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Evaluation of the level of LAG-3 gene expression among β-thalassemia major patients with and without alloantibodies in comparison with control group from patient in West Bank, Northern Palestine.

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This thesis was submitted in partial fulfilment of the requirements for the Master's degree in Immunohematology

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Thesis Approval

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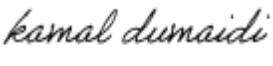
1. Dr. Kamal Dumaidi: Supervisor	
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Declaration

I declare that my dissertation, “Evaluation of the level of LAG-3 gene expression among β-thalassemia major patients with and without alloantibodies in comparison with control group from patient in West Bank, Northern Palestine.” submitted by me under the supervision of Dr. Kamal Dumaidi for the partial fulfillment of the award of a master's degree in immunohematology, is original and was written independently with no other sources than those quoted, and it is substantially my own work and has not been submitted for any other degree at the Arab American University or any other institution.

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Dedication

I dedicate this thesis to my parents, who have always inspired me: my mother and my father, Dr. Abdulraouf Jarrar, my family and my wonderful child, Khaled. Without their affection and encouragement, our project would not have been possible.

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Abstract

Background: The immune system maintains internal homeostasis by defending against pathogens while eliminating potentially harmful self-reactive cells. Among the regulatory molecules involved in this balance, lymphocyte-activation gene 3 (LAG-3) has emerged as a key player in both autoimmunity and tumor immune evasion. In patients reliant on chronic blood transfusions—such as those with β-thalassemia major or sickle cell disease—the development of alloantibodies and immune dysregulation is well documented. However, the specific contribution of LAG-3 expression in this clinical context remains poorly understood.

Objectives: The present study aims to evaluate the expression levels of lymphocyte activation gene-3 (LAG-3) in transfusion-dependent patients, including those with β-thalassemia major, compared to healthy controls. Additionally, the study seeks to assess LAG-3 expression in thalassemia patients with and without alloantibodies.

Methodology: The study enrolled 100 participants: 80 individuals diagnosed with β-thalassemia major, 20 with sickle cell disease, and 100 age- and sex-matched healthy controls. Peripheral blood mononuclear cells (PBMCs) were isolated, and total RNA was extracted for the assessment of LAG-3 gene expression. Quantitative real-time PCR (qRT-PCR) using the SYBR Green method was employed, with β-actin serving as the internal housekeeping gene. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. Data were entered and analyzed using IBM SPSS Statistics, with results expressed as medians and interquartile ranges (IQR) unless otherwise specified.

Results: Transfusion-dependent patients exhibited elevated LAG-3 transcript levels compared to age- and sex-matched healthy controls. However, no statistically significant difference in LAG-3 expression was observed between alloimmunized and non-alloimmunized subgroups. Notably, while total white blood cell counts and the number of transfused units approached conventional thresholds for significance, serum ferritin levels and prior splenectomy status showed no discernible effect on LAG-3 expression.

Conclusion: Chronic exposure to repeated blood transfusions appears to shift LAG-3 expression away from baseline levels, suggesting that the immune system may be undergoing stress-induced recalibration. Interestingly, the presence of alloantibodies does not correlate with further upregulation of this immune checkpoint marker. Longitudinal studies are warranted to determine whether LAG-3 acts merely as a passive indicator of transfusion-related immune changes or plays an active role in driving sustained immunologic dysregulation.

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List of Definitions of Abbreviations

Abbreviations	Title
AAUP	Arab American University Palestine
ADAM	Zinc-dependent a disintegrin and metalloproteinase
ADCC	Antibody-dependent cellular cytotoxicity
Allo (-)	Negative alloantibody
Allo (+)	Positive alloantibody
APC	Antigen presenting cell
Breg cells	B regulatory cells
CD137	Cluster of differentiation 137
CD27	Cluster of differentiation 27
CD28	Cluster of differentiation 28
CD3	Cluster of differentiation 3
CD4	Cluster of differentiation 4
CD47	Cluster of differentiation 47
CD8	Cluster of differentiation 8
cDNA	Complementary DNA
CRC	Colorectal cancer
CTL	Cytotoxic T-lymphocyte
CTLA-4	Cytotoxic T-lymphocyte–associated protein 4
CTLAG4	Cytotoxic T lymphocyte-associated antigen 4
DNA	deoxyribonucleic acid
FGL1	Fibrinogen-like protein 1
Hb	Hemoglobin
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCT	Hematocrit
HCV	Hepatitis C virus

HIV	Human immunodeficiency virus
HNSCC	Head and neck squamous cell carcinoma
ICD	Intracellular domain
ICIs	Immune checkpoint inhibitors
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL-12	Interleukin 12
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-7	Interleukin 7
IRB	Institutional Review Board
LAG-3	Lymphocyte activation gene-3
mAbs	Monoclonal antibodies
MDSC	Myeloid-derived suppressor cell
MHC	Major Histocompatibility Complex
MHC-II	Major histocompatibility complex II
NK	Natural killer cell
NKG2D	Natural killer group 2 member D
NSCLC	Non-small cell lung cancer
PD-1	Programmed cell death protein 1
pDCs	Plasmacytoid dendritic cells
PD-L1	Programmed death-ligand 1
pMHC	Major Histocompatibility Complex-bound
PTM	Post-translational modification
qRT-PCR	Quantitative real-time PCR
RBC	Red blood cell
RCC	Renal cell carcinoma

Rh	Directed against Rhesus
RNA	Ribonucleic acid
RT	Reverse Transcriptase
RT-PCR	Real-time polymerase chain reaction
SCD	Sickle cell disease
SD	Standard deviation
TCR	T cell receptor
Th	T helper cell
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIM-3	T cell immunoglobulin and mucin-domain containing-3
TNF-α	Tumor necrosis factor alpha
TNF-β	Tumor necrosis factor beta
Tr1 cells	Type 1 regulatory T cell
Tregs	Regulatory T cells
WBC	White blood cell count

Chapter One

1. Introduction:

1.1 Background:

The mammalian immune response is composed of a complex and interdependent network of chemicals, cells, and organs that work together to defend the body against an increasingly diverse and sophisticated range of microbial invaders. While immunology is a relatively modern field of study, societies have applied fundamental immunologic concepts to fight infectious diseases for over a thousand years.

Despite significant advances in our understanding of immunological mechanisms, the processes by which the immune system maintains a dynamic equilibrium between effector function and regulatory control remain incompletely elucidated. Current evidence positions the immune system not as an isolated defense apparatus, but as an intricately interconnected network that is highly responsive to environmental stimuli and inter-systemic signals. This paradigm shift challenges long-held assumptions and expands the conceptual boundaries of immunology, raising novel questions—many of which would not have previously been recognized as immunologically relevant—and presenting new opportunities for therapeutic innovation (Punt et al., 2018).

Currently, the immune system has emerged as a central focus for therapeutic innovation, particularly in the treatment of autoimmune diseases, malignancies, and infections that exhibit resistance to conventional therapies. Strategies under investigation include therapeutic vaccination employing adjuvants to induce specific immune profiles, immunomodulation through the administration of cytokines or their antagonists, and targeted manipulation of immune cell function via modulation of coreceptor signaling pathways.

Given the complexity of immune responses, alterations to one component can influence multiple interconnected pathways. This principle is exemplified by the cytokine network hypothesis, which posits that modulating the concentration of a single cytokine can trigger a cascade of changes across the broader cytokine milieu. Elevated cytokine levels often induce the shedding of their receptors from cell surfaces, thereby attenuating downstream signaling. These soluble receptors may sequester cytokines within tissue fluids, leading to reduced activity, enhanced clearance, or, paradoxically, stabilization and prolonged half-life. The clinical application of cytokines and their inhibitors thus necessitates a nuanced understanding of these regulatory dynamics. Furthermore, growing evidence indicates that the immune system operates in concert with other physiological systems, rather than in isolation. Elucidating these cross-system interactions could significantly deepen our understanding of how social, psychological, and environmental factors modulate immune function and influence disease susceptibility and progression (Parkin & Cohen, 2001).

The initiation, regulation, and resolution of immune responses are fundamentally mediated by immune checkpoint molecules, which play a pivotal role in maintaining immune homeostasis. Tumor cells often exploit these regulatory pathways to attenuate immune cell activity and facilitate immune evasion. Therapeutic interventions targeting immune checkpoints aim to restore antitumor immunity by inhibiting the interaction between checkpoint receptors and their corresponding ligands. Immune checkpoint inhibitors, particularly those targeting cytotoxic T-lymphocyte–associated protein 4 (CTLA-4) and programmed cell death protein 1/programmed death-ligand 1 (PD-1/PD-L1), have demonstrated substantial clinical efficacy. However, a significant proportion of patients exhibit limited therapeutic response or develop resistance. In response, there has been a rapid expansion of preclinical and clinical studies investigating novel immune checkpoint

targets—such as T cell immunoglobulin and mucin-domain containing-3 (TIM-3), lymphocyte activation gene-3 (LAG-3), T cell immunoreceptor with Ig and ITIM domains (TIGIT), natural killer group 2 member D (NKG2D), and CD47—and their associated ligands (Wang et al., 2024).

Recent studies have identified an early expansion of cytotoxic $CD3^+CD8^+$ T cells as a key feature contributing to both qualitative and quantitative alterations in the T-cell repertoire that drive disease progression. T-cell exhaustion, a state of functional impairment, arises from chronic antigen stimulation—particularly tumor-associated antigens—and persistent immune activation. This exhausted phenotype is characterized by the upregulation of multiple inhibitory immune checkpoint receptors, including programmed death-1 (PD-1), T cell immunoglobulin and mucin-domain containing-3 (Tim-3), lymphocyte activation gene-3 (LAG-3), and T cell immunoreceptor with Ig and ITIM domains (TIGIT). Interestingly, elevated expression of these markers—PD-1, Tim-3, LAG-3, or TIGIT—has been increasingly associated with favorable disease outcomes in recent investigations, suggesting a complex role for immune exhaustion markers in the context of disease recurrence and immune regulation (Ninkovic et al., 2023).

Currently, evidence indicates that patients with thalassemic disorders exhibit a range of immunological abnormalities that contribute to heightened infection risk and poor clinical outcomes. These include impaired innate immune function and a variety of functional and numerical defects across the immune system. Alterations in T cell subsets are particularly notable, such as increased levels and activity of cytotoxic/suppressor T cells ($CD8^+$), decreased helper T cell ($CD4^+$) counts and activity—leading to a reduced CD4/CD8 ratio—along with diminished T cell proliferation and natural killer (NK) cell activity. Collectively, these deficits reflect a state of immune dysregulation that may underlie the increased

susceptibility to infectious complications observed in this population (Gluba-Brzózka et al., 2021).

According to current studies, patients with thalassemia major exhibit distinct alterations in lymphocyte subpopulations, with one of the most prominent features being an increased proportion of T lymphocyte subsets involved in negative regulation of immune responses. These regulatory T cells contribute to the suppression of immune activity against both foreign antigens and self-antigens and exert inhibitory effects on other immune cells, including B and T lymphocytes, as well as antigen-presenting dendritic cells. This immunosuppressive phenotype may be driven, in part, by repeated blood transfusions and chronic immune system activation, which together contribute to the dysregulated and, at times, paradoxical immune responses observed in thalassemic individuals (Gluba-Brzózka et al., 2021).

1.2 Importance of the study:

The significance of this study is to elucidate the role and expression patterns of LAG-3 in transfusion-dependent patients, in order to clarify the immunological alterations associated with chronic blood transfusions and to explore their broader implications for immune regulation.

1.3 Study problem:

To date, only a single study has investigated LAG-3 gene expression as an immune checkpoint in thalassemia patients. However, the small sample size limits the robustness of the conclusions. Therefore, a more comprehensive assessment of LAG-3 expression is

necessary, incorporating critical variables such as patient age, clinical parameters, transfusion frequency, alloantibody status, genetic background of the cohort, and other pertinent factors.

