in resistance training.

Furthermore, BCAAs are essential for extended physical activity, particularly when glycogen reserves are exhausted. This function aids in preserving muscle mass and alleviating muscular fatigue. In addition to their role in muscle metabolism, BCAAs impact glucose metabolism and insulin sensitivity. Studies have shown that they can regulate insulin released by pancreatic beta cells, which is essential in controlling blood glucose levels [25]. Increased concentrations of BCAAs have been linked to insulin resistance, a condition frequently seen in individuals with obesity and T2D. This paradox implies that while BCAAs are essential for regular metabolic functioning, their imbalance may lead to metabolic diseases.

BCAAs also have a role in the production of neurotransmitters by traversing the blood-brain barrier and impacting brain activity [26]. They compete with aromatic amino acids, such as tryptophan, for transportation into the brain, which may influence the production of neurotransmitters like serotonin. This interaction could affect mood and cognitive function.

Moreover, BCAAs regulate lipid metabolism. They can affect the expression of genes involved in lipid production and breakdown, impacting overall metabolic health. BCAAs are crucial in dietary strategies for managing obesity and metabolic diseases (Fig. 2). Understanding their complex meta-

bolic processes is essential for developing effective dietary and pharmacological therapies. Elevated amounts of BCAAs, tyrosine, and phenylalanine, accompanied by decreased levels of glycine, have been linked to insulin resistance (IR) in individuals with obesity [27].

A study examining blood samples from individuals with obesity and insulin resistance, as opposed to those who are lean and have insulin sensitivity, has shown a significant association between elevated levels of BCAAs and IR [28]. Studies have also shown that fatty acids and their byproducts contribute to the onset of IR and T2D [29]. Studies on mice have demonstrated that the addition of BCAAs to a standard chow (SC) diet does not affect insulin levels. However, adding BCAAs to a high-fat diet (HFD) causes obesity and IR to develop [30]. These studies indicate that the presence of both BCAAs and lipids is required for the development of insulin resistance.

Research on an animal model of obesity found that the buildup of acyl-carnitine and acyl-CoA in muscles is caused by the incomplete breakdown of fatty acids and an excessive burden on the mitochondria [31]. An abundance of BCAAs leads to increased mitochondrial activity, altering lipid metabolism. Based on current understanding, a diet lacking in BCAAs may enhance the body's capacity to metabolize fats [32]. This process entails activating the general control nonderepressible 2 (GCN2) gene, which inhibits the expression of genes linked to fat creation and protein synthesis. Moreover, it restores glycine levels in the muscle to their initial condition and promotes acyl-glycine production. Excreting acyl-glycine helps decrease acyl-CoA concentrations.

The skeletal muscle of animal models with insulin resistance exhibits incompletely esterified lipids, namely acyl-carnitine and diacylglycerol [33]. IR may be caused by an overabundance of catabolic byproducts of BCAAs and the buildup of partially esterified lipids in the muscle [34]. Lipotoxicity occurs when lipids accumulate, leading to glucose intolerance, IR, and, ultimately, the onset of T2D. Metabolomics has also shown that elevated levels of BCAAs in the bloodstream may serve as an early indicator of the onset of T2D. Bariatric surgery results in a rapid reduction in BCAAs and aromatic amino acids (AAAs) [35]. Conversely, elevated amounts of BCAAs, AAAs, and their byproducts in pregnant women affect the amount of fat in the fetus [36]. Mendelian randomization research determined that BCAAs are causally linked to insulin resistance (IR) [37].

A hypothesis has been proposed suggesting that hypothalamic IR negatively affects BCAA metabolism in patients with T2D and obesity. These findings indicate that increased plasma BCAAs are a sign of insulin resistance in the hypothalamus rather than the primary source of insulin resistance in peripheral tissues [38]. Two comprehensive human studies have shown that consuming a large amount of BCAAs is associated with a higher likelihood of developing T2D [39]. The variations in results may be explained by the fact that some research examined the combined effects of all three BCAAs, while others specifically investigated the impact of leucine alone.

A recent study has shown that each BCAA has unique effects on metabolism. The three BCAAs activate mTORC1, an essential metabolic regulator, via separate mechanisms. After translation, they also modify various proteins, generating diverse intermediates and ultimate catabolic products [40]. Studies on leucine and isoleucine have shown that they enhance insulin sensitivity and facilitate the transformation of white adipose tissue (WAT) into brown adipose tissue in obese mice [41]. Leucine supplementation in obese individuals promotes weight reduction, decreases inflammation in WAT, and enhances mitochondrial function.

Currently, there is significant attention being given to the use of leucine supplementation as a potential therapy for T2D and obesity. A comprehensive investigation in this area [42] found that although the administration of leucine directly into the central nervous system reduces food consumption, this outcome is not consistently replicated. Ongoing research is focused on developing methods to more effectively analyze the many variables (such as nutrition, gut flora, and physical activity) that impact BCAA metabolism [43]. Therefore, it is reasonable to expect that leucine may soon be used for medicinal purposes.

Individuals diagnosed with T2D have increased skeletal muscle amounts of BCAAs and enhanced activation of the mTORC1/S6K1 pathway [44]. These patients exhibit defective insulin signaling through reduced activity of PI3K/Akt, resulting in insulin resistance [45]. The inadequate nourishment of muscle fibers may account for this behavior, as glucose absorption is suppressed due to the presence of leucine. Consequently, this impedes the insulin signaling pathway by inducing an elevation in S6K1 phosphorylation.

Furthermore, KIC, which results from the breakdown of leucine, has similar activity, suggesting that the suppression of insulin function is linked to an elevation in S6K1 phosphorylation [46]. The impact of this metabolite, known as KIC, is diminished in cells with a decreased abundance of BCAT2. The metabolite undergoes an additional conversion into leucine, which allows it to consistently hinder the entry of glucose into cells. Confirming these findings, skeletal muscles that lack the BCKDH enzyme exhibit decreased glucose absorption, whereas the phosphorylation status of S6K1 remains unchanged [47]. This suggests an alternative mechanism for impeding the activity of insulin [48].

The findings suggest that increased levels of the metabolite 3-hydroxybutyrate, produced when valine is broken down, play a role in insulin resistance by causing an abnormal buildup of fatty acids in skeletal muscles. Based on this discovery, limiting the intake of BCAAs in Zucker rats enhances insulin efficiency in skeletal muscle and simultaneously promotes the degradation of fatty acids [49]. While elevated levels of BCAAs have been associated with IR, athletes who follow diets abundant in BCAAs do not exhibit indications of IR [50]. This apparent paradox may be explained by the fact that athletes consume a significant amount of BCAAs via their diet, preventing the accumulation of BCAAs in their bodies.

BCAA on Dietary Intake Patterns

Multiple studies have shown the inhibitory impact of intravenous leucine infusion on feed consumption [51]. The mTOR signaling pathway is crucial in the brain for detecting nutritional availability and controlling energy balance. Yao et al. [52] experimented on rats and demonstrated that mTOR signaling in some areas of the hypothalamus is regulated by energy levels. They also found that mTOR signaling is present in the same locations as neurons in the arcuate nucleus, including neuropeptide Y and proopiomelanocortin [53].

However, the effect of leucine on food consumption varies depending on whether it is administered through the diet. Multiple experiments have shown that augmenting the diet with more leucine does not lead to increased feed consumption in animals. The varying outcomes observed when comparing oral and central leucine supplementation may be attributed to leucine's capacity to infiltrate the blood-brain barrier and access the central nervous system [54]. While additional BCAA supplementation in the diet did not increase feed consumption, it is important to acknowledge the role of a BCAA-deficient diet in reducing feed intake.

Biochemical Pathways into BCAA Metabolism

The link between BCAAs and obesity has recently been reaffirmed and confirmed by many studies using high-throughput metabolomics analysis [55]. Following the rise in the popularity of BCAAs, it was shown that plasma BCAAs and their derived intermediates, BCKAs, are not only elevated in individuals with insulin resistance or T2D, but they also act as dependable indicators for future susceptibility to these conditions, regardless of age and ethnicity [56].

Recent findings suggest that BCAAs or BCKAs are linked to the excessive stimulation of mTOR signaling, the production of oxidative stress, compromised mitochondrial function, apoptosis, and, importantly, the development of insulin resistance and poor glucose metabolism. These components are essential for the onset of diabetes. These findings are corroborated by the observation that including BCAAs in a high-fat diet (HFD) or inhibiting BCAA oxidation by eliminating methylmalonyl-CoA mutase in mice results in IR and decreased glucose tolerance. In a recent study, Nilsen et al. [57] showed that individuals with diabetes had higher amounts of 3-hydroxybutyrate, a valine catabolic precursor. This compound was found to enhance the transit of fatty acids in the blood vessels of muscles, resulting in reduced glucose tolerance in mice.

Ketoisocaproic acid, a byproduct of leucine metabolism, hinders the transportation of glucose triggered by insulin in L6 myotubes [58]. On the other hand, if BCAAs are removed from the regular chow diet or HFD in mice, or if a diet with lower amounts of all three BCAAs is provided to genetically diabetic Zucker fatty rats, insulin sensitivity improves and blood sugar levels are better regulated [59].

In a subsequent investigation, White et al. [22] confirmed these results by demonstrating an 80% reduction in glucose intolerance and insulin resistance when rats were exposed to BCAAs in a Western diet for four weeks. Studies have shown that drinking water with BCAA metabolites may decrease the active form of AKT (pAKT), which indicates insulin signaling in muscle [60]. Zhou et al. [61] demonstrated that supplementing an HFD with BCAAs leads to a decrease in pAKT levels and an increase in mTOR signaling activation in skeletal muscle compared to combining BCAAs with a conventional diet or HFD alone. Consequently, this results in a decline in the body's capacity to efficiently metabolize glucose.

