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Gene expression profiling in B-cell non-Hodgkin lymphomas

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Abstract:

BACKGROUND: Gene expression profiling has become a fundamental tool in cancer diagnosis and management. B-cell non-Hodgkin lymphoma (B-NHL) is a group of malignant neoplasms originating from the lymphoid tissues, mainly the lymph nodes and the gene expression technique was used to unravel its complexity and aid in clinical decision-making.

OBJECTIVES: The aims of this study were to find the significance of gene expression profiling focusing on colony-stimulating factor 1 receptor (*CSF1R*), myeloid differentiation factor 88 *(MyD88)*, and tumor necrosis factor-α (*TNF-*α) as a promising approach in B-NHL diagnosis and their comparison with healthy controls.

PATIENTS, MATERIALS AND METHODS: The current clinical prospective study was mediated from June 1, 2021, to December 30, 2022, of NHL patients in Kurdistan, Iraq. Seventy-three patients were recruited from Nanakali Hospital for Blood Diseases and Cancer, Erbil. The integration of gene expression biomarkers uses quantitative real-time polymerase chain reaction technique to diagnose B-NHL. Specifically, we focused on three key genes *MyD88*, *TNF*, and *CSF1R* whose expression profiles were analyzed in B-NHL patients and controls. We leveraged a dataset to explore gene expression patterns in B-NHL and applied classification algorithms to distinguish between B-NHL patients and controls.

RESULTS: The initial results show the overall lower *CSF1R* expression in B-NHL as compared to the controls and a significant reduction in *CSF1R* expression in females ($\leq$50 years and >50 years). The result considers lower *CSF1R* expression in B-NHL males ($\leq$50 years) and higher but not significant in males (>50 years).

CONCLUSIONS: These B-NHL-expressed genes may be considered potential diagnostic markers with their meaningful comparisons to control groups, and they could be proposed to guide the management of patients and facilitate their stratification into clinical trials.

Keywords:

B-cell non-Hodgkin lymphoma, colony-stimulating factor 1 receptor, gene expression profiling, myeloid differentiation factor 88, tumor necrosis factor

Introduction

Non-Hodgkin B-cell lymphomas are an extremely diverse category of mature B-cell cancers.[1] The classification of lymphoid malignancies has evolved from a purely morphological scheme to the current World Health Organization classification, which takes into consideration histological, immunophenotypic, genetic, and clinical information.[2]

Currently, biomedical researches attempt to explore gene expression to be a great resource to explain the mechanisms by which a particular disease develops.[3] Numerous genes control the cell cycle including apoptosis and lymphocyte development in B-cell non-Hodgkin lymphoma (B-NHL)-associated translocations.[4]

For individuals with follicular lymphoma, the use of immunologic treatments that alter the host immune response may be crucial to their prognosis.[5] Thus, the application of gene expression profiling in B-NHL provides the important prognostic

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information at the time of diagnosis and can be translated into therapeutic options that improve patient outcomes.[6]

Gene expression analysis mainly helps to study the gene quantification methods using various downstream detection approaches such as imaging, amplification, probe hybridization, and sequencing.[7] In contrast to deoxyribonucleic acid (DNA), which is less variable, mRNA levels change throughout time and among different cell types in various environments. Gene expression analysis is principally focused on the determination of mRNA levels transcribed from DNA.[8,9]

Myeloid differentiation factor 88 (*MyD88*) is involved in oncogene-induced cell intrinsic inflammation and in cancer-associated extrinsic inflammation, and *MyD88* contributes to skin, liver, pancreatic, and colon carcinogenesis, as well as sarcomagenesis.[10] Myeloid differentiation primary response protein 88 (*MYD88*) is a general adaptor protein that acts downstream of the toll-like receptor (TLR) and interleukin (IL)-1 or IL-18 receptors to mediate the activation of nuclear factor-κB (NF-κB).[11]

The multifunctional cytokine tumor necrosis factor (*TNF*) is involved in a variety of biological processes, including cell survival, proliferation, differentiation, and death.[12] *TNF* is a protein consisting of 157 amino acids and is synthesized as a membrane-bound protein (pro-*TNF*) that is released by *TNF*-converting enzyme-mediated cleavage. As a pro-inflammatory cytokine, *TNF* is secreted by inflammatory cells, which may be involved in inflammation-associated carcinogenesis.[13] *TNF-α* is a pleiotropic biomodulator and an important inducer of certain inflammatory immune reactions such as granuloma formation and septic shock. As *TNF-α* activates the NF-κB pathway, a central mechanism for inflammation, it is plausible that these genetic factors contribute to further chronic inflammation that ultimately leads to lymphomagenesis.[14]