1.4 Aim of the study

The present study aims to evaluate the expression levels of lymphocyte activation gene-3 (LAG-3) in transfusion-dependent patients, including those with β-thalassemia major, compared to healthy controls. Additionally, the study seeks to assess LAG-3 expression in thalassemia patients with and without alloantibodies.

1.5 Study questions:

1. Is there a significant difference in LAG-3 gene expression levels between transfusion-dependent β-thalassemia patients and healthy controls?
2. Is there a significant correlation between LAG-3 gene expression levels and alloimmunization status in patients with β-thalassemia?
3. How do clinical parameters—including hemoglobin (Hb), white blood cell count (WBC), serum ferritin levels, and other relevant factors—affect LAG-3 gene expression levels in transfusion-dependent patients?
4. How does the age at initiation of packed red blood cell (RBC) transfusion influence LAG-3 gene expression levels in β-thalassemia patients?
5. To what extent does the frequency of packed RBC transfusions affect LAG-3 gene expression levels in β-thalassemia patients?

1.6 Study hypothesis:

- The expression level of the LAG-3 gene is elevated in transfused β-thalassemia major patients compared to healthy controls.
- The expression level of the LAG-3 gene is higher in transfused patients with alloantibodies compared to those without alloimmunization.
- Patients with elevated serum ferritin levels exhibit higher LAG-3 gene expression compared to those with normal ferritin levels.
- White blood cell count (WBC) and other hematological parameters significantly influence the expression levels of the LAG-3 gene.

1.7 Definitions:

LAG-3: Lymphocyte activation gene-3, RBC's: Red blood cells, CTLA-4: cytotoxic T-lymphocyte-associated protein 4, PD-1: Programmed cell death 1, PD-L1: Programmed cell death 1 ligand 1, TIM3: T-cell immunoglobulin and mucin domain 3, TIGIT: T Cell Immunoreceptor With Ig And ITIM Domains, NKG2D: Natural killer group 2D, NK: Natural killer cell.

Chapter Two

2- Literature Review:

This chapter provides an extensive literature review that explains the immune system, regulation of immune responses in the periphery, immune checkpoint receptors, immune checkpoint inhibitors, immune checkpoint molecules, lymphocyte activation gene 3 (LAG-3) expression, benefits, and effects on the immune system and its relationship to diseases, β-thalassemia disease, and repeated blood transfusions.

2.1 Immune system:

The immune system is a network of cells, tissues, and their soluble byproducts that identify, target, and eliminate substances that may pose a threat to a person's health. Immunity, which comes from the Latin immunities, which means "to be exempt from," is the result of the immune system's regular operation. Prior to the understanding of the causes of sickness, this idea was first proposed in the 1500s (Mak et al., 2013).

The immune system reacts in an effort to maintain the body's homeostasis. This reaction entails locating and eliminating the body's deteriorated and dying cells. In order to combat the attack of infectious organisms, such as bacteria, viruses, parasites, and fungus, the body has developed more sophisticated defense mechanisms. The immune system is always busy controlling attacks from this sector since these alien invaders are essentially everywhere on Earth and are always looking for susceptible hosts (Mak et al., 2013).

White blood cells, or leukocytes, are the part of the immune system that is in charge of cell-mediated immunity. They travel via the lymphatic and circulatory systems and are mostly found in tissues and organs. Certain leukocyte types release soluble molecules called

antibodies, which are in charge of humoral immunity. A complicated signaling system mediated by cytokines, which are mostly generated by leukocytes, is necessary for the development of antibodies and cell-mediated immune responses (Mak et al., 2013). In order to moderate self-tolerance, prevent autoimmunity, and shield organs from immunological attack, immune checkpoints are negative regulators of the immune system (Xu, 2020). Immune checkpoints come in two different types: those that participate in co-stimulatory interactions with T cells (like 4-1BB and its ligands) and those which function as suppressive factors (like LAG-3) (Xu, 2020).

This study will discuss the suppressive factor or inhibitory molecule, particularly lymphocyte activation gene 3 (LAG-3), and how it interacts with recurrent blood transfusions especially in patients with B-thalassemia major in Northern Palestine.

2.2 Regulation of Immune Responses in the Periphery:

In a healthy person, the innate and adaptive immune responses that function in the body's peripheral tissues are very active and strictly regulated. Immune modulation and tolerance are the two mechanisms by which this control is implemented. Immune regulatory systems control the activities of effector cells, whereas tolerance mechanisms inhibit lymphocyte activation. Peripheral tolerance is demonstrated at the cellular level when a mature peripheral lymphocyte does not get activated upon interaction with its associated antigen. Either the lymphocyte is functionally inactivated or apoptosis is initiated. Maintaining tolerance to self-tissues is a key function of such lymphocyte inhibition. This peripheral "self-tolerance" is essential because it inhibits autoreactive cells from activating and staying in a variety of lymphocytes that have been otherwise formed to detect non-self-antigens after escaping

central tolerance mechanisms. Additionally, there is peripheral tolerance to harmless non-self-antigens, which aids in avoiding inflammatory reactions that may otherwise cause needless tissue damage (Mak et al., 2013).

To avoid or reduce collateral damage to nearby healthy tissues, a lymphocyte's response must eventually be suppressed, regardless of whether it detects a self or non-self-antigen. The body gradually reaches a stable state because of the cellular and biochemical regulating systems. These regulatory mechanisms must take action to prevent further harm to the healthy self-tissues that are the target of the reaction when an autoreactive lymphocyte is activated in spite of efforts to tolerate it. Furthermore, good health depends on the management of any reactions to harmless non-self-entities, such as the commensal bacteria in the digestive system, the proteins in our food, or the air we breathe. If tolerance and regulatory mechanisms are successfully implemented, the host will be able to concentrate the immune response's strength on dangerous non-self-antigens for the proper amount of duration; if they are not, unchecked tissue damage and the possibility of developing autoimmune disease will occur (Mak et al., 2013).

Cellular states depend critically on transcription control, and a wide spectrum of diseases have been linked to transcriptional dysregulation. Therefore, it is important to identify the genetic elements that contribute to the explanation of gene transcription levels in order to produce precise models that can further link changes in gene expression to particular illnesses. This is especially helpful for tissues that are difficult to reach in order to evaluate gene expression directly (Lu et al., 2022).

The expression of the immune checkpoint molecule (like LAG-3) and its impact on immune system regulation will be covered in this study.

2.3 Immune check point receptors:

The immune system incorporates pathways known as immunological checkpoints. However, T cell activation in an adaptive immune response requires two stimuli. Antigen-presenting cells use Major Histocompatibility Complex (MHC) peptides to present distinct antigens and provide the T cell response's selectivity. T cell activation is then either stimulated or inhibited by immune checkpoint receptors (Dong et al., 2021). Major Histocompatibility Complex (pMHC)-bound antigenic peptides are recognized by the T cell receptor (TCR), and CD3 molecules provide activation signals to the T cell. Because of the TCR–CD3 complex's remarkable sensitivity to pMHC, the host may react to even the smallest variations between self and foreign antigens. T cells, when activated by pMHC, are extremely effective in directing inflammatory reactions and eliminating pathogen-infected host cells directly. Although fighting invasive germs requires these actions, there is a chance that host tissue that has not been affected by the infection might also undergo damage. Furthermore, it is essential to have strategies for blocking improperly initiated T-cell responses (Mariuzza et al., 2024). In order to reduce tissue damage, they are essential for regulating the effectiveness of immune responses against pathogenic infection and preserving peripheral tissue's capacity for self-tolerance. They are separated into two categories: co-stimulatory checkpoint molecules, such as CD27, CD28, CD137, ICOS, 4-1BB, and OX-40, stimulate T cells, and co-inhibitory checkpoints, such as PD-1, LAG-3, and CTLA-4, elicit inhibitory signals that prevent T cell activation in order to prevent autoimmune attacks and excessive inflammation (Figure 2.1) (Taefehshokr et al., 2020).

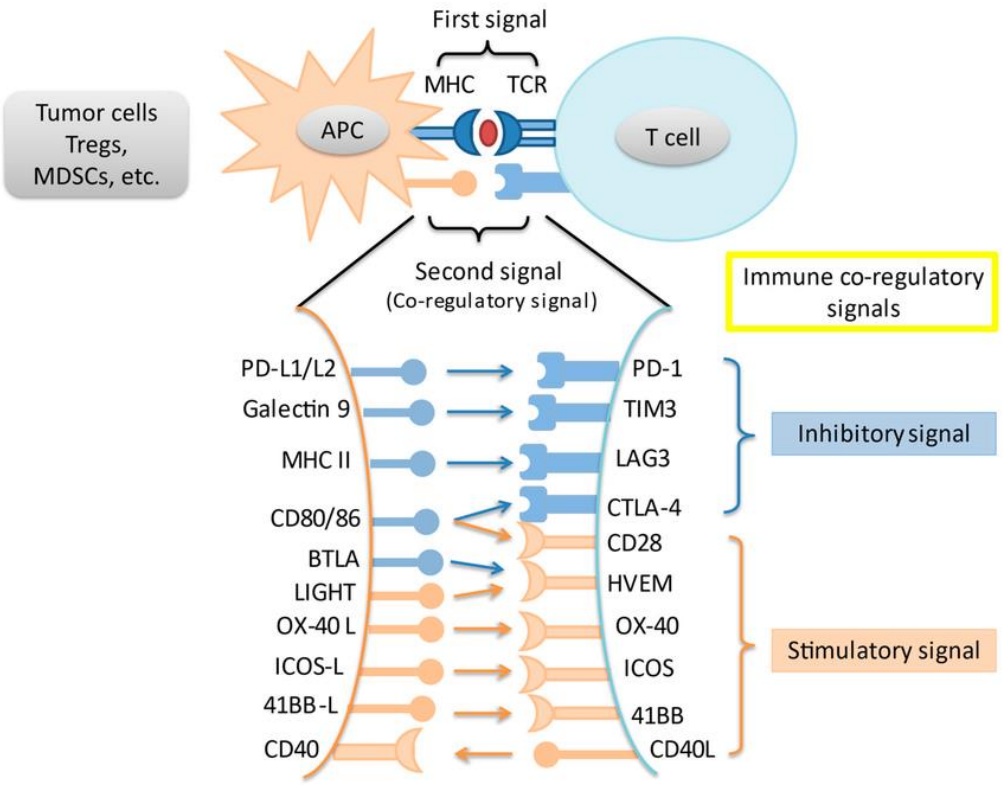

Figure 2.1: Interaction of main immune co-stimulatory/co-inhibitory molecules and their corresponding receptors. Cancer cells and other cells in the tumor microenvironment express a variety of inhibitory and stimulatory ligands that bind to their cognate receptors on immune cells, thereby leading to immune-modulation. These ligand-receptor pairs are known as immune checkpoints. Tregs = regulatory T cells; MDSC = myeloid-derived suppressor cell; APC = antigen presenting cell; TCR = T-cell receptor (Dong et al., 2021).

Immune checkpoint receptors play a major role in immune evasion strategies employed by cancer cells, which allow them to elude immune detection and assault. They are also essential in controlling the immune response and maintaining self-tolerance. The two most well-known checkpoint receptors are cytotoxic T lymphocyte-associated antigen 4 (CTLAG4) and programmed cell death protein 1 (PD-1). Targeted antibody antagonists targeting these receptors have significantly transformed cancer treatment. Similar to PD-1, LAG-3 (lymphocyte activation gene-3) is a checkpoint receptor that, upon T cell activation, is up-regulated. LAG-3 overexpression and knockout studies demonstrate that this receptor

negatively regulates the proliferation, activation, effector function, and homeostasis of both CD8+ and CD4+ T cells. As an "exhaustion" marker for CD8+ T lymphocytes, LAG-3 is typically co-expressed with PD-1 in malignancies and persistent viral infections as a response to repeated antigen stimulation. Anti-LAG-3 antibodies have shown promise in cancer therapy clinical trials; nevertheless, the exact mechanism by which LAG-3 works is yet unknown, which is the subject of this research (Silberstein et al., 2024).