Corroborating these findings, the absence of BCAAs in mice leads to elevated levels of pAKT in the liver and improved insulin sensitivity. This study provides evidence supporting the idea that BCAAs are not only associated with but also have a causative impact on the advancement of fat buildup, insulin resistance, and diabetes. The inhibition of the AKT signaling pathway in insulin-sensitive tissues may explain this behavior. The metabolic profiles derived from targeted and untargeted metabolomics approaches used to characterize obesity and insulin resistance indicate decreased BCKD activity in humans.

Cysteine is produced during the breakdown of cystathionine, which is formed from methionine/homocysteine by the action of cystathionase [62]. α-KB may also be generated from threonine through the activity of threonine hydratase. The liver BCKD, after partial purification, effectively catalyzes the oxidation of α-KB, resulting in propionyl-CoA production. This process is almost equivalent to the oxidation of BCAA-derived BCKA by the liver BCKD. α-KB can competitively inhibit the oxidation of BCKA by rat liver mitochondria and vice versa.

PDH similarly affects α-KB, while BCKD seems to have greater importance [63]. This is because, unlike pyruvate, the degradation of an α-ketobutyrate precursor known as α-aminobutyrate was not observed in fibroblasts derived from individuals with PDH deficiency [64]. The binding affinity of α-KB for partially purified PDH is four to five times lower than that of BCKD [65]. Thus, BCKD functions as the primary α-KB dehydrogenase.

This hypothesis is supported by the discovery that α-KB is one of the organic acids that acts as an indicator for maple syrup urine disease, a disorder caused by a defective BCKD complex, diagnosed by detecting increased levels of BCAAs in the blood or urine. Individuals with obesity and insulin resistance may have elevated levels of α-KB and its derivative, α-HB, due to reduced activity of BCKD (which is formed when LDH acts on α-KB), along with increased concentrations of BCAAs in the bloodstream. Theoretically, a biochemical obstruction at BCKD would lead to higher levels of methionine and cysteine-cystine upstream, resulting in greater concentrations of these compounds in the blood or tissues.

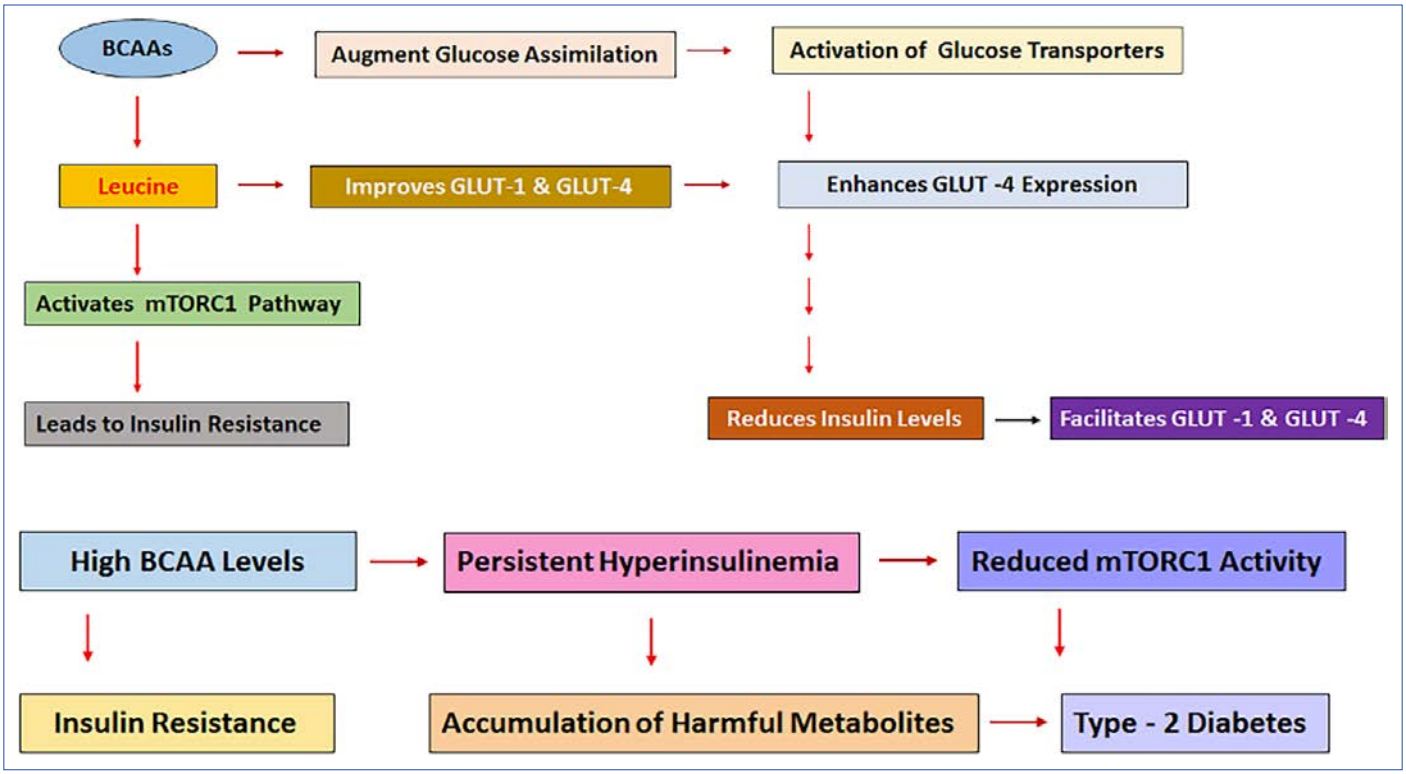

Figure 3. Leucine for diabetes treatment.

BCAAs: Branched-chain amino acids; GLUT: Glucose transporter; mTORC1: Rapamycin complex 1.

Leucine and Its Implications for Diabetes Treatment

Branched-chain amino acids (BCAAs) can augment the absorption and utilization of glucose. Amino acids can enhance glucose assimilation by activating glucose transporters. Leucine improves glucose absorption by facilitating the transportation of glucose transporter 1 (GLUT-1) and glucose transporter 4 (GLUT-4). Leucine may also enhance the expression of GLUT-4 [66]. Two theories explain the correlation between leucine and glucose transporters. One theory suggests that leucine reduces insulin levels and enhances the movement of GLUT-1 and GLUT-4. According to the second theory, leucine enhances glucose absorption in skeletal muscle by activating pathways related to GLUT-4 movement [67].

A strong correlation exists between insulin resistance and elevated levels of BCAAs, as reported in a study examining BCAA concentrations [68]. Studies have demonstrated the efficacy of BCAAs in stimulating insulin release. More specifically, leucine can induce the release of insulin. The presence of high amounts of BCAAs contributes to the onset of persistent hyperinsulinemia by fostering insulin resistance. Cuomo et al. [69] showed that consuming a diet deficient in BCAAs may improve insulin resistance. Similarly, Kim (2023) reported that dietary supplementation with leucine enhances glucose regulation in the body [70].

BCAAs in the blood are linked to insulin receptor substrate-1, which leads to insulin resistance and impaired glucose metabolism [71, 72]. BCAAs can enhance the expression of glucose transporters and stimulate insulin release [73, 74]. Elevated concentrations of these amino acids inside the body activate mechanistic target of rapamycin complex 1 (mTORC1) [75]. Activation of mTORC1 leads to reduced metabolic activity of BCAAs. This decrease is linked to the emergence of insulin resistance and type 2 diabetes, as it results in elevated BCAA levels in the blood and the accumulation of harmful metabolites.

Leucine is the principal amino acid with a branched chain that plays a crucial role in activating the mTORC signaling pathway. Li et al. [76] reported that leucine stimulates protein synthesis. Leucine, isoleucine, and valine may directly or indirectly affect metabolism (Fig. 3).

Clinical Perspectives on Therapeutic Potential

When managing a patient with cancer cachexia, potential treatment approaches may include implementing a diet low in protein to deprive the tumor of nutrients or a diet high in protein to alleviate the severe wasting associated with cachexia [77]. Several research studies have reported the effectiveness of targeted BCAA supplementation in preventing protein breakdown, promoting protein synthesis, and perhaps aiding in muscle repair, specifically for skeletal muscle [78]. Supplementing with BCAAs may enhance the body's protein synthesis needs while limiting the availability of amino acids for tumor growth. It is essential to assess the influence of BCAA supplementation on the progression

of insulin resistance during the formulation of treatment strategies. The insulin resistance was only detected within the framework of a high-fat diet. It indicates that the total nutritional condition of a person has a significant impact on the possibility of BCAA supplementation to cause diabetes. Branched-chain amino acid supplementation has been extensively studied for its therapeutic potential in treating advanced liver illnesses, namely hepatocellular carcinoma (HCC). Abe et al. [79] performed longitudinal research that focused on monitoring the progression of HCC in individuals with cirrhosis resulting from hepatitis C over several years. This study included a control cohort that received standard nutritional supplements and another administered with a BCAA supplement. The researchers noted a decrease in the incidence of HCC in the group that received BCAA. The Long Term Survival experiment (LOTUS) performed in Japan examined the impact of BCAA supplementation on the development of sequelae, including death, liver failure, and HCC, among those diagnosed with cirrhosis, as shown by a study involving 70 cases. The group that was administered BCAA saw a lower occurrence of problems when compared to the control diet. The hazard ratio was 0.67, with a 95% confidence range spanning from 0.49 to 0.93. By categorizing the participants based on their susceptibility to hepatocellular carcinoma (HCC), namely those with a body mass index over 25 kg/m2, it was shown that the inclusion of BCAA impeded the progression of HCC. Tsuchiya discovered that providing patients who had radical therapy for HCC with long-term BCAA supplementation led to a reduction in recurrence rates and an enhancement in the overall survival rate [80].