The single channel III transmembrane receptor tyrosine kinase, known as colony-stimulating factor 1 receptor (*CSF1R*), is involved in immunological modulation and the emergence of different forms of cancer. Numerous investigations have revealed a link between the poor prognosis of numerous malignant tumors and the overexpression of *CSF1R*.[15] *CSF1R*, a classic tyrosine kinase receptor, has been identified as a proto-oncogene in multiple cancers.[16]

In this study, gene expression profiling was conducted on B-NHLs, with a particular focus on three distinct genes: *CSF1R*, *MyD88*, and *TNF* within cancerous tissues. Additionally, this study aimed to perform a comparative analysis of gene expression levels in control tissues using real-time polymerase chain reaction (RT-PCR).

Materials and Methods

Sample preparation and group stratification

The current study was mediated from June 1, 2021, to December 30, 2022 of healthy controls and NHL patients from diverse ethnic backgrounds including Kurds, Arabs, Turks, and Christians. Patients were recruited from Nanakali Hospital for Blood Diseases and Cancer, Erbil. Medical records and formalin-fixed paraffin-embedded (FFPE) tissue blocks containing tumor tissue samples were retrieved from archival units of Rizgary Educatory Hospital, AL-Mufti Histo-Diagnostic Laboratory, and Luai Diagnostic Laboratory. The study exclusively enrolled newly diagnosed cases that had not undergone any prior medication or therapy. The cohort comprised 73 participants, including 24 healthy controls (HCs) (12 females and 12 males) and 49 NHL patients (22 females and 27 males).

This study was authorized and approved by the Human Ethics Committee of the College of Science, Salahaddin University, Erbil (Approval No: 45/147; Date: January 30, 2024). All patients provided written and informed consent for the publication of data in this study.

In the current study, we meticulously examined a cohort of 73 paraffin-embedded tissue samples, encompassing individuals diagnosed with NHL and individuals with normal tissue specimens. The sample selection process involved a comprehensive stratification based on two key demographic factors: gender and age. This stratification resulted in a total of eight distinct sample groups, allowing us to explore gene expression patterns across different subpopulations. The three genes of interest, *CSF1R*, *MyD88*, and *TNF*, were evaluated in comparison to Beta actin (ACTB) gene, which served as the reference housekeeping gene for normalization. The eight sample groups are served as NHL males over 50 years, normal males over 50 years, NHL females over 50 years, normal females over 50 years, NHL males under 50 years, normal males under 50 years, NHL females under 50 years, and normal females under 50 years.

Extraction of RNA from formalin-fixed paraffin-embedded tissue

Total RNA extraction was performed using the Magen HiPure FFPE RNA Plus Kit (R414303). Excess paraffin was carefully trimmed from tissue samples using a scalpel, and the collected paraffin was dissolved with DNA binding proteins from starved cells (DPS) buffer. The samples were then lysed through Proteinase K incubation at 80°C for 15 min. After digestion, the samples were transferred to adsorption columns and centrifuged. Following several washing steps, RNA was eluted. The concentration and purity of

the extracted RNA were evaluated using a Multiskan Go spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Any genomic DNA contamination in the RNA samples was eliminated using DNAse I enzyme (M0303S, New England BioLabs, NEB).[17,18]

Complementary deoxyribonucleic acid synthesis

Reverse transcription from RNA to complementary deoxyribonucleic acid (cDNA) was carried out using the Blirt TRANSCRIPTOME RNA Kit (RT31). Total RNA isolated from FFPE tissue was mixed with 1 µL of random hexamer primer and 1 µL of dNTP mix in a tube. The total volume was adjusted to 16 µL with nuclease-free distilled water, gently mixed, and centrifuged. After 5 min of incubation at 65°C, the mixture was cooled on ice. The following steps were taken: 2 µL of 10× reaction buffer, 1 µL of RNase inhibitor, and 1 µL of reverse transcriptase were added. After a gentle mixing, centrifugation, and 30-min incubation at 50°C, the tube was set aside. The resulting cDNA was stored at −20°C for future use.[19]