2.4 Immune check point inhibitors:

Immune checkpoint inhibitors (ICIs), which use the body's immune system to identify and eliminate tumor cells or infections, have completely changed the way that most diseases are treated. They function by disrupting immunological checkpoints that cancers utilize to avoid immune detection, such as lymphocyte activation gene 3 (LAG-3), programmed cell death ligand 1 (PD-L1), programmed cell death-1 (PD-1), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). ICIs improve the ability of immune cells to identify and combat cancer cells by blocking these checkpoints. For certain patients who had few therapy alternatives in the past, this strategy has shown long-lasting results in the treatment of diseases such as melanoma, non-small cell lung cancer, and renal cell carcinoma. These ICIs are monoclonal antibodies with high specificity and therapeutic efficacy. They can, however, also result in significant immunological resistance in hepatocellular carcinoma, hormone receptor positive breast cancer, microsatellite-stable colorectal cancer, and other cancers. This significantly reduces the effectiveness of medications and keeps many patients from benefiting from them. Combining ICIs with targeted treatments or chemotherapy has shown promise in recent years as a means of combating immune resistance (Guo et al., 2024).

Multiple immunological checkpoints are expressed by tumor-specific lymphocytes, and checkpoint inhibition can improve the efficacy of therapy. However, compared to inhibiting each checkpoint alone, synergistic inhibition of PD-1 and CTLA-4 produced significantly more anti-tumor immunity. Recent research has demonstrated that in models of both tumor tolerance and self-tolerance, LAG-3 is comparatively overexpressed on non-functional CD8 T cells. With indications of a cell-intrinsic impact on CD8 T cells, inhibiting LAG-3 alone led to a notable, if partial, recovery of function. When it comes to controlling T cell activity, LAG-3 and PD-1 may work in concert. Anti-LAG-3 monotherapy slowed down the growth of tumors and, unexpectedly, when attached, suppressed most cancers without causing any autoimmune adverse effects (Figure 2.2) (Nirschl & Drake, 2013).

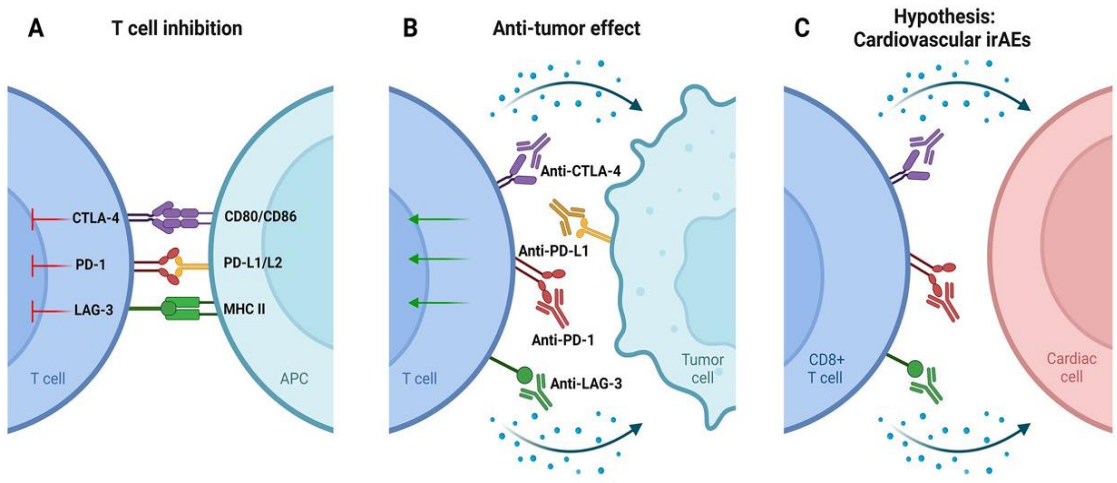

Figure 2.2: ICI mechanisms of action. A CTLA-4, PD-1, and LAG-3 binding leads to T cell inhibition. B Immune checkpoint inhibitors (ICI) block T cell inhibition, allowing for T cell activation in the tumor microenvironment (incl. cytokine release) and a potent anti-tumor response. C It is hypothesized that cardiovascular immune-related adverse events (irAEs) could occur through direct interaction between T cells and cardiac cells. CTLA-4, Cytotoxic T-lymphocyte-associated protein 4; PD-1, programmed cell death protein-1; PD-L1/L2, programmed cell death protein-1-ligand 1/2; LAG-3, lymphocyte activation gene-3; MHC-II, major histocompatibility complex II (Yousif et al., 2023).

2.5 Immune checkpoint molecules:

The immune system makes certain crucial choices, one of which is to control the extent and severity of a developing response. When the immune system is functioning effectively, it can store memory responses for future encounters and sterilize immunity against a wide range of diseases. Therefore, there are several systems in place to avoid autoimmune responses to self-proteins, and an immune response is strictly managed. These systems are crucial, as demonstrated by the devastation and permanent consequences of numerous autoimmune disorders. It has been discovered that a wide class of extracellular "checkpoint molecules" can alter T cell reactions to self-proteins throughout the last 20 years (Nirschl & Drake, 2013). The ability of chemicals that inhibit the interaction of immunological checkpoint molecules to enhance the effectiveness of tumor-directed immune responses. the potential for the opposite intervention, i.e., immunological checkpoint interaction stimulation to suppress immune responses to the cell surface-expressed MHC antigens. However, the ligation of several immune checkpoint molecules on the surface of immune cells causes co-stimulation, which is crucial in deciding whether T lymphocytes become activated or anergic upon antigen presentation (Righi et al., 2024).

Many immune checkpoint molecules have been discovered in recent decades, such as natural killer group 2, member D (NKG2D), cluster of differentiation 47 (CD47), T cell immunoglobulin domain and mucin domain 3 (TIM3), lymphocyte activation gene 3 (LAG3), T cell immunoreceptor with Ig and ITIM domains (TIGIT), programmed cell death protein-1 (PD-1), and cytotoxic T-lymphocyte-associated protein-4 (CTLA-4). These

molecules work together to create complex regulatory networks that precisely control the nature and extent of immune reactions (Wang et al., 2024).

To increase the effectiveness of immune checkpoint inhibitors (ICIs) in patients who are resistant to existing treatments, it is essential to understand the protein structure and regulatory mechanisms of these distinctive immune checkpoints. The process of adding or removing proteins, lipids, sugars, or functional groups from the side chains of amino acids is known as post-translational modification (PTM). Numerous enzymes, including as kinases, phosphatases, ligases, and transferases, catalyze this activity. As an alternative, it may occur chemically when hydrolases break peptide links, eliminating certain sequences or controlling subunits. Tumor pathogenesis and immunological function are two crucial cell physiological processes that are influenced by this crucial regulatory system. By modifying their activity, preserving protein structures, affecting localization, and regulating interactions with other molecules, PTM controls immunological checkpoint molecules (Wang et al., 2024).

Researchers have carefully examined various alterations impacting the expression of immunological checkpoint proteins and their ligands, despite the difficulties involved in investigating PTMs. Notably, important PTMs have been found to include ectodomain shedding, phosphorylation, ubiquitination, SUMOylation, neddylation, palmitoylation, and glycosylation. Determining the characteristics of these post-translational alterations reveals new targets and treatment pathways for disease immunotherapy, as well as important insights into novel immune checkpoint molecules (Wang et al., 2024).

2.6 Lymphocyte activation gene 3 (LAG3) expression, benefits, and effects on the immune system and its relationship to diseases:

Lymphocyte activation gene 3 (LAG-3) or CD 223 (NC_000012.12) is a surface molecule that is structurally similar to the CD4 co-receptor, yet shares less than 20% homology at the amino acid level. It is a member of the immunoglobulin superfamily (Xu, 2020) and a type I transmembrane protein that possesses four extracellular domains (D1 to D4) that resemble immunoglobulin (Ig) (Figure 2.3) (Silberstein et al., 2024). On chromosome 12, the human LAG-3 gene is situated next to the CD4 coding region. Furthermore, there is a strong structural homology between the D1 to D4 domains of LAG-3 receptor and CD4 (Tian et al., 2024). Due to the significant similarity between the LAG-3 and CD4, a glycoprotein that functions as a co-receptor for the T cell receptor, major histocompatibility class II (MHCII) was initially identified as a ligand for LAG-3 (Silberstein et al., 2024). LAG-3's cytoplasmic tail contains three distinct domains: the first domain functions as a putative serine phosphorylation site; the second domain is the KIEELE motif, which is crucial for regulating CD4+ T cell function; and the third domain is the glutamic acid–proline (EP) repeat, which binds to LAG-3-associated protein (LAP) (Xu, 2020). More recently, fibrinogen-like protein 1 (FGL1) was shown to be a newly emergent LAG-3 checkpoint ligand. It is believed that the primary binding domain of LAG-3 that interacts with MHCII and FGL1 is the D1 domain. It is believed that MHCII binds to a lengthy 30 amino acid loop (loop 1) on D1; MHCII binding is reduced by ablation of this loop. Reduction in MHCII binding is also observed with deletion of the D2 domain; however, the manner in which D2 interacts with MHCII remains unclear. On the other hand, Loop 2 on D1 is supposed to be bound by FGL1, and it is situated on the face of the D1 domain that faces the MHCII binding site. It has been

demonstrated that FGL1 similarly to MHCII needs the LAG-3 D2 domain for maximum binding to LAG-3 (Silberstein et al., 2024).

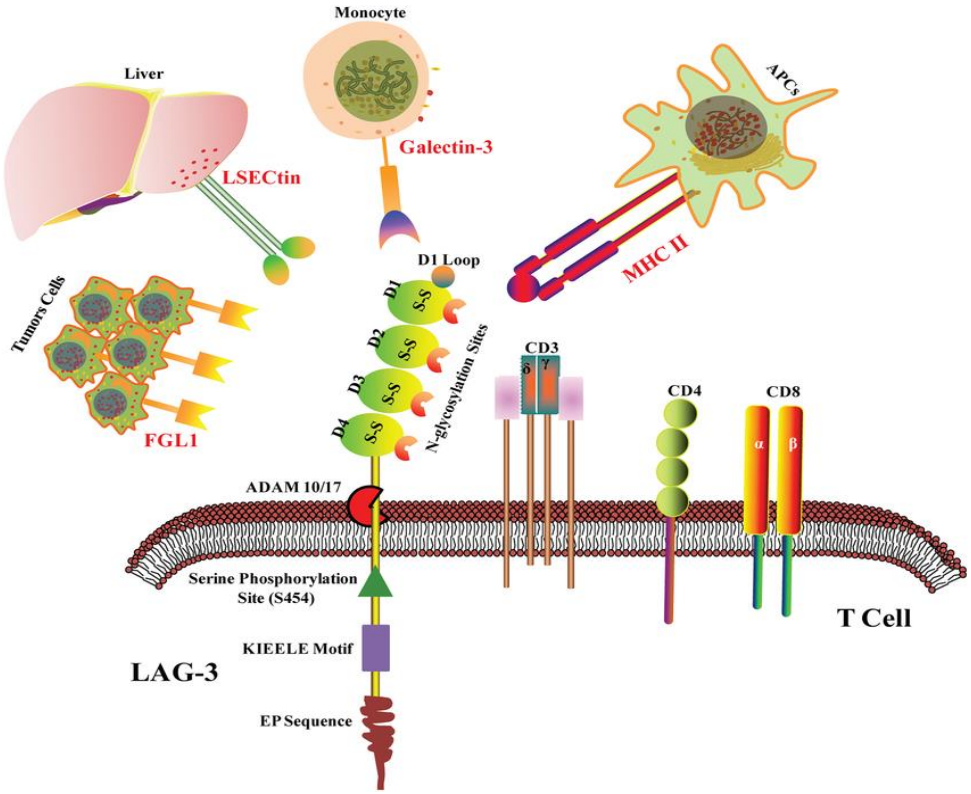

Figure 2.3: LAG-3 structure and ligands. LAG-3 consists of extracellular region, transmembrane region and cytoplasmic region. The extracellular domain is composed of four IgSF domains, namely D1, D2, D3 and D4. The D1 domain contains a loop domain rich in proline and an in-chain disulfide bond. In the transmembrane- cytoplasmic part, LAG-3 breaks away from the cell membrane under the action of metalloproteinases ADAM10/17. The cytoplasmic region of LAG-3 consists of three parts: the serine phosphorylation site S454, the highly conserved “KIEELE” motif and the glutamate-proline dipeptide repeat motif (EP sequence). MHC II, Galectin-3, LSECtin and FGL1 are the confirmed ligands of LAG-3 in tumor microenvironment (Huo et al., 2022).