A recent study has shown that supplementing with BCAA in persons with liver cirrhosis significantly improved their insulin sensitivity and lowered hyperinsulinemia, hence decreasing the likelihood of developing HCC. BCAA supplementation is believed to decrease insulin resistance by improving the uptake of glucose by skeletal muscle independently of insulin and boosting the body's glucose tolerance [81]. A comparative study investigating the impacts of enteral feeding and BCAA supplementation in liver failure showed that BCAA supplementation enhanced the preservation of blood albumin levels, which act as a marker for protein synthesis [82]. A significant discovery in this research revealed that the group receiving enteral nourishment saw an elevation in glycated hemoglobin and other indicators that suggest a decrease in glucose tolerance. In contrast, no such changes were seen in the BCAA group. Thus, the elevated dietary intake of BCAAs does not have the same impact on glucose regulation as sustained increases. Recent *in-vitro* research has elucidated the specific mechanisms by which branched-chain amino acids affect liver metabolism and cancer.

Research investigating hepatic stellate cells discovered that the presence of leucine resulted in the increased release of hepatocyte growth factor (HGF) [83]. HGF is regarded as a pleiotropic factor synthesized by cells in several organs and affects cell growth, function, and motility [84]. HGF, triggered by leucine, can enhance the process of liver regeneration in cases of liver diseases such as HCC. Recent research on HepG2 cells showed that VEGF mRNA degradation occurred in the presence of all three branched-chain amino acids [85]. Administering BCAAs to persons with cirrhosis may decrease the occurrence of HCC by inhibiting the synthesis of vascular endothelial growth factor (VEGF). A recent study on H4IIE hepatic cancer cell lines demonstrated that BCAAs impeded the growth of cells stimulated by insulin [86]. The observed outcome was ascribed to an augmentation in apoptosis rather than a decrease in cellular proliferation.

Mechanisms Connecting BCAAs to Insulin Resistance

The precise processes by which BCAAs contribute to insulin resistance are complex and are currently being investigated. The buildup of harmful metabolites, such as BCKAs, produced during the breakdown of BCAAs may disrupt insulin signaling pathways by blocking crucial enzymes such as pyruvate dehydrogenase (PDH) and insulin receptor substrate (IRS) [87]. The interference mentioned may disturb the movement of glucose transporters to the cell's outer layer, decreasing glucose absorption and adding to high blood sugar levels. BCKAs can stimulate stress-related enzymes, such as the c-Jun N-terminal kinase (JNK) and mTOR, which might affect ins could. Another method is modifying the gut microbiota. BCAAs may affect the makeup and operation of gut bacteria, resulting in alterations in the production of microbial substances such as lipopolysaccharides (LPS) and short-chain fatty acids (SCFAs). High levels of LPS may trigger an inflammatory reaction by activating toll-like receptors (TLRs), which affects insulin resistance [88]. Conversely, any deviation in the synthesis of SCFAs could interfere with the secretion of hormones such as GLP-1 and PYY. These hormones are crucial for regulating insulin secretion and appetite [89]. Moreover, BCAAs are linked to increased adiposity and visceral fat buildup, both risk factors for insulin resistance. BCAAs stimulate lipogenesis and hinder fatty acid oxidation via stimulating the mTOR pathway and inhibiting AMP-activated protein kinase (AMPK). This simultaneous process might result in fat buildup and a decrease in the breakdown of fatty acids, which can contribute to metabolic disruptions. Comprehending these intricate connections is essential for creating specific therapies to alleviate the negative consequences of increased BCAAs in metabolic diseases.

Mitochondrial Dysfunction in Obesity: The Role of BCAAs

Metabolic illnesses, such as obesity and diabetes, are distinguished by the existence of mitochondrial dysfunction. Mitochondria are vital in generating energy, and their operation is critical for maintaining metabolic balance. BCAAs, which consist of leucine, isoleucine, and valine, have a cru-

cial impact on the generation and operation of mitochondria by acting as key substances for the TCA cycle. This process is crucial in cellular respiration since it generates the high-energy molecules ATP, NADH, and FADH2, vital for several biological functions. BCAAs have a role in the TCA cycle, which helps produce ATP, an essential molecule for energy-intensive actions, especially in muscle cells [90]. An abundance of BCAAs might result in mitochondrial overload, in which the mitochondria become overburdened by the excessive intake of metabolic substrates [91]. This excessive burden may lead to increased reactive oxygen species (ROS) produced due to cellular metabolism and have harmful effects. An overabundance of ROS may result in oxidative stress, which occurs when the body's antioxidant defense systems cannot counteract the formation of unstable molecules. Oxidative stress can damage mitochondrial DNA, proteins, and lipids, compromising mitochondrial function. Cell death may occur due to the dysfunction of mitochondria's energy synthesis and the initiation of apoptotic pathways. The accumulation of ROS and subsequent damage to the mitochondria may exacerbate metabolic inefficiency, contributing to insulin resistance development [92]. Insulin resistance refers to a condition in which cells have diminished responsiveness to insulin, hindering glucose absorption and causing an increase in blood glucose levels. Hence, it is crucial to appreciate the impact of BCAAs on mitochondrial health to understand their dual function in enhancing energy generation and possibly inducing metabolic damage. Acquiring this information might advance targeted dietary and pharmacological strategies to mitigate the adverse effects of BCAAs in metabolic diseases.

Conclusion

BCAAs, particularly leucine, isoleucine, and valine, are vital components of human metabolism, playing critical roles in muscle protein synthesis, energy production, and glucose metabolism. While their essentiality in maintaining muscle health and regulating metabolic processes is well-established, recent research highlights the complex and dual nature of BCAAs in health and disease. Elevated levels of BCAAs have been linked to the development of insulin resistance, obesity, and type 2 diabetes (T2D), suggesting that an imbalance in BCAA metabolism could contribute to metabolic disorders. The mechanistic insights reveal that BCAAs influence the mTOR signaling pathway, lipid metabolism, and insulin sensitivity, with potential implications for therapeutic strategies. However, the paradoxical relationship between BCAA supplementation determines the need for further investigation. Understanding the nuanced effects of BCAAs in different physiological contexts is crucial for developing targeted dietary and pharmacological interventions to optimize metabolic health. As research continues to unravel the intricacies of BCAA metabolism, it becomes evident that both their benefits and risks must be carefully considered, especially in the context of metabolic diseases such as T2D and obesity.

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References

1. Zheng X, Zhu Y, Zhao Z, Chu Y, Yang W. The role of amino acid metabolism in inflammatory bowel disease and other inflammatory diseases. Front Immunol 2023;14:1284133. [CrossRef]
2. Pandey A, Vaduganathan M, Patel KV, Ayers C, Ballantyne CM, Kosiborod MN, et al. Biomarker-based risk prediction of incident heart failure in pre-diabetes and diabetes. Heart Fail 2021;9(3):215–23. [CrossRef]
3. Dai Z, Ramesh V, Locasale JW. The evolving metabolic landscape of chromatin biology and epigenetics. Nat Rev Genet 2020;21(12):737–53. [CrossRef]
4. Szwed A, Kim E, Jacinto E. Regulation and metabolic functions of mTORC1 and mTORC2. Physiol Rev 2021;101(3):1371–426. [CrossRef]
5. Holeček M. Branched-chain amino acids and branched-chain keto acids in hyperammonemic states: Metabolism and as supplements. Metabolites 2020;10(8):324. [CrossRef]
6. Mann G, Mora S, Madu G, Adegoke OA. Branched-chain amino acids: catabolism in skeletal muscle and implications for muscle and whole-body metabolism. Front Physiol 2021;12:702826. [CrossRef]
7. Kaspy MS, Hannaian SJ, Bell ZW, Churchward-Venne TA. The effects of branched-chain amino acids on muscle protein synthesis, muscle protein breakdown, and associated molecular signalling responses in humans: An update. Nutr Res Rev 2024;37(2):273–86. [CrossRef]
8. Lv X, Zhou C, Yan Q, Tan Z, Kang J, Tang S. Elucidating the underlying mechanism of amino acids to regulate muscle protein synthesis: effect on human health. Nutrition 2022;103:111797. [CrossRef]
9. Guo L, Tian H, Yao J, Ren H, Yin Q, Cao Y. Leucine improves α-amylase secretion through the general secretory signaling pathway in pancreatic acinar cells of dairy calves. Am J Physiol Cell Physiol 2020;318(6):C1284–93. [CrossRef]
10. Karvinen S, Fachada V, Sahinaho UM, Pekkala S, Lautaoja JH, Mäntyselkä S, et al. Branched-chain amino acid deprivation decreases lipid oxidation and lipogenesis in C2C12 myotubes. Metabolites 2022;12(4):328. [CrossRef]
11. Suzuki R, Sato Y, Obeng KA, Suzuki D, Komiya Y, Adachi SI, et al. Energy metabolism profile of the effects of amino acid treatment on skeletal muscle cells: Leucine inhibits glycolysis of