Quantitative real-time polymerase chain reaction

The expression levels of *CSF1R*, *MyD88*, and *TNF* genes in the cell lines were analyzed through quantitative RT-PCR (q-RT-PCR) using the Bio-Rad CFX96 Touch System (Bio-Rad, Richmond, CA, USA). A 20 µL final reaction mixture was prepared, containing 100 ng cDNA, 1 mM of both reverse and forward primers, 10X Luna® SYBR Green qPCR Master Mix (M3003S, NEB), and RNase-free distilled water. To check for contamination, no template control was included. β-actin was used as the housekeeping gene. The q-RT-PCR program consisted of an initial denaturating step at 95°C for 10 min, followed by 45 cycles of amplification with denaturation at 95°C for 20 s, annealing at 60°C for 20 s, and extension at 72°C for 20 s. Following cycling, software for melting curve analysis was implemented, utilizing a heating rate of 0.1°C/s and continuous fluorescence monitoring between 50°C and 99°C. Melting curve analysis verified that the PCR product had been found.[20] Quantification of specific mRNAs in the samples was carried out using the respective gene and the relative standard curve method. According to the Livak method, the study determined relative mRNA expression based on cycle threshold values.

Statistical analysis

A comprehensive comparison of gene expression by gender and age was used regarding means and standard error means (SEMs) of the results. The obtained data were analyzed with one-way ANOVA to compare B-NHL with controls, and the significant level was fixed at 0.05 using GraphPad Prism 8; GraphPad software, La Jolla, CA, USA.

Results

Healthy controls and non-Hodgkin lymphoma characteristics

The patient's demographics and baseline characteristics are summarized in Table 1. Patients with NHL were shown to have different distributions, with several different subtypes. A greater number of cases were categorized as diffuse large B-cell lymphoma (DLBCL) of all NHL cases. In addition, NHL patients were further subgrouped into high-grade B-cell lymphoma, low-grade B-cell lymphoma, follicular lymphoma, and Burkitt lymphoma. These results consider that DLBCL is significantly overrepresented among the patients. The frequency of high-grade B-cell lymphoma, follicular lymphoma, and Burkitt lymphoma was lower than that of low-grade B-cell lymphoma [Table 1].

The extent of tumor invasion, lymph node involvement in the regional area, and the presence of distant metastasis were all revealed by the Ann Arbor staging system. The most common was Stage IV. Stage III was the second most common staging pattern. In addition, patients were classified into Stage I and Stage II. The differences between the phases are indicative of factors such as tumor size, lymph node involvement, and the presence or absence of distant metastasis, as shown in Table 1.

Furthermore, the comparison results of the expression levels of the three genes (*CSF1R, MyD88,* and *TNF-α*) between HCs and NHL patients, segmented by gender (female and male) and age groups (≤50 years and >50 years) [Table 2].

The results of *CSF1R* gene in females (≤50 years) of HCs and NHL presented with $P = 0.023$ which is a

Table 1: Demographic and baseline characteristics of healthy controls and Non-Hodgkin lymphoma patients

Characteristic	Control	NHL
Female, *n* (%)	12 (16.43)	22 (30.13)
Male, *n* (%)	12 (16.43)	27 (36.98)
Age ≤50 (female/male), *n* (%)	5 (6.84)/7 (9.58)	10 (13.69)/13 (17.80)
Age >50 (female/male), *n* (%)	7 (9.58)/5 (6.84)	12 (16.43)/14 (19.17)
Lymphoma subtypes (%)		
DLBCL		59.18
High-grade B-cell lymphoma		8.16
Low-grade B-cell lymphoma		14.28
Follicular lymphoma		8.16
Burkitt lymphoma		8.16
Ann arbor staging (%)		
I		4.08
II		26.53
III		32.65
IV		36.73

NHL=Non-Hodgkin lymphoma, DLBCL=Diffuse large B-cell lymphoma

statistically significant difference. In addition, there is also a significant difference in the females (>50 years) of HC with NHL with $P = 0.010$. While, for males, there are no significant differences between HCs and NHL in both age groups [Table 2].

The results of *MyD88* gene in females of both ages for the HC compared with NHL have no significant difference.

The males of both ages for the HC have no significant difference with NHL.

Additionally, the *TNF-α* gene for females and males of both ages for the HC with NHLs has no significant differences [Table 2].