Through its intracellular domain (ICD), LAG-3 adversely regulates T lymphocytes upon ligand binding. In contrast to the cytoplasmic tails of PD-1 and other checkpoint receptors that control T cell function via an immunoreceptor tyrosine-based inhibition motif, LAG-3 is believed to transmit its inhibitory signal via a FxxL motif in conjunction with a tandem negatively charged glutamic acid-proline repeat. According to recent data, rather than preventing an extracellular interaction between CD4 and MHCII, the negatively charged part of the LAG-3 cytoplasmic tail is essential for preventing the association of activating kinases with the cytoplasmic tails of the CD4 and CD8 co-receptors (Silberstein et al., 2024).

Since certain monoclonal antibodies (mAbs) that do not bind to the D1 domain can enhance T cell activity, it is unclear how important the MHCII/LAG-3 association is to LAG-3's immunosuppressive effect. For instance, in animal tumor models and cell culture, C9B7W, a monoclonal antibody against the murine LAG-3 D2 domain, increases T cell proliferation and effector activities. However, this anti-body's capacity to inhibit LAG-3 binding to MHCII varies depending on the model system working, ranging from total incapacity to partial or full capacity. C9B7W has an impact on T cells that is comparable, if not the same, as that caused by the LAG-3 genetic deficit. F7, an anti-LAG-3 mAb that does not inhibit MHCII binding, was shown in different research to be able to rescue T cells from LAG-3-mediated inhibition and promote T cell activation (Silberstein et al., 2024). Additionally, it has been suggested that. LAG-3 directly inhibits T cell receptor (TCR) signaling in the immune response by binding to MHC-II on antigen-presenting cells (APCs) with a far higher affinity than CD4 does. Type 1 regulatory T cell (Tr1 cells), B cells, Plasmacytoid dendritic cells (pDCs), activated CD4+ and CD8+ effector T cells, CD4+ Foxp3+ regulatory T cells (Treg), and a fraction of Natural killer cells (NK cells) all express it. By blocking calcium

ion fluxes, cross-linking of LAG-3 and CD3 may hinder T cell proliferation and cytokine release (Xu, 2020).

LAG-3 binds to two more proteins, LSECtin and galectin-3, in addition to MHC-II. Galectin-3 is a soluble lectin that, in contrast to LSECt, is expressed in a variety of cell types, including tumor cells. It may interact with LAG-3 to increase LAG-3's immune-regulatory effects on tumor-infiltrating CD8+ T cells within the tumor microenvironment (TME). LSECtin is a member of the DC-sign family and is primarily expressed in the liver and on tumor cells. The LAG-3 intrinsic signaling transition is mediated by the cytoplasmic KIEELE motif, which stops T cells from entering the S-phase and, as a result, suppresses T cell proliferation (Xu, 2020).

The LAG-3/MHC-II association inhibits the growth of CD4+ T cells and inhibits the cytokine response. LAG-3 significantly upregulates in inflammatory circumstances due of its high affinity for MHC-II. In contrast to IL-4, IL-6, TNF-α, and TNF-β, IL-2, IL-7, and IL-12 may increase the expression level of LAG-3 on human-activated CD4+ T cells. Specifically, IL-12 is known to be the strongest inducer of IFN-γ; once stimulated, it will significantly increase the expression of LAG-3 and increase the quantity and frequency of T cells and NK cells that are positive for LAG-3. Furthermore, zinc-dependent a disintegrin and metalloproteinase (ADAM) also promotes LAG-3 production via TCR signaling-dependent pathways. Furthermore, LAG-3 and PD-1 are frequently co-expressed and increased on T cell ILs in the tumor microenvironment, which subsequently causes immunological fatigue and tumor progression (Xu, 2020).

Because LAG-3 blockade has several modes of action, mostly by preventing cell cycle progression, it not only enhances anticancer immune responses but also strengthens other types of immunotherapy. LAG-3 expression is extremely low in inactivated CD8+ T cells,

but it noticeably increases in response to antigenic stimuli. T cell activation causes LAG-3 expression on CD8+ T cells, and similar to CD4+ T cells, CD8 T-cell or cytotoxic T-lymphocyte (CTL) proliferation and effector activity are enhanced when LAG-3 is blocked. Crucially, fatigued CD8+ T cells in persistent viral infections and cancer, including ovarian cancer, Hepatocellular carcinoma (HCC), Renal cell carcinoma (RCC), and other solid tumors, also exhibit elevated expression of LAG-3. Galectin-3 and LSECtin are two more LAG-3 ligands that may be present in the tumor microenvironment and serve as a supplement to regulate CD8+ T cells. According to recent reports, these ligands may be able to prevent the Antibody-dependent cellular cytotoxicity (ADCC) of CD8+ T cells via LAG-3 for tumor immunity privilege. Moreover, it has been discovered that LAG-3 may simultaneously express many co-inhibitory immunological checkpoints, including PD-1, by CD8+ T cells. This might be interpreted as a biomarker of CD8+ TIL malfunction. In addition, LAG3 has been shown to be strongly expressed in Foxp3+ Tregs and regulatory IL-10 generating Tr1 cells. Additionally, LAG-3 has been shown to recognize IL-10 producing Tr1 cells in both people and mice in conjunction with CD49b. While forced expression of LAG-3 gave effector T cells the ability to inhibit, deletion of LAG-3 in Tregs decreased their suppressive activity. Moreover, it was demonstrated that LAG-3 cross-linking of MHC-II on DCs suppresses the priming of effector T cell responses by preventing DCs from differentiating. A wide range of human malignancies, including melanoma, Non-small cell lung cancer (NSCLC), Colorectal cancer (CRC), breast cancer, HCC, Head and neck squamous cell carcinoma (HNSCC), and others, have been shown to have abnormal LAG-3 expression, which is strongly linked to aggressive tumor growth and clinicopathological features (Xu, 2020).

The important factor in autoimmune disorders is LAG-3. The development of autoimmune disorders may be connected to LAG-3 deficiency. By blocking T helper cell (Th) activation and effector function, membrane surface LAG-3 lowers T cell autoreactivity and delays the onset of type I diabetes. Additionally, lag-3− /− displays significantly accelerated diabetes development, and enhanced CD4+ and CD8+ T cell infiltration and proliferation indicate that LAG-3 specifically suppresses antigenic reactive T cell infiltration and expansion. Patients with rheumatoid arthritis had lower levels of LAG3+Tregs, particularly those with high clinical disease activity ratings. Moreover, regulatory B cells known as LAG3+ B regulatory cells (Breg cells) impede the growth of T cells. In induced arthritis, therapy with LAG-3+Breg cells significantly reduces the extent of joint lesions and systemic inflammation. LAG-3 has a role in autoimmune diseases by suppressing autoimmunity; downregulating LAG-3 can change immunological tolerance and enhance immunity (Tian et al., 2024), prevents autoimmunity by working in concert with PD-1 and/or other immune regulators (Okazaki et al., 2011).

2.7 β-thalassemia disease and repeated blood transfusion:

The β-thalassaemias occur widely in a broad belt, ranging from the Mediterranean and parts of North and West Africa through the Middle East and Indian subcontinent to South east Asia. This disease is most prevalent in Southeast Asia, where it affects populations in a line that begins in southern China and extends through Thailand, Indonesia, the Malay Peninsula, and certain Pacific island nations. Gene frequencies vary from 2 to 30% in this region and in several Mediterranean island and mainland nations. In Palestine, it was estimated that the

prevalence rate of thalassemia carriers in Palestine was around 4% with a total number of symptomatic thalassemia patients of 847 (Aldwaik et al., 2021).

The β-thalassaemias are classified as autosomal recessive diseases because a clinically observable phenotype requires the inheritance of two defective β-globin genes, whereas carriers of one abnormal β-gene are asymptomatic. The globin gene promoter regions are affected by deletions and point mutations, which are alterations that disrupt transcription of globin. Numerous mutations obstruct the main mRNA transcript's processing. A frameshift mutation or single-base alteration to a nonsense codon renders the gene totally inactive in around half of the β-thalassaemia variants (Hoffbrand et al., 2016). In β-thalassaemia, the synthesis of β-chain is either missing or decreased due to molecular abnormalities. There are too many α-chains as a result of the imbalance in globin chain synthesis. Since the unbound α-globin chains are so fragile, they precipitate in red cell precursors and create intracellular inclusions that impede the formation of red blood cells. All β-thalassaemias are characterized by varying degrees of intramedullary loss of erythroid precursors, also known as ineffective erythropoiesis. The α-chain inclusions seen in mature red blood cells that enter the circulation obstruct their ability to move through the microcirculation, especially in the spleen. However, β-thalassaemia causes more than only mechanical damage to red cell progenitors and their offspring. Significant anomalies in electrolyte balance and membrane deformability are among the many harmful consequences that the breakdown products of excess α-chains, especially haem and iron, have on the proteins and lipids that make compose red cell membranes. An extremely rigid red cell with a shorter period of survival is the ultimate result. Therefore, inefficient erythropoiesis and hemolysis together lead to the anemia of β-thalassaemia patients. It increases the synthesis of erythropoietin, which causes the bone marrow to swell and can result in severe skull and long bone abnormalities. The

spleen hypertrophies as a result of the aberrant red cells that are continuously bombarding it. Along with bone marrow enlargement, the resultant splenomegaly leads to a significant rise in plasma volume, which further exacerbates the anemia (Hoffbrand et al., 2016).

One of the most prevalent hematologic diseases is β-thalassemia major. According to epidemiologic research, between 1 to 20% of people are carriers of β-thalassemia, and 23,000 children worldwide are born with β-thalassemia major each year. Patients with β-thalassemia major have been shown to exhibit a variety of clinical characteristics, including growth retardation, hepatosplenomegaly, pallor, jaundice, skeletal abnormalities, and impairments in organ function, particularly in the liver, heart, and endocrine glands, as a result of iron overload (Shokrgozar et al., 2021).

Frequent and early blood transfusion decreases the complications of severe anemia and extends survival age (Vichinsky et al., 2014). Therefore, long life blood transfusion is considered as the main supportive management to sustain growth and development during childhood and to maintain acceptable quality of life. However, repeated blood transfusions stimulate the patient's immune system to produce antibodies against foreign erythrocyte antigens (alloimmunization) (Pandey et al., 2014) (Singer et al., 2000) (Stiegler et al., 2001) (Chou et al., 2013). Red blood cells alloimmunization is considered as the most important adverse effect because it leads to an increased need for transfusion due to hemolysis and restriction in obtaining further blood transfusion, both of which can significantly complicate transfusion management for thalassemic patients (Khan & Delaney, 2018) (Ogedegbe, 2002) (Rostamian et al., 2022).