myotubes. Nutrition 2020;77:110794. [CrossRef]
12. Polis B, Samson AO. Role of the metabolism of branched-chain amino acids in the development of Alzheimer's disease and other metabolic disorders. Neural Regen Res 2020;15(8):1460–70. [CrossRef]
13. Mansoori S, Ho MY, Ng KK, Cheng KK. Branched-chain amino acid metabolism: Pathophysiological mechanism and therapeutic intervention in metabolic diseases. Obes Rev 2024;26(2):e13856. [CrossRef]
14. Scagliola A, Mainini F, Cardaci S. The tricarboxylic acid cycle at the crossroad between cancer and immunity. Antioxid Redox Signal 2020;32(12):834–52. [CrossRef]
15. McGarrah RW, White PJ. Branched-chain amino acids in cardiovascular disease. Nat Rev Cardiol 2023;20(2):77–89. [CrossRef]
16. Dimou A, Tsimihodimos V, Bairaktari E. The critical role of the branched chain amino acids (BCAAs) catabolism-regulating enzymes, branched-chain aminotransferase (BCAT) and branched-chain α-keto acid dehydrogenase (BCKD), in human pathophysiology. Int J Mol Sci 2022;23(7):4022. [CrossRef]
17. Toyokawa Y, Koonthongkaew J, Takagi H. An overview of branched-chain amino acid aminotransferases: Functional differences between mitochondrial and cytosolic isozymes in yeast and human. Appl Microbiol Biotechnol 2021:105(21–22):8059–72. [CrossRef]
18. Deon M, Guerreiro G, Girardi J, Ribas G, Vargas CR. Treatment of maple syrup urine disease: Benefits, risks, and challenges of liver transplantation. Int J Dev Neurosci 2023;83(6):489–504. [CrossRef]
19. Gilbert M. Role of skeletal muscle lipids in the pathogenesis of insulin resistance of obesity and type 2 diabetes. J Diabetes Investig 2021;12(11):1934–41. [CrossRef]
20. De Bandt JP, Coumoul X, Barouki R. Branched-chain amino acids and insulin resistance, from protein supply to diet-induced obesity. Nutrients 2022;15(1):68. [CrossRef]
21. Imierska M, Kurianiuk A, Błachnio-Zabielska A. The influence of physical activity on the bioactive lipids metabolism in obesity-induced muscle insulin resistance. Biomolecules 2020;10(12):1665. [CrossRef]
22. White PJ, McGarrah RW, Herman MA, Bain JR, Shah SH, Newgard CB. Insulin action, type 2 diabetes, and branched-chain amino acids: A two-way street. Mol Metab 2021;52:101261. [CrossRef]
23. Holeček M. The role of skeletal muscle in the pathogenesis of altered concentrations of branched-chain amino acids (valine, leucine, and isoleucine) in liver cirrhosis, diabetes, and other diseases. Physiol Res 2021;70(3):293. [CrossRef]
24. Rehman SU, Ali R, Zhang H, Zafar MH, Wang M. Research progress in the role and mechanism of leucine in regulating animal growth and development. Front Physiol 2023;14:1252089. [CrossRef]
25. Campbell JE, Newgard CB. Mechanisms controlling pancreatic islet cell function in insulin secretion. Nat Rev Mol Cell Biol 2021;22(2):142–58. [CrossRef]
26. Singh S, Vellapandian C. Structure of the blood brain barrier and its role in the transporters for the movement of substrates across the barriers. Curr Drug Metab 2023;24(4):250–69. [CrossRef]
27. Lo EK, Felicianna, Xu JH, Zhan Q, Zeng Z, El-Nezami H. The emerging role of branched-chain amino acids in liver diseases. Biomedicines 2022;10(6):1444. [CrossRef]
28. Putha S. AI-driven metabolomics: Uncovering metabolic pathways and biomarkers for disease diagnosis and treatment. Distrib Learn Broad Appl Sci Res 2020;6:354–91.
29. Li N, Li J, Wang H, Liu J, Li W, Yang K, et al. Branched-chain amino acids and their interactions with lipid metabolites for increased risk of gestational diabetes. J Clin Endocrinol Metab 2022;107(7):e3058–65. [CrossRef]
30. Arjmand B, Ebrahimi Fana S, Ghasemi E, Kazemi A, Ghodssi-Ghassemabadi R, Dehghanbanadaki H, et al. Metabolic signatures of insulin resistance in non-diabetic individuals. BMC Endocr Disord 2022;22(1):212. [CrossRef]
31. Mthembu SX, Dludla PV, Nyambuya TM, Kappo AP, Madoroba E, Ziqubu K, et al. Experimental models of lipid overload and their relevance in understanding skeletal muscle insulin resistance and pathological changes in mitochondrial oxidative capacity. Biochimie 2022;196:182–93. [CrossRef]
32. Gojda J, Cahova M. Gut microbiota as the link between elevated BCAA serum levels and insulin resistance. Biomolecules. 2021 Sep 28;11(10):1414. [CrossRef]
33. Galarregui C, Cantero I, Marin-Alejandre BA, Monreal JI, Elorz M, Benito-Boillos A, Herrero JI, de la O V, Ruiz-Canela M, Hermsdorff HH, Bressan J. Dietary intake of specific amino acids and liver status in subjects with nonalcoholic fatty liver disease: fatty liver in obesity (FLiO) study. European Journal of Nutrition. 2021 Jun;60:1769–80. [CrossRef]
34. Supruniuk E, Żebrowska E, Chabowski A. Branched chain amino acids-friend or foe in the control of energy substrate turnover and insulin sensitivity. Crit Rev Food Sci Nutr 2023;63(15):2559–97. [CrossRef]
35. Abidi W, Nestoridi E, Feldman H, Stefater M, Clish C, Thompson CC, et al. Differential metabolomic signatures in patients with weight regain and sustained weight loss after gastric bypass surgery: A pilot study. Dig Dis Sci 2020;65:1144–54. [CrossRef]
36. Yang J, Wu J, Tekola-Ayele F, Li LJ, Bremer AA, Lu R, et al. Plasma amino acids in early pregnancy and midpregnancy and their interplay with phospholipid fatty acids in association with the risk of gestational diabetes mellitus: Results from a longitudinal prospective cohort. Diabetes Care 2023;46(4):722–32. [CrossRef]
37. Mosley JD, Shi M, Agamasu D, Vaitinadin NS, Murthy VL, Shah RV, et al. Branched-chain amino acids and type 2 diabetes: A bidirectional Mendelian randomization analysis. Obesity 2024;32(2):423–35. [CrossRef]
38. Ye Z, Wang S, Zhang C, Zhao Y. Coordinated modulation of energy metabolism and inflammation by branched-chain amino acids and fatty acids. Front Endocrinol 2020;11:617. [CrossRef]