Furthermore, the results of this study show the expression levels of *CSF1R*, *MyD88*, and *TNF-α* between all HCs and all NHL patients. The study reveals significant differences in gene expression between non-Hodgkin lymphoma (NHL) patients and healthy controls, for both *CSF1R* and *MYD88* gene [Figure1].

A similar pattern, NHL patients generally exhibit lower CSF1R expression and being more pronounced in females than males for those under 50 years [Figures 2 and 3].

Furthermore, significantly reduction in *CSF1R* and *MYD88* were presented in females over 50 years old [Figure 2].

The results of *CSFIR* gene expression increased in males compared to females [Figure 4].

The results from multiple comparisons illustrate changes in CSF1R, MyD88, and *TNF-α* expression. Specifically, CSF1R expression was higher in males over 50 years than other subgroups, and slightly increased *TNF-α* expression in females over 50 years, whereas, MyD88 do not demonstrate notable differences. This analysis underscores the importance of both gender and age when evaluating gene expression in NHL patients [Figure 5].

The detailed comparisons have shed light on the influencing gene expression in NHL patients regarding gender and age, ultimately providing valuable insights into the molecular mechanisms associated with this disease.

Discussion

The analysis of differential gene expression remains a cornerstone in molecular biology, providing profound

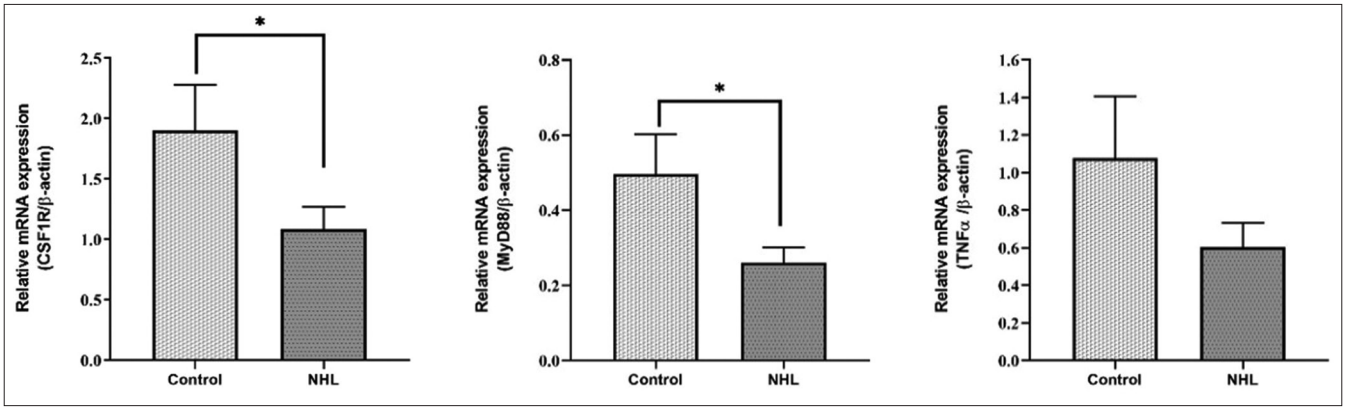

Figure 1: The comparison expression of CSF1R, MyD88 and TNF-α between controls and NHL. CSF1R = Colony-stimulating factor 1 receptor, MyD88= Myeloid differentiation factor 88, and TNF-α = Tumor necrosis factor-alpha, NHL = Non-Hodgkin lymphoma

Table 2: The Comparison of gene expression levels in healthy controls and Non-Hodgkin lymphoma

Gene names	Female		*P*	Male		*P*
	HC	NHL		HC	NHL	
CSF1R						
≤50	2.586±0.946	0.743±0.220	0.023	1.206±0.505	0.826±0.248	NS
>50	2.552±0.821	0.700±0.151	0.010	1.147±0.615	1.910±0.515	NS
MyD88						
≤50	0.604±0.155	0.286±0.114	NS	0.358±0.187	0.264±0.076	NS
>50	0.872±0.254	0.294±0.096	NS	0.130±0.045	0.204±0.037	NS
TNF α						
≤50	1.098±0.446	0.637±0.276	NS	0.502±0.236	0.920±0.610	NS
>50	2.179±0.961	0.802±0.275	NS	0.313±0.275	0.651±0.295	NS