Risk factors of alloimmunization include female sex, history of pregnancy, duration of transfusion therapy, genetic and environmental factors. In addition, patients with a history of antibodies after a few blood transfusions present a greater risk for additional

alloantibodies and hemolytic transfusion reactions. The main alloantibodies reported in thalassemic patients are directed against Rhesus (Rh), Kell, Kidd, and Duffy system (Gehrie & Tormey, 2014) (da Cunha Gomes et al., 2019) (Strobel, 2008).

Recently, co-inhibitory or immune checkpoint receptors play a role in the maintenance of immune homeostasis: therefore, their expression on effector and regulatory T (Treg) cells guarantees the proper function to control effector T cells. Accordingly, their function in regulating pro-inflammatory T cell responses and the maintenance of self-tolerance has been most widely studied including their role as co-inhibitory receptors in cancer and chronic viral infection where these receptors are highly expressed and are being targeted clinically to improve anti-tumor and anti-viral T cell responses (Anderson et al., 2016). A recent report with small number of thalassemia patients showed that LAG-3 as an immune check point was significantly increased in thalassemia patients compared to the control group (Shokrgozar et al., 2021).

The main objective of this research was to investigate the prevalence of alloantibodies and their types in thalassemia patients and to evaluate the expression of lymphocyte activation gene 3 (LAG-3) as an immune checkpoint molecule in β-thalassemia patients with and without alloantibody as well as in comparison to healthy individuals.

Chapter Three

3- Materials and methods:

This chapter presents a comprehensive description of the research methodology, encompassing the study design, study setting, ethical considerations, sampling methods and sample size determination, inclusion and exclusion criteria, data collection procedures, blood sample collection and RNA extraction, RNA quantification, complementary DNA (cDNA) synthesis, real-time polymerase chain reaction (RT-PCR), gene expression analysis, and statistical methods employed.

3.1 Study design:

This research adopts a quantitative, prospective, cross-sectional, multi-center case-control study design.

3.2 Study Participants and setting:

This multi-center study was conducted across three major tertiary care hospitals in northern Palestine: Jenin Governmental Hospital (Jenin), Thabet Thabet Governmental Hospital (Tulkarm), and the National Government Hospital (Nablus). The study population comprised patients diagnosed with β-thalassemia major and sickle cell disease who were receiving regular packed red blood cell transfusions. Eligible patients were enrolled between August 2023 and December 2023.

A total of 100 transfusion-dependent patients were recruited based on predefined inclusion and exclusion criteria. Additionally, 100 healthy individuals, matched for age and sex, were enrolled as a control group to enable comparative analysis of LAG-3 gene expression levels. Depending on complete blood count (CBC) and serum ferritin level tests the control participants had no history of hemoglobinopathy, prior blood transfusions, or immunological disorders.

3.3 Ethical Consideration:

This study was approved by the Institutional Review Board (IRB) of Arab American University (AAUP) on June 23, 2023, under archive number 2023/B/72/N (Appendix 1). In

addition, the Palestinian Ministry of Health provided official authorization through a letter of facilitation issued on August 15, 2023, to support the collection of blood samples and access to relevant patient laboratory data stored within the Ministry's records (Approval Number: 2023/1616/162; Appendix 2). Written informed consent was obtained from all participants. For pediatric participants, consent was obtained from legal guardians (Appendix 3 – 4). The study also employed a structured questionnaire to collect relevant medical history and demographic data (Appendices 5–8).

The patient samples were analyzed at the Genetics Department at the Central Specialized Laboratories in the Jenin Governorate, operating under the General Directorate of Paramedical Services of the Palestinian Ministry of Health. This laboratory analysis was officially authorized by the Assistant Deputy of Allied Health Professions and Blood Banks on July 30, 2024, under authorization number 2024-855 (Appendix 9).

3.4 Sampling and Sample Size Determination:

The required sample size for this study was calculated using OpenEpi software, based on the following parameters: a two-sided p-value of <0.05, 95% confidence interval, 80% study power, and an estimated 17.6% prevalence of high LAG-3 gene expression among controls. An odds ratio (OR) of 9.75 was assumed, with an equal allocation ratio of 1:1 for cases and controls. The sample size was computed using the equations of Kelsey, Fleiss, and Fleiss with continuity correction (Hong et al., 2022) (Sullivan & Soe, 2007).

A total of 100 transfusion-dependent patients were enrolled, including 80 with β-thalassemia major and 20 with sickle cell disease. Additionally, 100 healthy individuals matched by age and sex were recruited as the control group. Blood samples were collected by trained staff nurses working in thalassemia care units, under the direct supervision of the research team.

3.5 Study Population: Inclusion and Exclusion Criteria:

This study included patients with confirmed diagnoses of β-thalassemia major or sickle cell disease residing in northern Palestine. Participants were identified and recruited through the Palestinian Ministry of Health, based on their documented medical histories. All patients were matched by age, sex, age at initiation of blood transfusion, and splenectomy status.

Each received regular packed red blood cell transfusions every 1 to 4 weeks and was maintained on appropriate iron chelation therapy. A total of 100 patients meeting these inclusion criteria were enrolled in the study.

Exclusion criteria included the presence of human immunodeficiency virus (HIV), hepatitis B virus (HBV), or hepatitis C virus (HCV) infections, as well as the use of immunosuppressive medications, hydroxyurea, or deferiprone (L1). The control group consisted of 100 healthy individuals with no history of anemia, blood transfusion, or chronic medical conditions. Controls were not biologically related to any of the patients and were matched for age and sex.

3.6 Data Collection and Consent:

Clinical and laboratory data were collected from healthcare providers between August and December 2023. The collected data included blood group, type of anemia, date of first diagnosis, date of first blood transfusion, and results for HCV antibodies, HBV surface antigen, HIV, hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), white blood cell count (WBC), red blood cell count (RBC), GPT, GOT, ALP, albumin, ferritin levels, and the indirect antiglobulin test.

3.7 Blood Sample Collection and RNA Extraction:

Approximately 3 mL of peripheral blood was collected in EDTA tubes from each participant. Genomic RNA was extracted from the buffy coat using the QIAamp Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer's protocol. Briefly, in a sterile 1.5 mL microcentrifuge tube, 200 µL of AL lysis buffer was mixed with 2 µL of Carrier RNA. Then, 200 µL of the buffy coat sample was added to the mixture, which was incubated at 56°C for 15 minutes. Following incubation, 250 µL of absolute ethanol was added to the tube and mixed thoroughly by vortex. The lysate was then incubated at room temperature (15–25°C) for 5 minutes and subsequently transferred to a QIAamp MinElute spin column. The column was centrifuged at 8000 rpm for 1 minute. Washing was performed sequentially with buffers AW1 and AW2, followed by an additional wash with 500 µL of 96–100% ethanol, centrifuged at 8000 rpm for 1 minute.

Finally, RNA was eluted using 50 µL of Buffer AVE into a sterile microcentrifuge tube and stored at –20°C until further use.

3.8 RNA Quantification:

RNA concentration and purity were assessed using a NanoDrop spectrophotometer (IMPLEN, Germany). Briefly, one microliter (1 µL) of Buffer AVE was used as a blank, followed by 1 µL of the extracted RNA sample. The OD260/OD280 ratio was calculated to evaluate RNA purity, with acceptable values ranging between 1.8 and 2.2, indicating minimal protein contamination and suitability for downstream molecular analysis.

3.9 cDNA synthesis and Real-time PCR:

First, total RNA was reverse-transcribed into complementary DNA (cDNA). Quantitative real-time PCR (qRT-PCR) was then conducted using the SYBR Green detection method. LAG-3 gene expression levels were normalized to the β-actin housekeeping gene, and relative expression was determined using the $2^{-\Delta\Delta CT}$ method.

3.9.1 Real-Time PCR Amplification:

Real-time PCR amplification was carried out in a total reaction volume of 20 µL, consisting of the following components: 10 µL of iTaq™ Universal SYBR® Green Supermix (Bio-Rad, USA), 0.8 µL each of forward and reverse primers specific for either the LAG-3 or β-actin genes (primer sequences and PCR product sizes are listed in Table 3.1), 0.2 µL of QuantiTect Reverse Transcription enzyme (QIAGEN, Hilden, Germany) for cDNA synthesis, 5 µL of the cDNA template, and 3.2 µL of nuclease-free water. Detailed reaction setup and thermocycling conditions are provided in Tables 3.2 and 3.3.

Table 3.1: The primer sequences and PCR products of LAG-3 and the β-actin genes are as below:

Gene	**Primer sequences (5′–>3′)**	**PCR product length (bp)**
LAG-3 forward primer:	*5′-CTTCTTGGAGCAGCAGTG-3′*	*133*
LAG-3 reverse primer:	*5′-AAAGGAGCAGAGAAAGGAC-3′*	
β-Actin forward primer:	*5′-ATCGTGCGTGACATTAAGGAG-3′*	*177*
β-Actin reverse primer:	*5′-GAAGGAAGGCTGGAAGAGTG-3′*	

Table 3.2: Master mix preparation for LAG-3 gene is as below:

Component	**Volume per 20 µL reaction for 1 rxn**	**Final concentration**
iTaq Universal SYBR Green Supermix (2x)	10 µL	1 x
Reverse Transcriptase (RT)	0.2 µL	
Forward Primer LAG-3	0.8 µL	400 nM
Reverse Primer LAG-3	0.8 µL	400 nM
Nuclease-free water	3.2 µL	---
Nucleic acid template	5 µL	
Total reaction mix volume	20 µL	

Table 3.3: Master mix preparation for β-Actin gene are as below:

Component	**Volume per 20 µL reaction for 1 rxn**	**Final concentration**
iTaq Universal SYBR Green Supermix (2x)	10 µL	1 x
Reverse Transcriptase (RT)	0.2 µL	
Forward Primer β-Actin	0.8 µL	400 nM
Reverse Primer β-Actin	0.8 µL	400 nM
Nuclease-free water	3.2 µL	---
Nucleic acid template	5 µL	
Total reaction mix volume	20 µL	

3.9.2 PCR Protocol:

Real-time PCR was performed using the CFX Opus 96 Real-Time PCR System (Bio-Rad, USA) with SYBR Green chemistry. The protocol included the following steps: an initial reverse transcription step at 45 °C for 15 minutes, followed by polymerase activation and initial cDNA denaturation at 95 °C for 25 seconds. This was followed by 40 amplification cycles consisting of denaturation at 95 °C for 5 seconds and annealing/extension at 60 °C for 30 seconds, with fluorescence data acquisition at the end of each cycle. The run concluded with a melt curve analysis from 65 °C to 95 °C, increasing by 0.5 °C every 5 seconds, with fluorescence recorded at each increment to confirm product specificity. Full thermocycling parameters are detailed in Table 3.4.

Table 3.4: Thermal cycling protocol for amplification of LAG-3 and B-Actin genes separately are as below:

Step	**Reverse Transcriptase (RT)**	**Polymerase activation & cDNA denaturation**	**Amplification**			**Melt Curve Analysis**		
			cDNA denaturation	**Annealing/Extension + Plate Read**				
Temperature	45 ℃	95 ℃	95 ℃	60 ℃	**GOTO 2**	65 ℃	95 ℃ 0.5 ℃	
Time	15 Min	25 sec	5 sec	30 sec		5 sec		
			40 times + Plate read			Plate read		

3.10 Gene expression:

Following the determination of the CT of the housekeeping gene (β-Actin) and the target gene (LAG-3), the relative expression of the targeted gene was eventually determined using the $2^{-\Delta\Delta CT}$ method (Rao et al., 2013).