39. Vanweert F, Schrauwen P, Phielix E. Role of branched-chain amino acid metabolism in the pathogenesis of obesity and type 2 diabetes-related metabolic disturbances BCAA metabolism in type 2 diabetes. Nutr Diabetes 2022;12(1):35. [CrossRef]
40. Bo T, Fujii J. Primary Roles of Branched Chain Amino Acids (BCAAs) and Their Metabolism in Physiology and Metabolic Disorders. Molecules. 2024 Dec 27;30(1):56. [CrossRef]
41. Zhou X, Chen J, Sun B, Wang Z, Zhu J, Yue Z, et al. Leucine, but not isoleucine or valine, affects serum lipid profiles and browning of WAT in mice. Food Funct 2021;12(15):6712–24. [CrossRef]
42. Zhang L, Li F, Guo Q, Duan Y, Wang W, Zhong Y, et al. Leucine supplementation: A novel strategy for modulating lipid metabolism and energy homeostasis. Nutrients 2020;12(5):1299. [CrossRef]
43. Cella V, Bimonte VM, Sabato C, Paoli A, Baldari C, Campanella M, et al. Nutrition and physical activity-induced changes in gut microbiota: Possible implications for human health and athletic performance. Foods 2021;10(12):3075. [CrossRef]
44. Nikparast A, Razavi M, Sohouli MH, Hekmatdoost A, Dehghan P, Tohidi M, Rouhani P, Asghari G. The association between dietary intake of branched-chain amino acids and the odds of nonalcoholic fatty liver disease among overweight and obese children and adolescents. Journal of Diabetes & Metabolic Disorders. 2024 Dec 20;24(1):19. [CrossRef]
45. Tong C, Wu Y, Zhang L, Yu Y. Insulin resistance, autophagy and apoptosis in patients with polycystic ovary syndrome: Association with PI3K signaling pathway. Front Endocrinol 2022;13:1091147. [CrossRef]
46. Mann GS. Effect of inflammation on ketoisocaproic acid induced insulin resistance in skeletal muscle cells. Master of Science, York University.
47. David J, Dardevet D, Mosoni L, Savary-Auzeloux I, Polakof S. Impaired skeletal muscle branched-chain amino acids catabolism contributes to their increased circulating levels in a non-obese insulin-resistant fructose-fed rat model. Nutrients 2019;11(2):355. [CrossRef]
48. Choi BH, Hyun S, Koo SH. The role of BCAA metabolism in metabolic health and disease. Experimental & Molecular Medicine. 2024 Jul;56(7):1552–9. [CrossRef]
49. Sjögren RJ, Rizo-Roca D, Chibalin AV, Chorell E, Furrer R, Katayama S, Harada J, Karlsson HK, Handschin C, Moritz T, Krook A. Branched-chain amino acid metabolism is regulated by ERRα in primary human myotubes and is further impaired by glucose loading in type 2 diabetes. Diabetologia. 2021 Sep;64(9):2077–91. [CrossRef]
50. Lee J, Vijayakumar A, White PJ, Xu Y, Ilkayeva O, Lynch CJ, Newgard CB, Kahn BB. BCAA supplementation in mice with diet-induced obesity alters the metabolome without impairing glucose homeostasis. Endocrinology. 2021 Jul 1;162(7):bqab062. [CrossRef]
51. Erukainure OL, Atolani O, Banerjee P, Abel R, Pooe OJ, Adeyemi OS, et al. Oxidative testicular injury: Effect of l-leucine on redox, cholinergic and purinergic dysfunctions, and dysregulated metabolic pathways. Amino Acids 2021;53:359–80. [CrossRef]
52. Yao J, Yan X, Xiao X, You X, Li Y, Yang Y, et al. Electroacupuncture induces weight loss by regulating tuberous sclerosis complex 1-mammalian target of rapamycin methylation and hypothalamic autophagy in high-fat diet-induced obese rats. Front Pharmacol 2022;13:1015784. [CrossRef]
53. Benite-Ribeiro SA, Rodrigues VA, Machado MR. Food intake in early life and epigenetic modifications of pro-opiomelanocortin expression in arcuate nucleus. Mol Biol Rep 2021;48(4):3773–84. [CrossRef]
54. Khandpur GK. Investigating the mechanism of cellular toxicity during aberrant branched-chain amino acid metabolism. Dissertation, Kaiserslautern: Technische Universität Kaiserslautern; 2021.
55. Hameed A, Mojsak P, Buczynska A, Suleria HA, Kretowski A, Ciborowski M. Altered metabolome of lipids and amino acids species: A source of early signature biomarkers of T2DM. J Clin Med 2020;9(7):2257. [CrossRef]
56. Pabla P. Development of a liquid chromatography-mass spectrometry method to investigate branched chain amino acid and acylcarnitine metabolism in type 2 diabetes. Dissertation, Nottingham: University of Nottingham.
57. Nilsen MS, Jersin RÅ, Ulvik A, Madsen A, McCann A, Svensson PA, et al. 3-Hydroxyisobutyrate, a strong marker of insulin resistance in type 2 diabetes and obesity that modulates white and brown adipocyte metabolism. Diabetes 2020;69(9):1903–16. [CrossRef]
58. Moghei M. The role of leucine and its metabolite (KIC) in insulin signalling and glucose transport in L6 myotubes. Master of Science, York University.
59. Roquetto AR, Moura CS, de Almeida Santos-Junior V, Oliveira PO, Machado KI, et al. Moderate intake of BCAA-rich protein improves glucose homeostasis in high-fat-fed mice. J Nutr Biochem 2020;80:108332. [CrossRef]
60. Rivera ME, Rivera CN, Vaughan RA. Excess branched-chain amino acids alter myotube metabolism and substrate preference which is worsened by concurrent insulin resistance. Endocrine 2022;76(1):18–28. [CrossRef]
61. Zhou M, Shao J, Wu CY, Shu L, Dong W, Liu Y, et al. Targeting BCAA catabolism to treat obesity-associated insulin resistance. Diabetes 2019;68(9):1730–46. [CrossRef]
62. Muthuraman A, Ramesh M, Shaikh SA, Aswinprakash S, Jagadeesh D. Physiological and pathophysiological role of cysteine metabolism in human metabolic syndrome. Drug Metab Lett 2021;14(3):177–92. [CrossRef]
63. Gaspar VP, Ibrahim S, Zahedi RP, Borchers CH. Utility, promise, and limitations of liquid chromatography-mass spectrometry-based therapeutic drug monitoring in precision medicine. J Mass Spectrom 2021;56(11):e4788. [CrossRef]
64. LeClair RJ. Amino acid metabolism and specialized products. Neurosci Pre-Clin Students. Available at: https://pressbooks.lib.vt.edu/neuroscience/chapter/amino-acid-metabolism-and-specialized-products/. Accessed Feb 5, 2025.

65. Prudent R, Annis DA, Dandliker PJ, Ortholand JY, Roche D. Exploring new targets and chemical space with affinity selection-mass spectrometry. Nat Rev Chem 2021;5(1):62–71. [CrossRef]
66. Wang T, Wang J, Hu X, Huang XJ, Chen GX. Current understanding of glucose transporter 4 expression and functional mechanisms. World J Biol Chem 2020 Nov 11;11(3):76. [CrossRef]
67. O'rielly R, Li H, Lim SM, Yazbeck R, Kritas S, Ullrich SS, et al. The effect of isoleucine supplementation on body weight gain and blood glucose response in lean and obese mice. Nutrients 2020;12(8):2446. [CrossRef]
68. Cheng L, Wang J, Dai H, Duan Y, An Y, Shi L, Lv Y, Li H, Wang C, Ma Q, Li Y. Brown and beige adipose tissue: a novel therapeutic strategy for obesity and type 2 diabetes mellitus. Adipocyte. 2021 Jan 1;10(1):48–65. [CrossRef]
69. Cuomo P, Capparelli R, Iannelli A, Iannelli D. Role of branched-chain amino acid metabolism in type 2 diabetes, obesity, cardiovascular disease and non-alcoholic fatty liver disease. Int J Mol Sci 2022;23(8):4325. [CrossRef]
70. Kim J, Kim J, Kwon YH. Leucine supplementation in maternal high-fat diet alleviated adiposity and glucose intolerance of adult mice offspring fed a postweaning high-fat diet. Lipids Health Dis 2023;22(1):50. [CrossRef]
71. Abdul-Ghani M, Maffei P, DeFronzo RA. Managing insulin resistance: The forgotten pathophysiological component of type 2 diabetes. Lancet Diabetes Endocrinol 2024;12(9):674–80. [CrossRef]
72. Yu L, Song P, Zhu Q, Li Y, Jia S, Zhang S, et al. The dietary branched-chain amino acids transition and risk of type 2 diabetes among Chinese adults from 1997 to 2015: Based on seven cross-sectional studies and a prospective cohort study. Front Nutr 2022;9:881847. [CrossRef]
73. Takahara T, Amemiya Y, Sugiyama R, Maki M, Shibata H. Amino acid-dependent control of mTORC1 signaling: A variety of regulatory modes. J Biomed Sci 2020;27:1–6. [CrossRef]
74. Trillos-Almanza MC, Martinez-Aguilar M, Arroyave-Ospina JC, van Vilsteren F, Blokzijl H, Moshage H. Clinical and Therapeutic Implications of BCAAs Metabolism during Chronic Liver Disease in Humans: Crosstalk between Skeletal Muscle and Liver. Muscles. 2024 Mar 4;3(1):71–87. [CrossRef]
75. Frias MA, Mukhopadhyay S, Lehman E, Walasek A, Utter M, Menon D, et al. Phosphatidic acid drives mTORC1 lysosomal translocation in the absence of amino acids. J Biol Chem 2020;295(1):263–74. [CrossRef]
76. Li S, Zhong H, Wang Z, Chen J, Huang Z, Zou T, et al. Dietary protein restriction regulates skeletal muscle fiber metabolic characteristics associated with the FGF21-ERK1/2 pathway. Iscience 2024;27(3):109249. [CrossRef]
77. Natto HA, Sahoo D, Muneera N. Benefits of personalized diet, nutrition, and exercise programs for cancer survivors. Int J Trends OncoSci 2024;2(2):12–22. [CrossRef]
78. Kaspy MS, Hannaian SJ, Bell ZW, Churchward-Venne TA. The effects of branched-chain amino acids on muscle protein synthesis, muscle protein breakdown, and associated molecular signalling responses in humans: An update. Nutr Res Rev 2023;37(2):1–36. [CrossRef]
79. Abe H, Midorikawa Y, Higaki T, Yamazaki S, Aramaki O, Nakayama H, et al. Magnetic resonance elastography-based prediction of hepatocellular carcinoma recurrence after curative resection. Surgery 2021;170(1):167–72. [CrossRef]
80. D'Avola D, Granito A, De La Torre-Alaez M, Piscaglia F. The importance of liver functional reserve in the non-surgical treatment of hepatocellular carcinoma. J Hepatol 2022;76(5):1185–98. [CrossRef]
81. Guo F, Chen R, Kong L, Wei P, Liu Z, Wang X, Hao H, Lu Y, Hu W. Effects of serum branched-chain amino acids on nonalcoholic fatty liver disease and subsequent cardiovascular disease. Hepatology International. 2022 Dec;16(6):1424–34. [CrossRef]
82. Mukmin S, Riviati N, Saleh MI. Egg white extract supplementation improves albumin and IGF-1 levels in malnourished elderly patients: A randomized controlled trial. Bioscientia Med J Biomed Transl Res 2024;9(2):6236–48. [CrossRef]
83. Tomiya T, Inoue Y, Yanase M, Arai M, Ikeda H, Tejima K, et al. Leucine stimulates the secretion of hepatocyte growth factor by hepatic stellate cells. Biochem Biophys Res Commun 2002;297(5):1108–11. [CrossRef]
84. Vincenti F, Kim J, Gouveia D, Pelle G, Mayne TJ, Neylan JF. Phase 3 trial design of the hepatocyte growth factor mimetic ANG-3777 in renal transplant recipients with delayed graft function. Kidney Int Rep 2021;6(2):296–303. [CrossRef]
85. Park JG, Tak WY, Park SY, Kweon YO, Chung WJ, Jang BK, et al. Effects of branched-chain amino acid (BCAA) supplementation on the progression of advanced liver disease: A Korean nationwide, multicenter, prospective, observational, cohort study. Nutrients 2020;12(5):1429. [CrossRef]
86. Rajesh Y, Sarkar D. Molecular mechanisms regulating obesity-associated hepatocellular carcinoma. Cancers 2020;12(5):1290. [CrossRef]
87. Abdualkader AM, Karwi QG, Lopaschuk GD, Al Batran R. The role of branched-chain amino acids and their downstream metabolites in mediating insulin resistance. J Pharm Pharm Sci 2024;27:13040. [CrossRef]
88. Li B, Leung JC, Chan LY, Yiu WH, Tang SC. A global perspective on the crosstalk between saturated fatty acids and Toll-like receptor 4 in the etiology of inflammation and insulin resistance. Prog Lipid Res 2020;77:101020. [CrossRef]
89. Lis M, Stańczykiewicz B, Liśkiewicz P, Misiak B. Impaired hormonal regulation of appetite in schizophrenia: A narrative review dissecting intrinsic mechanisms and the effects of antipsychotics. Psychoneuroendocrinology 2020;119:104744. [CrossRef]
90. Sjögren RJ, Rizo-Roca D, Chibalin AV, Chorell E, Furrer R, Katayama S, et al. Branched-chain amino acid metabolism is regulated by ERRα in primary human myotubes and is further impaired by glucose loading in type 2 diabetes. Diabetologia 2021;64(9):2077–91. [CrossRef]