CSF1R=Colony-stimulating factor 1 receptor, *MyD88*=Myeloid differentiation factor 88, *TNF-α*=Tumor necrosis factor-α, NS=Not significant, NHL=Non-Hodgkin lymphoma, HC=Healthy control

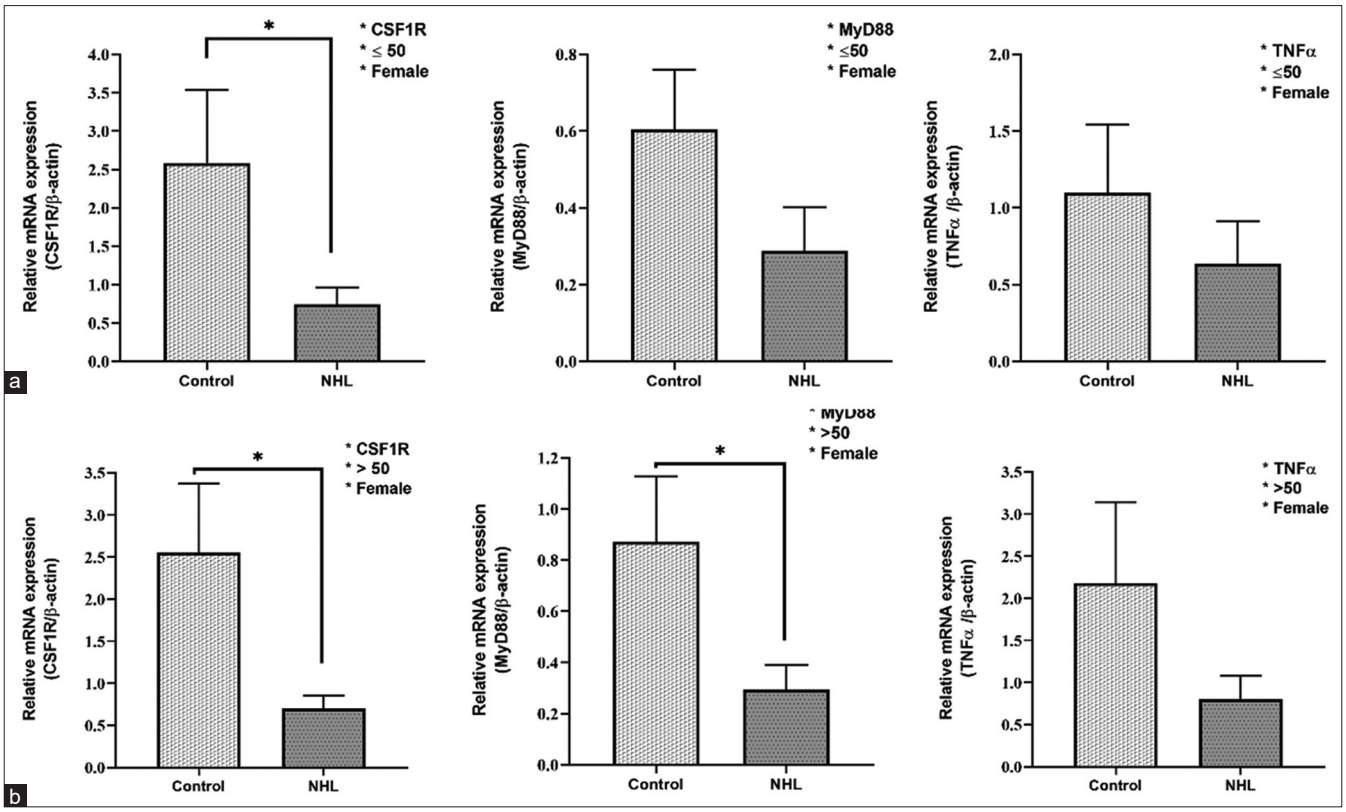

Figure 2: The comparison of the expression levels of colony-stimulating factor 1 receptor, myeloid differentiation factor 88, and tumor necrosis factor in female individuals diagnosed with non-Hodgkin lymphoma, stratified by age, (a) The comparison between healthy controls and females of/under 50 years, (b) The comparison between healthy controls and females over 50 years. *CSF1R* = Colony stimulating factor 1 receptor, *MyD88*= Myeloid differentiation factor 88, *TNF-α* = Tumor necrosis factor-α, NHL = Non-Hodgkin lymphoma

insights into the relationships between transcription products, biological processes, and diseases. Small changes in RNA expression can have significant implications.[21] The role of gene regulation in evolution and adaptation has underscored its importance in understanding species divergence and environmental adaptation.[22]