The $2^{-\Delta\Delta CT}$ calculation formula as the following:

$$\Delta CT = CT \text{ (a target gene)} - CT \text{ (a reference gene)}$$

The ΔΔCT is the difference in ΔCT as described in the above formula between the target and reference samples, which is:

$$\Delta\Delta CT = \Delta CT \text{ (a target sample)} - \Delta CT \text{ (a reference sample)}$$

$$\Delta\Delta CT = \Delta CT_{\text{Patients}} - \Delta CT_{\text{Control}}$$

$$\text{e.g.: } \Delta\Delta CT = (\text{LAG-3 CT} - \beta\text{-Actin CT})_{\text{Patients}} - (\text{LAG-3 CT} - \beta\text{-Actin CT})_{\text{Control}}$$

$$(\Delta\Delta CT) \times -1 = -\Delta\Delta CT$$

The expression of the target gene equal $2^{-\Delta\Delta CT}$

When comparing a target sample to a reference sample, the method's end result is displayed as the fold change in target gene expression, normalized to a reference gene. For reference samples, the relative gene expression is often adjusted to one since ΔΔCT equals zero and 2^0 equals one.

3.11 Statistical Analysis:

Data entry and statistical analysis were performed using IBM SPSS Statistics, Version 26. To compare quantitative and qualitative variables across different groups, a one-way ANOVA test was employed, followed by the Bonferroni post hoc correction for multiple comparisons. Pearson correlation analysis was conducted to assess the relationship between LAG-3 gene expression and associated clinical or laboratory factors. A p-value of less than 0.05 was considered statistically significant.

Chapter Four

4- Results:

4.1 Demographic and Clinical Characteristics of the Study Population:

A total of 100 transfusion-dependent patients were enrolled in the study, comprising 80 individuals diagnosed with β-thalassemia major and 20 with sickle cell disease (SCD). An age- and sex-matched control group of 100 apparently healthy individuals was also included. The demographic and clinical characteristics of the patient cohort are shown in Table 4.1. Within the patient group, 58% (n = 58) were male and 42% (n = 42) were female, with a mean age of 22.25 years (range: 2–51 years). The control group exhibited the same sex distribution—58% male and 42% female—with a mean age of 22.6 years (range: 10–60 years). Comparative analyses of demographic variables using two-sided unpaired t-tests revealed no statistically significant differences in gender (P = 1.00) or age (P = 0.788), confirming appropriate matching between cases and controls. Among the transfusion-dependent patients, 39% had undergone splenectomy. Patients with β-thalassemia major and sickle cell disease (SCD) exhibited significantly lower hemoglobin (Hb) levels compared to healthy controls (P = 0.001). Serum ferritin levels and white blood cell (WBC) counts were also significantly elevated in the patient group relative to controls (P = 0.001). Importantly, LAG-3 gene expression was significantly higher in the transfusion-dependent patient group (mean ± SD: 5.05 ± 2.28) compared to healthy individuals (9.8 ± 2.14) (P = 0.001). Furthermore, 22% of transfusion-dependent patients were positive for alloantibodies, a statistically significant difference compared to the control group (P = 0.001).

Table 4.1: Demographic and Clinical Features of Transfusion-Dependent Patients (SCD and β-Thalassemia Major) and Control Individuals.

Variable	Dependent blood transfusion n=100	β-thalassemia major n=80	SCD n=20	Controls n=100	P value
Age (years) mean ± SD	22.25±9.7	21.8±9.4	24.1±10.9	22.6±9.7	0.788
Sex (n):					
Male	58	46	12	58	1.00
Female	42	34	8	42	
Marital status (n):					
Single	92	73	19	36	1.00
Married	8	7	1	64	
Place of resident (n):					
Jenin district	42	32	10	40	1.00
Nablus district	27	21	6	30	
Tolkarim district	31	27	4	30	
Splenectomy (n):					
Yes	39	36	3	0	--
No	61	44	17	100	
HGB level (g/dl) ±SD	7.7±1.3	7.8±1.3	7.1±1.1	14.2±1.5	0.001
WBC's count $\times 10^9$/l ±SD	16.61±26.23	18.67±28.8	8.38±6.71	6.4±1.8	0.001
Serum ferritin level (ng/mL) ±SD	3906±3715	4086±3907	3188±2782	120±25	0.001

Num. of blood units proposed (mean±SD)	500±401	521±400	416±403	0	--
Num. of blood transfusions/year (mean±SD)	25±15	26±15	22±16	0.0	--
Alloantibody (n):					
Positive	22	15	7	0	0.001
Negative	78	65	13	100	
LAG-3 gene expression	5.05±2.28	5.0±2.13	5.24±2.85	9.8±2.14	0.001

4.2: LAG-3 Expression and Clinical Parameters in Transfusion-Dependent Patients:

White blood cell (WBC) count demonstrated a significant association with LAG-3 gene expression. Patients with normal WBC counts exhibited lower LAG-3 expression levels (8.7 ± 2.3), whereas those with either leukopenia or leukocytosis showed elevated LAG-3 expression (5.1 ± 2.2 and 5.1 ± 2.4, respectively; $p = 0.001$). Conversely, serum ferritin levels and splenectomy status did not significantly impact LAG-3 expression ($p = 0.799$ and $p = 0.944$, respectively). Furthermore, patients receiving blood transfusion units (<250) exhibited significantly lower LAG-3 expression (8.6 ± 2.5), while those transfused with high number of blood units (>600 units) showed increased LAG-3 expression (4.7 ± 2.1) ($p = 0.001$). These findings are presented in Table 4.2**.**

Table 4.2: LAG-3 Gene Expression with Clinical and Laboratory Parameters in Transfusion-Dependent Patients (Sickle Cell Disease and β-Thalassemia Major):

Variable:	**n**	**LAG-3 gene expression for dependent blood transfusion mean ±SD**	***P*-value**
WBC's count ×10^9/l:			
Low < 5	30	5.1±2.2	0.001
Normal 5 – 10	30	8.7±2.3	
High > 10	40	5.1±2.4	
Serum ferritin level (ng/mL):			
< 2000	32	5.1±2.3	0.799
2000 – 4000	33	4.8±2.3	
> 4000	35	5.2±2.2	
Splenectomy (n):			
Yes	39	5.1±2.4	0.944
No	61	5±2.2	
Num. of blood units proposed:			
< 250	34	8.6±2.5	0.001
250 – 600	33	5.3±2	
> 600	33	4.7±2.1	

4.3: Comparison of LAG-3 Gene Expression Between β-Thalassemia Major and Sickle Cell Disease Patients:

Hemoglobin levels (7.1 ± 1.1 g/dL) and white blood cell (WBC) counts (8.38 ± 6.71 ×10^9/L) were significantly lower in sickle cell disease (SCD) patients compared to those with β-thalassemia major (p=0.015 and p = 0.005, respectively). Additionally, the prevalence of splenectomy was significantly higher in β-thalassemia patients (45%) compared to SCD patients (p = 0.014). No significant differences were observed between the groups regarding age, serum ferritin levels, or LAG-3 gene expression (Table 4.3).

Table 4.3: Comparison of Demographic Characteristics and Clinical Parameters between Patients with β-Thalassemia Major and Sickle Cell Disease (SCD):

Variable:	**β-thalassemia major n=80**	**SCD n=20**	**P value**
Age (years) mean ± SD	21.8±9.4	24.1±10.9	0.391
Sex (n): **Male** **Female**	 46 34	 12 8	1.00
HGB level (g/dl) mean ± SD	7.8±1.3	7.1±1.1	0.015
WBC's count ×10^9/l mean ± SD:	18.67±28.8	8.38±6.71	0.005
Serum ferritin level (ng/mL) mean ±SD	4086±3907	3188±2782	0.412

Splenectomy (n): **Yes** **No**	 36 44	 3 17	 0.014
Num. of blood units proposed (mean ±SD)	521±400	416±403	0.308
LAG-3 gene expression	5.0±2.13	5.24±2.85	0.733

4.4: Alloimmunization Among Transfusion-Dependent Patients:

Table 4.4 presents the demographic characteristics and clinical parameters of patients stratified by alloimmunization status. No statistically significant differences were observed between the alloantibody-positive and -negative groups with respect to age, sex, hemoglobin levels, white blood cell (WBC) count, ferritin levels, splenectomy status, number of transfused blood units, or LAG-3 expression.

Table 4.4: Demographic data and clinical parameters between alloantibody negative and positive patients (β-thalassemia major and SCD):

Variable	**None-alloimmunized (n=78)**	**Alloimmunized (n=22)**	**P-value**
Age (years) mean ± SD	22.32±10.06	22±8.7	0.883
Sex (n): **Male** **Female**	 47 31	 11 11	 0.392

HGB level (g/dl) mean ±SD	7.76±1.26	7.29±1.36	0.155
WBC's count ×10^9/l mean ±SD:	15.59±18.3	20.22±44.7	0.640
Serum ferritin level (ng/mL) mean ±SD	3447.32±2871.2	5533.24±5591.5	0.379
Splenectomy (n): **Yes** **No**	 29 49	 10 12	0.484
Num. of blood units proposed (mean±SD)	476±415	585±340	0.214
LAG-3 gene expression	5.08±2.36	4.94±2.01	0.776

4.5: Demographic Characteristics and Clinical Profiles: β-Thalassemia Major vs. Controls:

According to Table 4.5, patients with β-thalassemia exhibited significantly lower hemoglobin levels and significantly higher ferritin and white blood cell (WBC) counts compared to controls ($p = 0.001$). Moreover, LAG-3 expression was significantly reduced in patients (5.0 ± 2.13) compared to controls (9.8 ± 2.14) ($p = 0.001$).

Table 4.5: Demographic data and clinical parameters for β-thalassemia major patients and control individuals:

Variable	**β-thalassemia major n=80**	**Controls n=100**	**P-value**
Age (years) mean ± SD	21.8±9.4	22.6±9.7	0.505
Sex (n): **Male** **Female**	 46 34	 58 42	 0.703
HGB level (g/dl) mean ± SD	7.8±1.3	14.2±1.5	0.001
WBC's count ×10^9/l mean ± SD:	18.67±28.8	6.4±1.8	0.001
Serum ferritin level (ng/mL) mean ±SD	4086±3907	120±25	0.001
Splenectomy (n): **Yes** **No**	 36 44	 0 100	 --
Num. of blood units proposed (mean ±SD)	521±400	0	--
LAG-3 gene expression	5.0±2.13	9.8±2.14	0.001

4.6: Alloantibody-Negative vs. Positive β-thalassemia Major Patients:

As shown in Table 4.6, there were no statistically significant differences between the groups in any of the following parameters: age, sex, hemoglobin (HGB), white blood cell count (WBC), ferritin levels, history of splenectomy, number of blood units transfused, or LAG-3 expression.

Table 4.6: Demographic data and clinical parameters of alloantibody positive and negative β-thalassemia major patients:

Variable	**None-alloimmunized (n=65)**	**Alloimmunized (n=15)**	**P-value**
Age (years) mean ± SD	22.1±9.6	40.47±8.7	0.528
Sex (n): **Male** **Female**	 40 25	 6 9	 0.131
HGB level (g/dl) mean ±SD	7.8±1.3	7.9±1.1	0.768
WBC's count ×10^9/l mean ±SD:	16.7±19.7	27.0±53.1	0.597
Serum ferritin level (ng/mL) mean ±SD	3598.8±2968.1	6195.9±6324.8	0.556
Splenectomy (n): **Yes** **No**	 27 38	 9 6	 0.198
Num. of blood units proposed (mean±SD)	523.2±433.3	510.7±211.5	0.872
LAG-3 expression	5.01±2.13	4.98±2.22	0.968

4.7: LAG-3 Expression in Alloimmunized Patients:

Significant variations in LAG-3 expression were observed based on white blood cell (WBC) count, ferritin levels, and splenectomy status. Higher LAG-3 expression was associated with elevated WBC and ferritin levels ($p = 0.001$). Additionally, LAG-3 expression was significantly higher in splenectomized patients compared to non-splenectomized individuals (5.4 ± 1.5 vs. 4.4 ± 2.4; $p = 0.005$). Increased LAG-3 expression was also correlated with a higher number of blood transfusions ($p = 0.011$), as detailed in Table 4.7.