91. Buondonno I, Sassi F, Carignano G, Dutto F, Ferreri C, Pili FG, et al. From mitochondria to healthy aging: The role of branched-chain amino acids treatment: MATeR a randomized study. Clin Nutr 2020;39(7):2080–91. [CrossRef]

92. Frangos SM, Bishop DJ, Holloway GP. Revisiting the contribution of mitochondrial biology to the pathophysiology of skeletal muscle insulin resistance. Biochem J 2021;478(21):3809–26. [CrossRef]

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Opinion Paper

Artificial intelligence-based novel wearables for non-invasive point-of-care assessment of high sensitivity cardiac troponins in patients with acute coronary syndrome

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Abstract

The timely and accurate diagnosis of acute coronary syndrome (ACS) is essential for improving patient outcomes, as delayed treatment can result in irreversible myocardial damage and increased mortality. Many studies investigated the feasibility and efficacy of artificial intelligence (AI)-based wearable technologies for the non-invasive, point-of-care assessment of high-sensitivity cardiac troponins (hs-cTn), a critical biomarker for myocardial injury. These wearables combine advanced biosensors with machine learning algorithms to deliver real-time, accurate hs-cTn measurements, enabling faster and more effective clinical decision-making. Clinical trials reveal that AI-powered wearables achieve diagnostic accuracy comparable to traditional laboratory assays while significantly reducing diagnostic time and resource burden. Additionally, their portability and cost-effectiveness make them suitable for diverse healthcare settings, including remote and resource-limited environments. This opinion paper seeks to elaborate on the insights gained from this study, emphasizing the transformative potential of AI-driven wearables in enhancing ACS diagnosis, streamlining patient care, and reducing strain on healthcare systems.

Keywords: Artificial intelligence, machine learning, non-invasive diagnostics, point-of-care testing, wearable technology

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Acute coronary syndrome (ACS) is one of the leading causes of death globally. Rapid advancements in healthcare technology have made rapid detection and treatment more effective [1]. However, the incidences of out-of-hospital deaths are still at alarming levels. With the facility of point-of-care (POC) assessment, we can drastically bring down these concerning numbers. Timely diagnosis allows for prompt interventions, reduces long-term complications, and enhances overall patient outcomes.

Cardiac biomarkers, including high-sensitivity cardiac troponins (hs-cTn), are essential for the early detection and diagnosis of ACS. Creatine kinase-MB, myoglobin, and troponins serve as indicators of myocardial damage, with troponins being highly specific markers. High-sensitivity troponin assays, a significant advancement, enable the detection of even minor cardiac injuries, facilitating early intervention and improved patient outcomes. Serial testing of hs-cTn levels helps assess changes over time, aiding in risk stratification. The precision and specificity of these assays, coupled with their ability to rule out ACS, have transformed clinical approaches, providing a valuable tool for timely and accurate diagnosis in cardiovascular care.

However, traditional laboratory-based testing faces challenges in achieving early detection, particularly in cases like ACS, where timeliness is critical. The emergence of artificial intelligence (AI)-based wearable devices offers a transformative approach to non-invasive, POC diagnostics for ACS. These smart wearables leverage AI algorithms to analyze biosignals and biomarkers in real time, providing immediate insights into cardiac health [2]. By enabling the continuous monitoring of

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high-sensitivity cardiac troponin (hs-cTn) levels, these devices could facilitate the early detection of myocardial injury, even in asymptomatic individuals or those at high risk of ACS.

A pioneering study was conducted by Sengupta et al. [3] on the effectiveness of a wrist-worn transdermal infrared spectrophotometric sensor (transdermal-ISS) and a machine learning algorithm to predict elevated high-sensitivity cardiac troponin-I (hs-cTnI) levels in ACS patients. The study, encompassing 238 patients across five sites, demonstrates the clinical feasibility of this innovative method, showcasing high sensitivity and specificity in predicting elevated hs-cTnI levels. This breakthrough holds promise for rapid, bloodless biomarker diagnosis in real-world settings, potentially revolutionizing point-of-care assessments for acute myocardial infarction and facilitating efficient triaging of patients with suspected ACS (Fig. 1).

Discussion

The discussions surrounding this novel but nascent approach are currently focused on its potential applications and the ongoing testing required for future clinical trials. There is an ongoing United States study enrolling participants to assess the device's value in emergency rooms, with prospects of extending its use to chest pain units, urgent care settings, and even ambulances by trained paramedics, eliminating the need for blood draws [4]. In addition to wrist wearables, more wearables, such as skin patches and other sensors, can also be developed with transparent algorithms. These innovations offer diverse options for continuous monitoring and early detection of high-sensitivity cardiac troponins in patients with ACS.

Another study by Li et al. [5] confirms that Mindray's hs-cTnI using CLIA (Chemiluminescence Immunoassay), a laboratory-based assay, is precise, sensitive, and specific in measuring cardiac troponin, making it a valuable diagnostic tool for myocardial infarction. Its comparable performance to the ARCHITECT hs-cTnI assay suggests it could be a reliable alternative, offering clinicians confidence in accurate diagnoses [6]. This positive outcome may influence healthcare practices, stimulate further research, and impact regulatory considerations for cardiac troponin assays. While the study extensively discusses the analytical and diagnostic performance of the hs-cTnI (CLIA) assay, it does not explicitly address the crucial need for early detection of acute coronary syndrome (ACS). Timely identification of ACS is vital for prompt intervention and improved patient outcomes.

While the potential of these technologies is significant, their impact on laboratory medicine must also be considered. In many countries, medical biochemists play a central role in managing POC devices, including their procurement, verification, and quality control [7]. The introduction of AI-based wearables into clinical practice could significantly alter these responsibilities. Laboratory medicine specialists might need