The necessity of delineating gene expression profiles within specific demographic populations is underscored by a comprehensive study conducted on individuals of Egyptian descent. This investigation aimed to elucidate distinctive gene expression patterns inherent to NHL cases within this demographic subset, employing a microarray-based approach. The outcomes of this study unveiled notable associations, with eight genes demonstrating upregulated expression and four genes exhibiting downregulation in correlation with NHL.[23] Among these genes, the *CSF1R* emerges as a novel prognostic indicator and a promising therapeutic target within the context of follicular lymphoma.[24]

In the current investigation, we undertook a comprehensive analysis encompassing not only the comparison of gene expression between cancerous and normal tissue samples but also scrutinized variations based on gender and age. Interestingly, we found no visible differences in gene expression patterns between males and females.[25,26] Moreover, upon examining the interaction between gender and age, no notable alterations or disparities in gene expression profiles were observed. The distinctive feature of our study is the comprehensive examination of three pivotal genes within NHLs, accomplished through precise measurements of gene expression using RT-PCR. The rigorous structure of our study incorporated two distinct sets of samples, encompassing both cancerous and normal tissues.[27] The selection of active cycle of breathing technique as a housekeeping gene proved to be a suitable and reliable choice for gene expression assessment.[28]

The majority of patients had NHL, and the advanced stages that were found in them highlight the need of early diagnosis and the need for improved diagnostic markers. The research that now indicates DLBCL as the most frequent form of NHL, marked by aggressive behavior and a bad prognosis if treatment is delayed, is supported by the greater incidence of DLBCL among the subtypes.[29]

The current results showed that a gender-specific decrease in *CSF1R* expression indicates that females,