Table 4.7: LAG-3 gene expression associated with multiple medical test parameters for alloimmunized patients (SCD and β-thalassemia major).

Variable:	**n=22**	**LAG-3 expression for alloimmunized patients mean ±SD**	**P-value**
WBC's count $\times 10^9$/l:			
Low < 5	**8**	5.6±1.0	0.001
Normal 5 – 10	**6**	4.7±2.4	
High > 10	**8**	4.48±2.5	
Serum ferritin level (ng/mL):			
< 2200	**8**	6±0.72	0.001
2200 – 6000	**7**	5.2±1.7	
> 6000	**7**	3.55±1.5	
Splenectomy (n):			
Yes	**10**	4.4±2.4	0.005
No	**12**	5.4±1.5	

Num. of blood units proposed:			
< 450	**9**	5.2±2.1	0.011
450 – 700	**9**	4.8±2.0	
> 700	**4**	4.5±2.4	

Chapter Five

5- Discussion, Conclusions, Limitations and Recommendations:

This chapter presents an analysis of the study's results to offer comprehensive insights and highlight key findings.

5.1 Discussion:

Lymphocyte activation gene 3 (LAG-3) expression is an important consideration in patients with β-thalassemia major and sickle cell disease who require chronic blood transfusions. Multicenter study has identified several transfusion-related complications, including splenectomy, hyper-ferritinemia, alloimmunization, and leukocytosis (Faranoush et al., 2023). The present study evaluated the expression of the LAG-3 gene in transfusion-dependent patients compared to a healthy control group. It also examined the association between LAG-3 expression and key clinical complications, highlighting the potential role of immune checkpoint receptors in transfusion-related immune modulation. The study also compared the clinical and demographic characteristics of 100 healthy control individuals with those of 100 transfusion-dependent patients, including 20 with sickle cell disease (SCD) and 80 with β-thalassemia major. Both, the study and control groups were well-matched across key demographic variables, including age, sex, marital status, and place of residence, with no statistically significant differences observed ($p = 0.788$, 1.000, 1.000, and 1.000, respectively).

Among the study patients, 39% of transfusion-dependent individuals had undergone splenectomy, and 22% were alloimmunized. Additionally, these patients showed elevated

white blood cell counts ($16.61 \pm 26.23 \times 10^9$/L) and high serum ferritin levels (3906 ± 3715 ng/mL).

Primary finding of this study was that transfusion-dependent patients exhibited significantly higher LAG-3 gene expression (5.05 ± 2.28) compared to healthy controls (9.8 ± 2.14; $p = 0.001$), indicating a possible link between chronic transfusion exposure and immune modulation. Notably, there was no statistically significant difference in LAG-3 expression between patients with sickle cell disease (SCD) (5.24 ± 2.85) and those with β-thalassemia major (5.00 ± 2.13). This finding aligns with a previous study reporting elevated LAG-3 expression in β-thalassemia major patients, although LAG-3 expression in SCD had not been previously investigated (Shokrgozar et al., 2021).

In this study, a statistically significant difference in white blood cell (WBC) count was observed between transfusion-dependent patient groups, with SCD patients showing a mean WBC count of $8.38 \pm 6.71 \times 10^9$/L and β-thalassemia major patients exhibiting a higher mean count of $18.67 \pm 28.8 \times 10^9$/L ($p = 0.005$). Previous studies have also reported elevated WBC counts in individuals with both sickle cell disease and β-thalassemia major (Ahmed et al., 2017) (Khawaji et al., 2020). In SCD patients, elevated WBC levels have been attributed to factors such as infection or pharmacologic response (Ahmed et al., 2017). Analysis of the association between white blood cell (WBC) count and LAG-3 gene expression revealed that both low ($<5 \times 10^9$/L) and elevated ($>10 \times 10^9$/L) WBC counts were associated with significantly higher LAG-3 expression levels (5.1 ± 2.2 and 5.1 ± 2.4, respectively). In contrast, patients with WBC counts within the normal range ($5–10 \times 10^9$/L) exhibited significantly lower LAG-3 expression (8.7 ± 2.3) ($p = 0.001$). These findings suggest a potential relationship between abnormal WBC levels and upregulation of the LAG-3 immune checkpoint.

Previous studies have shown that LAG-3 functions as a negative regulator competing with CD4, playing a critical role in controlling the expansion of memory T cells and the proliferation of activated primary T cells (Workman et al., 2004). Dysregulation of this pathway can have significant immunological consequences. Excessive expansion of the T cell pool may lead to autoimmunity or lymphoproliferative disorders, whereas impaired immune regulation can result in lymphopenia, diminishing the T cell response to external infections. Moreover, overexpression of LAG-3 has been reported to enhance WBC activity and inhibit WBC apoptosis, contributing to leukocytosis and immune exhaustion (El Dosoky et al., 2024). This immunosuppressive environment has been associated with the persistence of chronic infections and tumor progression (Konnai et al., 2013).

Lymphocyte activation gene 3 (LAG-3) expression was observed among alloimmunized individuals with varying white blood cell (WBC) counts: low ($<5 \times 10^9$/L), normal ($5–10 \times 10^9$/L), and elevated ($>10 \times 10^9$/L), with corresponding mean expression levels of 5.6 ± 1.0, 4.7 ± 2.4, and 4.48 ± 2.5, respectively. A statistically significant association was found between WBC count and LAG-3 expression in alloimmunized patients receiving frequent blood transfusions ($p = 0.001$). These findings align with previous study indicating that LAG-3 exerts an immunosuppressive effect, which may contribute to both the development and severity of alloimmunization by modulating T-cell responses and overall immune regulation (Shokrgozar et al., 2020).

Serum ferritin levels below 2200 ng/mL, between 2200–6000 ng/mL, and above 6000 ng/mL were associated with progressively decreasing LAG-3 gene expression in alloimmunized patients (6.0 ± 0.72, 5.2 ± 1.7, and 3.55 ± 1.5, respectively). A statistically significant association was observed between serum ferritin levels and LAG-3 expression among alloimmunized individuals receiving frequent blood transfusions ($p = 0.001$). Previous study

reported that iron overload and elevated ferritin levels resulting from chronic transfusions contribute to oxidative stress and the generation of reactive oxygen species. This promotes immune-senescence, characterized by reduced antigen responsiveness and impaired T cell function (Shokrgozar et al., 2020). Consistent with these findings, serum ferritin levels in our patient group were significantly higher than those in the control group.

Additional study has also demonstrated that serum ferritin is a key regulatory factor in immune modulation (Wang et al., 2023). Elevated ferritin levels have been shown to influence the expression of immune checkpoint genes and suppress immune function by promoting the induction of regulatory T cells (Tregs). Patients with β-thalassemia major exhibit increased levels of Tregs and heightened expression of immune checkpoint genes compared to healthy controls (Shokrgozar et al., 2020), further supporting the link between iron overload and immune dysregulation in chronically transfused individuals.

Alloimmunized, transfusion-dependent patients who had undergone splenectomy demonstrated significantly higher LAG-3 gene expression compared to those with intact spleens (5.4 ± 1.5 vs. 4.4 ± 2.4; $p = 0.005$). This observation is consistent with previous findings suggesting that the spleen plays a vital role in the activation and differentiation of regulatory T cells (Tregs), which may explain the upregulation of immune checkpoint genes such as LAG-3 following splenectomy (Shokrgozar et al., 2020).

Supporting this, a study involving Asian patients in the United States identified splenectomy as a significant risk factor for red blood cell alloimmunization. The authors hypothesized that the absence of splenic filtration impairs the clearance of antigens and damaged erythrocytes, thereby increasing the risk of alloantibody production. Additionally, splenectomy has been linked to changes in both humoral and cell-mediated immunity,

reinforcing its contribution to immune dysregulation (Jansuwan et al., 2015). Despite these associations, the direct relationship between splenectomy and LAG-3 gene expression remains insufficiently studied. Further research is needed to elucidate the underlying immunological mechanisms and to better understand the role of LAG-3 in post-splenectomy immune modulation.

An analysis of the relationship between the number of transfused blood units and LAG-3 gene expression in transfusion-dependent patients revealed the patients who received fewer than 250 units exhibited the lowest LAG-3 expression rate (8.6 ± 2.5), while those receiving 250–600 units and more than 600 units showed progressively higher expression levels (5.3 ± 2.0 and 4.7 ± 2.10), respectively; ($p = 0.001$). A similar trend was observed among alloimmunized patients, where LAG-3 expression level increase as the number of transfused units increased. Expression levels were 5.2 ± 2.1 in those receiving fewer than 450 units, 4.8 ± 2.0 in those receiving 450–700 units, and 4.5 ± 2.4 in those receiving more than 700 units ($p = 0.011$). These findings suggest that cumulative transfusion exposure may influence LAG-3 gene expression, potentially reflecting immunomodulatory changes, particularly in alloimmunized individuals.

Previous research has demonstrated that repeated exposure to foreign antigens through chronic blood transfusions can activate the immune system, potentially leading to alloimmunization. While genetic differences between alloimmunized and non-alloimmunized patients may contribute to this risk, immunologic variability is also considered a significant factor in the development of alloimmunization (Shokrgozar et al., 2021).

Additionally, studies have reported that patients with β-thalassemia major who receive frequent transfusions tend to exhibit reduced regulatory T cell (Treg) counts—both in

percentage and absolute number—which may increase their susceptibility to alloantibody formation and alloimmunization (Shokrgozar et al., 2020).

Transfusion-dependent patients consistently exhibited higher LAG-3 expression compared to control subjects. A correlation was observed between LAG-3 levels and alloimmunization, suggesting that LAG-3 may serve as a potential immunological marker. The elevated expression of LAG-3 appears to be influenced by transfusion-related factors and immune-related parameters, such as white blood cell (WBC) count and ferritin levels. These findings indicate a possible role for LAG-3 in immune regulation among individuals receiving frequent blood transfusions.

5.2 Conclusions:

This study highlights the pivotal role of LAG-3 gene expression in maintaining immune homeostasis among chronically transfused patients. Individuals with sickle cell disease (SCD) and β-thalassemia major exhibited significantly higher LAG-3 expression levels compared to healthy controls, though no significant difference was observed between the two patient groups.

Key clinical variables—including splenectomy, abnormal white blood cell (WBC) counts, elevated serum ferritin levels, alloimmunization status, and the cumulative number of transfusions—were associated with variations in LAG-3 expression. Notably, LAG-3 levels were elevated in patients with both low and high WBC counts, indicating possible immune dysregulation. Increased expression was also observed in alloimmunized individuals, especially those with splenectomy or iron overload, further linking LAG-3 to transfusion-related immune modulation.

In alloimmunized individuals, an inverse relationship between LAG-3 expression and transfusion units may indicate immunological exhaustion or responsiveness over time. Collectively, these findings support the immunoregulatory function of LAG-3 in transfusion-dependent patients and suggest its potential role in transfusion-related immunopathology. Further research is needed to fully elucidate the mechanisms by which immune checkpoint receptors like LAG-3 contribute to alloimmunization and immune dysfunction in chronically transfused populations.

5.3 Limitations:

It is important to note that certain environmental, social, and cultural factors unique to this population may influence the study's findings. As such, the results may not be fully generalizable to other geographic or cultural contexts. The study population included patients diagnosed with either sickle cell disease or β-thalassemia major residing in the northern West Bank. Logistical challenges, particularly poor road infrastructure, posed limitations to travel and data collection.