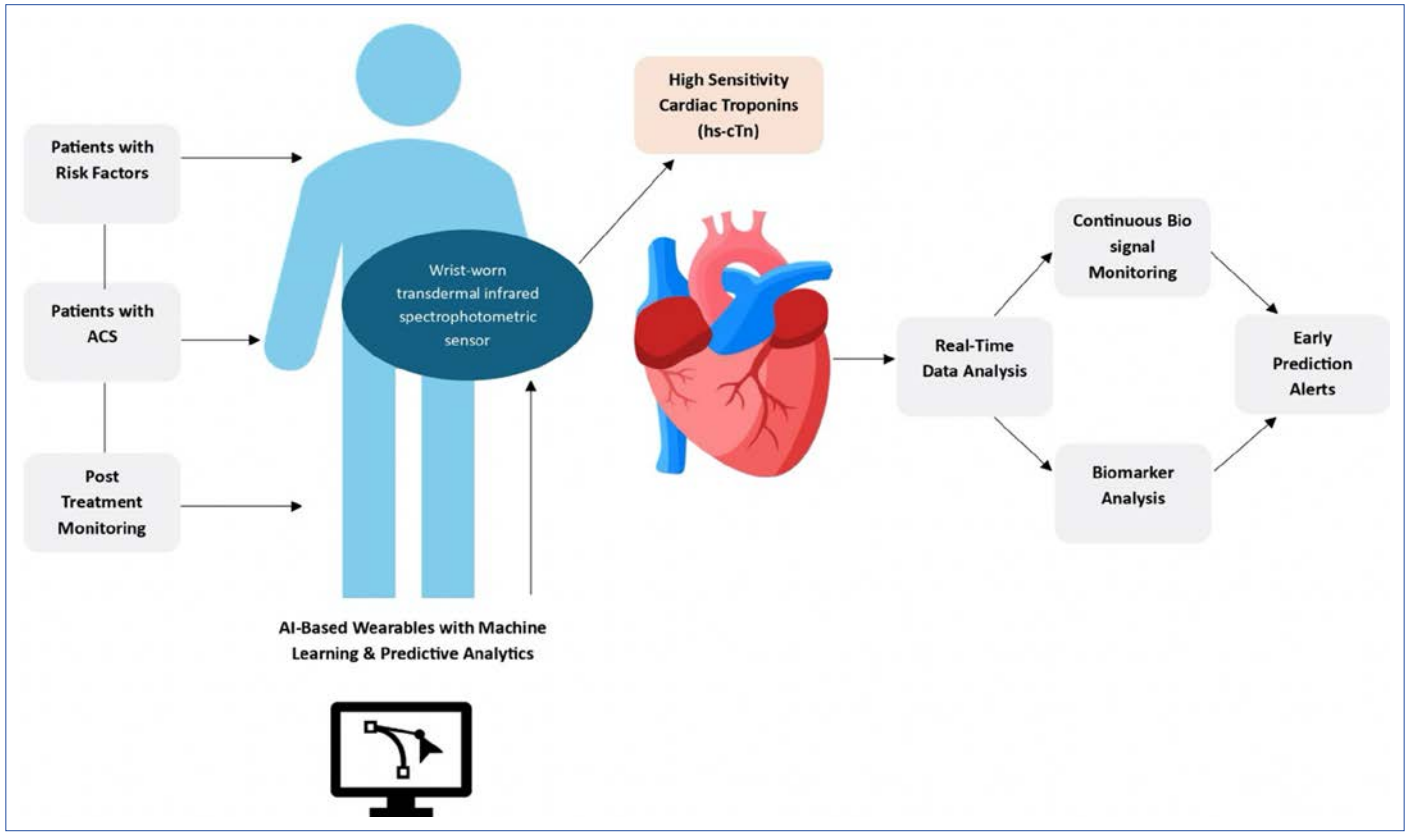

Figure 1. Illustration of how AI-based point-of-care testing wearables such as wrist-worn transdermal infrared spectrophotometric sensor (transdermal-ISS) helps in predicting elevated high-sensitivity cardiac troponin-I (hs-cTnI) levels.

ACS: Acute coronary syndrome.

Table 1. Comparison of lab-based and AI-based detection of cardiac biomarkers

Aspect	Lab-based detection	AI-based detection (transdermal-ISS)
Accuracy	Gold standard with high accuracy and reliability	High sensitivity and specificity demonstrated in clinical settings
Invasiveness	Requires blood sample collection	Non-invasive; uses a wrist-worn sensor
Time to results	Typically requires several hours for processing	Rapid, real-time predictions
Point-of-care utility	Limited by need for specialized lab infrastructure	Portable and suitable for bedside or out-of-hospital use
Scalability	Limited by equipment and trained personnel	Highly scalable with minimal hardware requirements
Cost efficiency	High costs due to reagents, equipment, and labor	Potential for lower costs over time with widespread adoption
Patient comfort	May cause discomfort due to blood draw	Comfortable and non-invasive for patients
Application in remote areas	Challenging due to infrastructure requirements	Feasible with minimal infrastructure
Real-time monitoring	Not feasible for continuous monitoring	Enables continuous or repeat assessments over time

to adapt their expertise to include oversight of these novel devices, from validating AI algorithms to ensuring the accuracy of data generated by wearables [8].

Methodologically, laboratory-based hs-cTn assays benefit from well-established protocols for calibration, quality control, and standardization, ensuring consistent results across diverse patient populations and settings. In contrast, wearable devices face challenges such as variations in skin properties (e.g., pigmentation, hydration, and thickness), environmental factors (e.g., temperature and movement artifacts), and signal processing limitations [9]. A comparison of both methods is given in Table 1. To address these challenges, robust testing of wearables in real-world scenarios is essential. For example, longitudinal studies could compare hs-cTnI trends obtained via wearables and laboratory assays under identical clinical conditions, assessing concordance and reliability.

Practical examples of implementation can further highlight the feasibility and integration of wearable technologies into the healthcare system. For instance, in pre-hospital settings such as ambulances or urgent care centers, wearables could provide real-time troponin trends, enabling paramedics to stratify ACS risk before reaching the hospital. However, seamless data integration with centralized laboratory systems is crucial to ensure continuity of care. A standardized reporting framework, where wearable-generated data can be directly compared or correlated with laboratory assay results, would facilitate such integration and support clinical decision-making [10].

The challenges highlighted, including cost, accessibility, and the need for clinical validation, merit deeper analysis. For instance, cost-effectiveness studies should evaluate whether wearables reduce overall healthcare expenses by minimizing hospital admissions, reducing emergency department congestion, and preventing unnecessary invasive procedures [11]. To overcome accessibility barriers, especially in resource-limited settings, partnerships with governmental health programs or subsidized models could promote equitable adoption. Regarding validation, multicenter trials across diverse populations should assess the wearables' sensitivity and specificity against laboratory assays to ensure global applicability and reliability [12].

Finally, addressing regulatory and operational considerations is paramount. Unlike laboratory assays, wearable devices operate under conditions where data quality can be influenced by user adherence and device variability [13]. Regulatory agencies such as the FDA or EMA should collaborate with developers to establish robust standards for wearable validation, ensuring safety, efficacy, and reproducibility. Furthermore, laboratory professionals, such as medical biochemists, must play a key role in developing protocols for periodic calibration, data verification, and integration into existing diagnostic workflows [14]. This collaboration would bridge the gap between wearable technology and traditional laboratory medicine, ensuring complementary use rather than competition. By integrating a direct comparison, technical specifics, practical use cases, and actionable strategies, this letter can offer a balanced and scientifically robust perspective that fosters meaningful dialogue within the academic and clinical communities.

Conclusion

AI-powered wearable technologies represent a groundbreaking shift in diagnosing and managing acute coronary syndrome (ACS). By providing rapid, non-invasive, and real-time insights into high-sensitivity cardiac troponin (hs-cTn) levels, these innovations have the potential to redefine point-of-care diagnostics, particularly in pre-hospital and remote settings. They promise not only faster triaging and earlier interventions but also improved accessibility for patients who might otherwise face barriers to traditional laboratory testing.

The road ahead involves translating these promising technologies into clinical practice. Future efforts should prioritize large-scale validation across diverse populations, ensuring reliability and inclusivity. Beyond clinical accuracy, addressing practical

challenges like cost, user adoption, and system integration will be key to their success. Collaboration between regulatory bodies, healthcare providers, and technology developers will be critical to establishing standards that ensure safety and efficacy.

With continued innovation and careful implementation, AI-based wearables could bridge critical gaps in ACS care, offering a scalable solution that aligns with the demands of modern healthcare systems. Their integration has the potential to not only enhance clinical workflows but also alleviate pressure on emergency departments, ultimately advancing the future of cardiovascular care.

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References

1. Atwood J. Management of acute coronary syndrome. Emerg Med Clin North Am 2022;40(4):693–706. [CrossRef]
2. Garg P, Morris P, Fazlanie AL, Vijayan S, Dancso B, Dastidar AG, et al. Cardiac biomarkers of acute coronary syndrome: From history to high-sensitivity cardiac troponin. Intern Emerg Med 2017;12(2):147–155. [CrossRef]
3. Sengupta S, Biswal S, Titus J, Burman A, Reddy K, Fulwani MC, et al. A novel breakthrough in wrist-worn transdermal troponin-I-sensor assessment for acute myocardial infarction. Eur Heart J Digit Health 2023;4(3):145–154. [CrossRef]
4. McKeown LA. Wrist-worn sensor may help detect elevated troponin in ACS. Available at: https://www.tctmd.com/news/wrist-worn-sensor-may-help-detect-elevated- troponin-acs. Accessed Jan 2, 2023.
5. Li L, Shu X, Zhang L, Xu A, Yang J, Jing Y, et al. Evaluation of the analytical and clinical performance of a new high-sensitivity cardiac troponin I assay: hs-cTnI (CLIA) assay. Clin Chem Lab Med 2023;62(2):353–360. [CrossRef]
6. Warner JV, Marshall GA. High incidence of macrotroponin I with a high-sensitivity troponin I assay. Clin Chem Lab Med 2016;54(11):1821–1829. [CrossRef]
7. Peacock WF, Soto-Ruiz KM, Jaffe AS, Tiffany BR, Mahler SA, Patterson BW, et al. A rapid non-invasive wearable device for assessing cardiac troponin. Clin Exp Emerg Med 2024;24:294. [CrossRef]
8. Shufelt CL, Kim A, Joung S, Barsky L, Arnold C, Cheng S, et al. Biometric and psychometric remote monitoring and cardiovascular risk biomarkers in ischemic heart disease. J Am Heart Assoc 2020;9(18):e016023. [CrossRef]
9. Wang Y, Dai L, Wang N, Zhu Y, Chen M, Wang H. Rapid rule out of acute myocardial infarction in the observe zone using a combination of presentation N-terminal pro-B-type natriuretic peptide and high-sensitivity cardiac troponin I. Clin Biochem 2019;70:34–38. [CrossRef]
10. Kaier TE, Twerenbold R, Puelacher C, Marjot J, Imambaccus N, Boeddinghaus J, et al. direct comparison of cardiac myosin-binding protein c with cardiac troponins for the early diagnosis of acute myocardial infarction. Published correction appears in Circulation 2017;136(25):e469. [CrossRef]
11. Tscherny K, Kienbacher C, Fuhrmann V, Schreiber W, Herkner H, Roth D. Early identification of patients with chest pain at very low risk of acute myocardial infarction using clinical information and ECG only. Int J Clin Pract 2020;74(8):e13526. [CrossRef]
12. Lopez-Ayala P, Boeddinghaus J, Koechlin L, Nestelberger T, Mueller C. Early rule-out strategies in the emergency department utilizing high-sensitivity cardiac troponin assays. Clin Chem 2021;67(1):114–123. [CrossRef]
13. Suran M. Highlights from the american college of cardiology's 2023 scientific session: The ketogenic diet and cardiac events, a wearable sensor to predict troponin levels, bempedoic acid for statin intolerance, and more. JAMA 2023;329(14):1139–1141. [CrossRef]
14. Anbuselvam B, Gunasekaran BM, Srinivasan S, Ezhilan M, Rajagopal V, Nesakumar N. Wearable biosensors in cardiovascular disease. Clin Chim Acta 2024;561:119766. [CrossRef]

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Opinion Paper

Telemedicine in urinalysis: Future is now

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Abstract

Telemedicine is namely the practice of medicine using electronic and telecommunication technologies at a distance. Urinalysis may be the latest unit to meet automation in the Clinical Pathology Laboratory. Microscopic evaluation of urine samples has been a challenge for manufacturers in this field. We report an application of telemedicine in this field. Digital images taken by Sysmex UD-10 device built in a city were revised by a pathologist in another city.