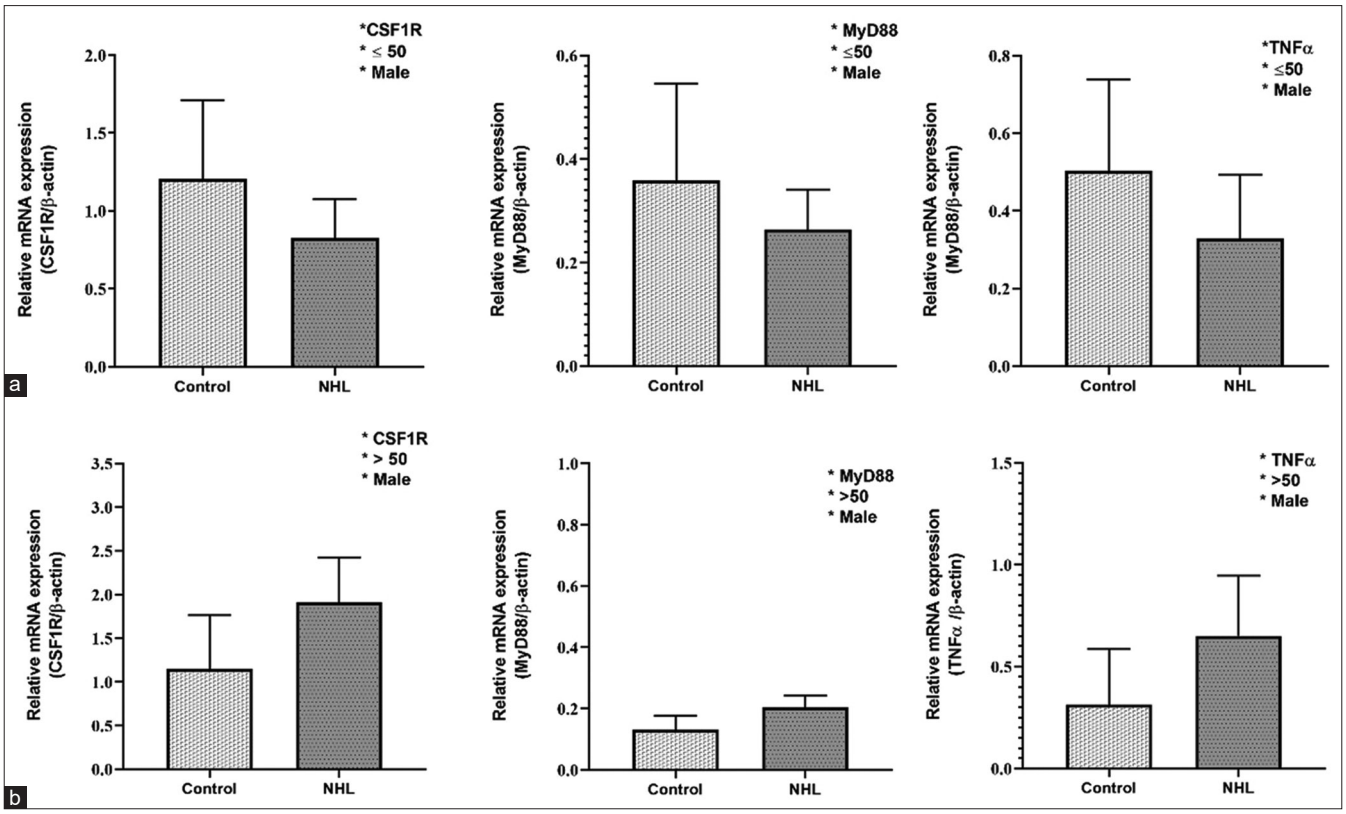

Figure 3: Comparison of the expression levels of colony-stimulating factor 1 receptor, myeloid differentiation factor 88, and tumor necrosis factor in male individuals diagnosed with non-Hodgkin lymphoma, stratified by age, (a) The comparison between healthy controls and males of/under 50 years, (b) The comparison between healthy controls and males over 50 years. *CSF1R* = Colony stimulating factor 1 receptor, *MyD88*= Myeloid differentiation factor 88, *TNF-α* = Tumor necrosis factor-α, NHL = Non-Hodgkin lymphoma

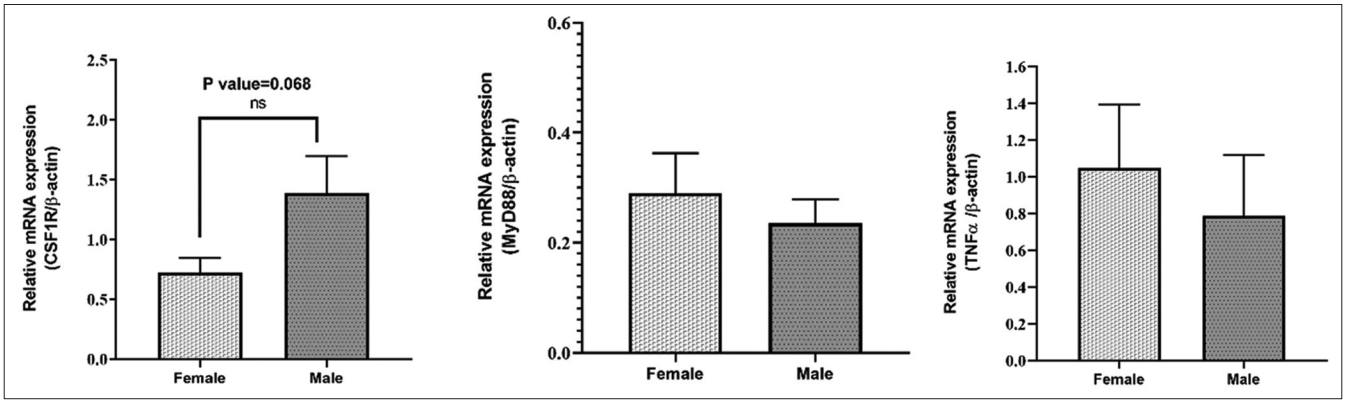

Figure 4: Comparison of the expression levels of colony-stimulating factor 1 receptor, myeloid differentiation factor 88, and tumor necrosis factor in individuals diagnosed with non-Hodgkin lymphoma generally, stratified by gender. *CSF1R* = Colony stimulating factor 1 receptor, *MyD88*= Myeloid differentiation factor 88, *TNF-α* = Tumor necrosis factor-α

especially those over 50, may have a distinct biological response to NHL involving *CSF1R*. This can be explained by the genetic and hormonal differences that exist between males and females, which affect immunological response and gene expression. The expression of immune-related genes, such as *CSF1R*, may be modulated by estrogen, according to research, which might account for the observed variances. The significant decrease in *CSF1R* expression in female NHL patients, particularly those over 50 years, suggests that *CSF1R* plays a crucial role in the pathogenesis and progression of NHL. *CSF1R* is a key regulator of macrophages within the tumor microenvironment, influencing tumor growth and metastasis.[30] This finding is consistent with studies highlighting the involvement of *CSF1R* in various cancers, where it regulates the differentiation and function of macrophages, essential components of the tumor stroma.