Keywords: Healthcare, laboratory, telemedicine, urinalysis

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Telemedicine is namely the practice of medicine using electronic and telecommunication technologies at a distance. As started with transforming information and images between telephones, the ultimate rise of the digital age has brought a plethora of applications. In hospitals, medical departments using digital imaging technologies, like radiology or surgical and clinical pathology, enjoy the comfort of sharing all sorts of data including real-time interactions. Compared to the surgical pathology, clinical pathology laboratory has been more convenient to automation. Accordingly, modern laboratories run to a future without any human interventions [1].

Urinalysis may be the latest unit to meet automation in the clinical pathology laboratory. Microscopic evaluation of urine samples has been a challenge for manufacturers in this field. Besides many articles reporting good correlation between instruments and manual microscopy, it should be considered as a field to be improved even in the most advanced systems. Meanwhile, automated urine analyzers have been accepted with exuberant welcome in laboratories as they are fast and precise. Particularly considering automated urine analyzers, it is not odd to call these instruments artificial intelligence: These machines "decide" on the nature of particles they "see" due to the characteristics of signal patterns [2]. As a state of wisdom, instruments classify particles "unknown" to them to be edited by human users. Actually, all instruments in the market need user editing to some degree that manual microscopy with a light microscope remain a compulsory component of the urine laboratory. New generation devices are equipped with adequate hardware components, so that developmental efforts on urinalysis automation focus mostly on software systems [3]. From the point of automation, hardware and software corresponds to human eye and neural system, respectively. The instruments using flow-cytometry or built-in microscopes have superior vision to human eye. However, a technician with average experience in urinalysis is still favored to the most advanced instrument. Obviously, superiority of the technician over the machine is due to the level of intelligence. On the other hand, as the instruments evolve, the need for human interference has decreased the capability of technicians in urinalysis. Education of the young ones is getting worse and as the experienced ones get retired, we observe a growing deficit of on-board editing of urine analyzers. An unusual crystal, for example, is reported undefined, unless consulted to an

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experienced supervisor. We have been using e-mail options for such consultations. We here describe a more efficient way of consultation that should be classified as telemedicine.

We have been dealing with the experimental parameter "atypical cells" in Sysmex UN automated urine analyzer for some time [3–5]. Our search aimed to evaluate the performance of the instrument in this parameter. In our latest study, a modular unit composed of Sysmex UF-5000 flow cytometry instrument and Sysmex UD-10 digital imaging instrument, was built in the laboratory [5]. While the counting of atypical cells by Sysmex UF-5000 was completely automated, digital images taken by Sysmex UD-10 had to be revised by a user [6]. Particularly, the evaluation of digital images by Sysmex UD-10 needed extra experience in this specific issue. In our study, while the instrument, the urologist ant other members of the study were located in one city (in Ankara), the expert pathologist to review the images of the Sysmex UD-10 unit joined the study from another city (Antalya). The pathologist was able to reach the instrument on-line and perform any action possible on-board. At the end of the day every patient had a report composed flow-cytometric analysis performed by the instrument located in Ankara and a decision comment performed by the pathologist located in Antalya.

We witness the rise of the artificial intelligence in all fields of life sciences and clinical pathology is no exception. It does not to be the most advanced applications; starting from the very beginning of laboratory information systems, we have been working with the artificial intelligence. Recents on-line applications like auto-verification of laboratory test results cause major changes in the management of modern laboratories [7]. 'Digital accessibility' brings many future aspects of laboratory medicine to date. We believe this particular experience has many applications that will influence the future aspects of telemedicine.

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References

1. Pallua JD, Brunner A, Zelger B, Schirmer M, Haybaeck J. The future of pathology is digital Pathol Res Pract 2020;216(9):153040. [CrossRef]
2. Aydin O. Atypical cells parameter in an automated urine analyzer: Does it have a future? Anal Biochem 2020;600:113763. [CrossRef]
3. Aydin O. Atypical cells parameter in Sysmex UN automated urine analyzer: Feedback from the field. Diagn Pathol 2021;16:1–5. [CrossRef]
4. Aydin O, Yapıcı O, Copuroglu R. Atypical cells in Sysmex UN automated urine particle analyzer: A case report and pitfalls for future studies. Turk J Biochem 2020;45(5):617–9. [CrossRef]
5. Aydin O, Sarikaya S, Caglar E, Ayas R, Ozgurtas T. Atypical cells parameter in Sysmex UN automated urine analyzer: A single center study. Turk J Biochem 2025;50(1):32–6. [CrossRef]
6. Aydin O. Sysmex UD-10 should accompany UF-5000 in managing atypical cells in urine. J Bioanal Methods Tech 2020;1(1):102.
7. Gul BU, Ozcan O, Dogan S, Arpaci A. Designing and validating an autoverification system of biochemical test results in Hatay Mustafa Kemal University, clinical laboratory. Biochemia Medica 2022;32(3):385–95. [CrossRef]

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Letter to the Editor

Impact of preanalytical storage on the accuracy of CD3, CD4, CD8 testing results using the BD FACSLyric™ clinical flow cytometry system

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Dear Editor,

We eagerly and thoroughly enjoyed reading your journal's first edition of the new year. We were particularly interested in the flow cytometry-related paper and the letter to the editor in this issue [1, 2]. We would like to take this opportunity to express our appreciation to the participating researchers and technical collaborators.

Various theoretical and practical approaches are involved in the interpretation of flow cytometry analyses. The analysis is subject to interpretation because it might differ based on the scientific discipline and methodology used, even though the basic ideas are always the same.

In keeping with the aforementioned concepts, we would like to provide some remarks and contributions to illustrate our methodology through the insightful article of the International Journal of Medical Biochemistry titled "Impact of preanalytical storage on the accuracy of CD3, CD4, and CD8 testing results using the BD FACSLyric™ Clinical Flow Cytometry System."

We shall try to draw conclusions through some remarks because the article did not go into detail about the testing procedures. For example, how was the absolute count of T lymphocyte subsets determined using the gating strategy? First, we assume that the flow was monitored (SS-Time), then the doublets were removed (FS Peak- FS Int), followed by the use of a viability dye (7-AAD) to identify live cells, and finally, we think that the CD45 pan-leukocyte marker (CD45 against SSC) was used for gating.

We were unable to draw firm conclusions regarding the fluorescent signal strength of the cells and the tube design, which may differ depending on the laboratory, because we were unable to observe the tube design of the lab (CD45-FITC/CD4-PE/CD3-PerCp, etc.). Thus, we can add that APC is more stable among the fluorescent dyes, whereas FITC is more light-sensitive. Consequently, it should either be evaluated right away after application or left in the dark until examination is complete. Although the platform utilized to calculate absolute cell counts is not explicitly mentioned in the article, it appears that the researchers employed the single-platform approach with beads (fluorescent microspheres) to accomplish so. A precise and exact WBC count is crucial when employing the dual-platform approach to provide absolute counts. WBC count × lymphocyte × antibody positivity is the formula for absolute count/µL. The gating technique is crucial in this situation. If there are issues with the WBC count, the dual-platform method is not recommended [3].

Giving the blood-to-bead ratio (1:1, vol/vol, etc.) could have given more information about the researchers' analysis approach if their approach had been single-platform. However, if the article's approach was dual-platform, the Australasian Cytometry Society recommends that the T-cell absolute count be determined using lymphocytes for samples older than 48 hours and WBC counts for samples younger than 48 hours [4]. The "Lymphosum" method is advised to increase accuracy in dual-platform analysis of absolute T-cell counts. The formula T cells+B cells+NK cells=100%±5 is used to confirm this [5].

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References

1. Ilardo C, Tabone D, Becker M, Deloison L, Arias Rojas N, Dirat M. Impact of preanalytical storage on the accuracy of CD3, CD4, CD8 testing results using the BD FACSLyric™ Clinical Flow Cytometry System. Int J Med Biochem 2023;6(2):109-13. [CrossRef]
2. Arash M. Impact of preanalytical storage on the accuracy of CD3, CD4, CD8 testing results using the BD FACSLyric™ clinical flow cytometry system. Int J Med Biochem 2025;8(1):53. [CrossRef]
3. Clinical and Laboratory Standards Institute. Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline, 2nd ed. CLSI document H42-A2. Wayne:Clinical and Laboratory Standards Institute; 2007.
4. Australasian Cytometry Society. ACS guideline for lymphocyte subset immunophenotyping. Available at: https://cytometry.org.au/wp-content/uploads/2018/08/acs-guideline-for-lymphocyte-subset-immunophenotyping.pdf. Accessed Feb 14, 2025.
5. Demirel GY, Deniz G, Demiralp EE. Guidelines for lymphocyte immunotyping. Turk Soc Immunol Flow Cell Meter Subgroup [Article in Turkish] 2010;2010:1-19.