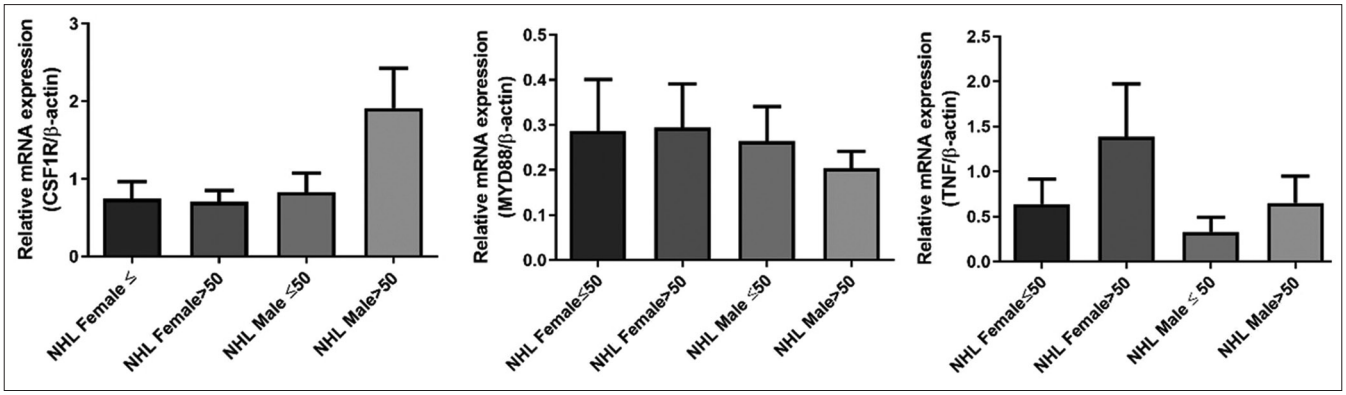

Figure 5: Multiple comparisons of the expression levels of colony-stimulating factor 1 receptor, myeloid differentiation factor 88, and tumor necrosis factor in individuals diagnosed with non-Hodgkin lymphoma, regarding gender and age. *CSF1R* = Colony stimulating factor 1 receptor, *MyD88*= Myeloid differentiation factor 88, *TNF-α* = Tumor necrosis factor-α, NHL = Non-Hodgkin lymphoma

Colony stimulating factor, a receptor for colony-stimulating factor 1, is crucial for the recruitment and activation of tissue macrophages. In the context of tumors, macrophages, known as tumor-associated macrophages (TAMs), contribute significantly to the tumor microenvironment, aiding in tumor progression and metastasis. Aberrant expression of *CSF1R* has been documented in various lymphomas, including NHL, suggesting its potential as a therapeutic target. *CSF1R* is primarily expressed on the surface of macrophages and their precursors. In the tumor microenvironment, *CSF1R* signaling promotes the survival and proliferation of TAMs, which are known to support tumor growth by suppressing immune responses and enhancing angiogenesis. The reduced expression of *CSF1R* in female NHL patients might impair TAM function, leading to less effective tumor support and potentially slower disease progression.[31]

Nonetheless, a compelling finding emerged, revealing a substantial decrease in *MyD88* expression, specifically among males aged 50 and under 50 compared to their older counterparts. Myeloid differentiation primary response 88 is an adaptor protein that plays a pivotal role in the innate immune response and inflammation through the TLR and IL-1 receptor pathways. The reduced expression of *MyD88* in NHL patients might reflect a compromised immune response, which could facilitate tumor growth and immune evasion.[32]

The study acknowledges a limitation due to limited available samples, highlighting the need for a larger, diverse cohort to gain a more comprehensive understanding of cancer development roles.

Conclusions

Real-time q-RT-PCR is a powerful tool for measuring gene expression in NHL. The targeted expressed genes and their changes through meaningful comparisons with controls can serve as potential diagnostic markers, guiding patient management and facilitating stratification for clinical trials. Further research should explore the underlying mechanisms driving these gender-specific differences and their implications for personalized treatment approaches in NHL.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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