5.4 Recommendations:

Further exploration of LAG-3's role in immune modulation may be enhanced by integrating cytokine profiling and T-cell functional assays. Assessing LAG-3 expression could support the development of personalized transfusion protocols aimed at minimizing immune exhaustion and reducing the risk of alloimmunization in transfusion-dependent patients. Given its immunoregulatory function, LAG-3 also presents as a potential therapeutic target for modulating immune responses and mitigating transfusion-related complications.

To strengthen the validity and generalizability of these findings, future studies should include larger and more ethnically diverse populations across multiple centers. Additionally, patients who have undergone splenectomy should be closely monitored for immunological alterations, including shifts in LAG-3 expression, to better manage their risk profile. Continued research into LAG-3 may also yield valuable insights for addressing other immune-related conditions, such as malignancies and autoimmune diseases.

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Appendices

Appendix 1: Institutional Review Board (IRB) of Arab American University (AAUP).

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عمادة البحث العلمي
لجنة أخلاقيات البحث العلمي
تلفون: 04-241-8888 ext 1196
البريد الالكتروني: irb.aaup@aaup.edu

IRB Approval Letter

Study Title: Evaluation of the level of LAG-3 gene expression among β-thalassemia major patients with and without alloantibodies in comparison with control group from patient in West Bank, Northern Palestine.

Submitted by: Ahmed Abd Alraouf Ahmed Jarrar

Date received: 08/04/2023

Date reviewed: 01/05/2023

Date approved: 23/06/2023

Your Study titled "**Evaluation of the level of LAG-3 gene expression among β-thalassemia major patients with and without alloantibodies in comparison with control group from patient in West Bank, Northern Palestine.**" With archived number 2023/B/72/N was reviewed by the Arab American University IRB committee and was approved on 23rd June 2023.

Reham Khalaf-Nazzal, MD, PhD
IRB committee chairman
Arab American University of Palestine

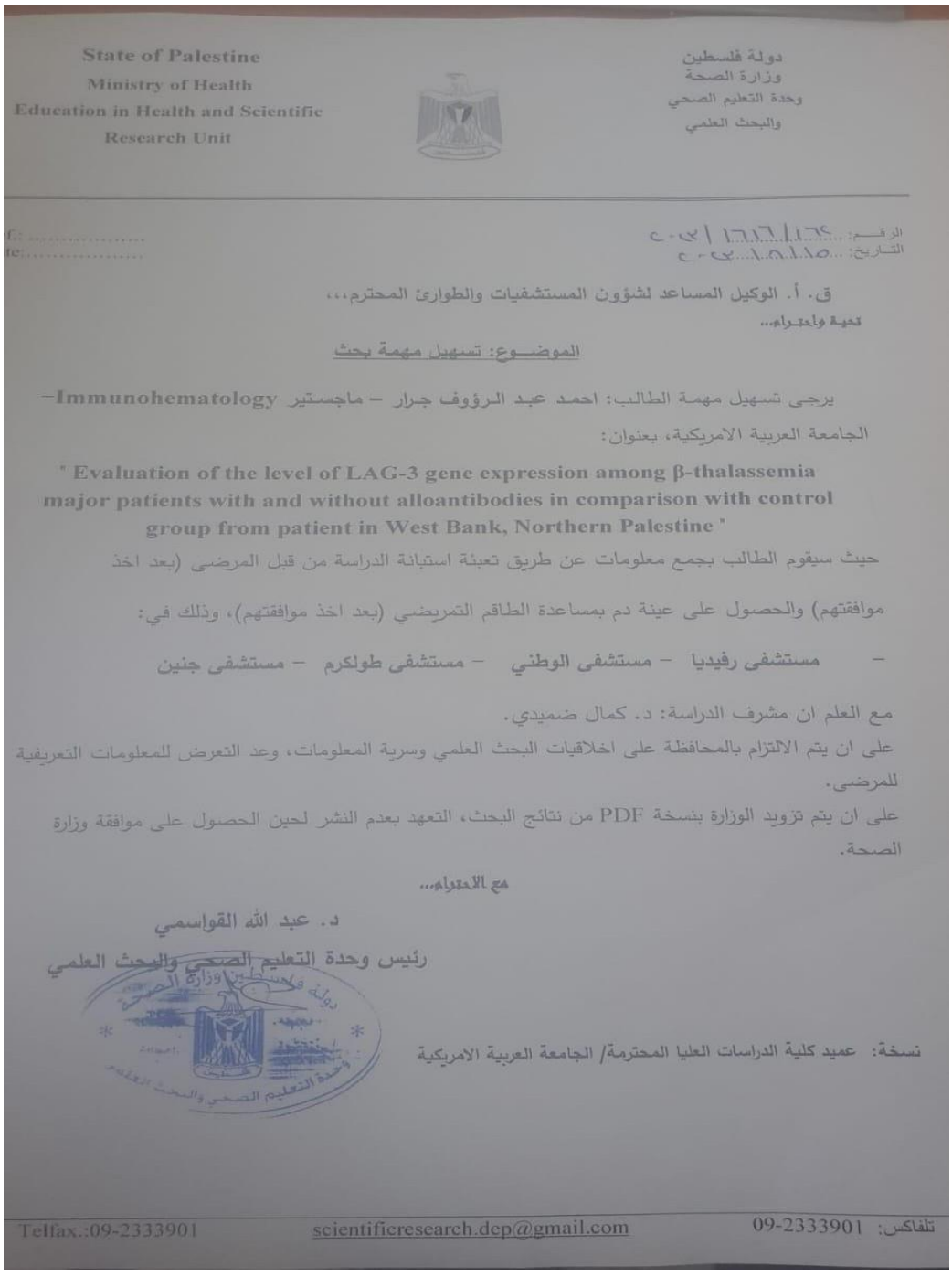
General Conditions:

1. Valid for 6 months from date of approval.
2. It is important to inform the committee with any modification of the approved study protocol.
3. The committee appreciates a copy of the research when accomplished.

لجنة أخلاقيات البحث العلمي في الجامعة العربية الامريكية

IRB at Arab American University

Appendix 2: Approval to allow the collection of patient samples and the results of their previous tests registered with the Palestinian Ministry of Health.



State of Palestine
Ministry of Health
Education in Health and Scientific Research Unit

دولة فلسطين
وزارة الصحة
وحدة التعليم الصحي والبحث العلمي

الرقم: ٠٢/١٦٦٦/١٦٩
التاريخ: ٢٠٢٢/٥/١٨

ق. أ. الوكيل المساعد لشؤون المستشفيات والطوارئ المحترم،،،

تحية واحترام...

الموضـــوع: تسهيل مهمة بحث

يرجى تسهيل مهمة الطالب: احمد عبد الرؤوف جرار – ماجستير Immunohematology– الجامعة العربية الامريكية، بعنوان:

" Evaluation of the level of LAG-3 gene expression among β-thalassemia major patients with and without alloantibodies in comparison with control group from patient in West Bank, Northern Palestine "

حيث سيقوم الطالب بجمع معلومات عن طريق تعبئة استبانة الدراسة من قبل المرضى (بعد اخذ موافقتهم) والحصول على عينة دم بمساعدة الطاقم التمريضي (بعد اخذ موافقتهم)، وذلك في:

– مستشفى رفيديا – مستشفى الوطني – مستشفى طولكرم – مستشفى جنين

مع العلم ان مشرف الدراسة: د. كمال ضميدي.

على ان يتم الالتزام بالمحافظة على اخلاقيات البحث العلمي وسرية المعلومات، وعد التعرض للمعلومات التعريفية للمرضى.

على ان يتم تزويد الوزارة بنسخة PDF من نتائج البحث، التعهد بعدم النشر لحين الحصول على موافقة وزارة الصحة.

مع الاحترام...

د. عبد الله القواسمي

رئيس وحدة التعليم الصحي والبحث العلمي

نسخة: عميد كلية الدراسات العليا المحترمة/ الجامعة العربية الامريكية

Telfax.:09-2333901 scientificresearch.dep@gmail.com تلفاكس: 09-2333901

Appendix 3: English informed consent.

Arab American University
Scientific Research Deanship
Ethical Review Committee

الجامعـــة العربيـــة الأمريكيـــة
عمادة البحث العلمي
لجنة اخلاقيات البحث العلمي

INFORMED CONSENT

AAUP-IRB Code No.: .....
AAUP-IRB Date: .....

I, ... *(Name of Participant / optional)* hereby agree to take part in the clinical research (clinical study/questionnaire study/drug trial) specified below:

Title of Study: ....., Fulfillment of Master degree, in Immunohematology, in AAUP.
(Name of program)

The nature and purpose of which has been explained to me by ....., and interpreted by ..... to the best of his/her ability in English.

I have been told about the nature of the research in terms of methodology, possible adverse effects and complications (as per Participant Information Sheet).
After knowing and understanding all the possible advantages and disadvantages of this research, I voluntarily consent of my own free will to participate in the clinical research specified above.
I understand that I can withdraw from this research at any time without assigning any reason whatsoever.

Date: ..... **Signature:** .....
(Participant)

IN THE PRESENCE OF:

Name: .....

Designation: . ..... **Signature:** .....

(Witness for Signature of Participant)

I confirm that I have explained to the patient the nature and purpose of the above-mentioned research.

Date: ..... **Signature:** .....

(Attending investigator)

Appendix 4: Arabic informed consent.

موافقة مسبقة

رقم كود AAUP-IRB: .....

تاريخ AAUP-IRB: .....

انا.....اوافق بموجبه على المشاركة في البحث السريري (الدراسة السريرية / دراسة الاستبيان / تجربة الدواء) المحددة أدناه:

عنوان الدراسة:.....

إتمام الماجستير في علم الدم المناعي بالجامعة العربية الأمريكية

تم شرح طبيعتها والغرض منها من خلال .....، وتفسيرها بواسطة ..... على أفضل وجه في اللغة الإنجليزية.

لقد تم إخباري عن طبيعة البحث من حيث المنهجية والآثار السلبية المحتملة والمضاعفات (حسب ورقة معلومات المشارك) بعد معرفة وفهم جميع المزايا والعيوب المحتملة لهذا البحث ، أوافق طواعية بمحض إرادتي على المشاركة في البحث السريري المحدد أعلاه. أفهم أنه يمكنني الانسحاب من هذا البحث في أي وقت دون إبداء أي سبب على الإطلاق.

التوقيع.....**التاريخ:**.....

(المشارك)

في حضور:

الاسم:.....

التسمية.....**التوقيع:**.....

(شاهد على توقيع المشارك)

أؤكد أنني أوضحت للمريض طبيعة وهدف البحث المذكور أعلاه.

التوقيع.....**التاريخ:**.....

(بحضور الباحث)

Appendix 5: English Research Questionnaire for patients more than 10 Years age.

Research questioner for " Evaluation of the level of LAG-3 gene expression among β-thalassemia major patients with and without alloantibodies in comparison with control group from patient in West Bank, Northern Palestine".

Prepared by:

Ahmed Abd Al Rou'f Jarrar.

2022-2023

Research Questionnaire

Dear brother;

We are working on research entitled "**Evaluation of the level of LAG-3 gene expression among β-thalassemia major patients with and without alloantibodies in comparison with control group from patient in West Bank, Northern Palestine**". You have been selected to participate in this study.

We are delighted to ask you to fill this questionnaire by yourself. There is no right or wrong answer. Your responses are completely confidential

Please answer every question and may stop filling at any time if you want to do so. The survey will take about 5 minutes to fill this questionnaire.

Please read each question carefully and show your answers by circling or filling your answers that apply to you **if you are more than 10 years old.**

Student name:

Ahmed Abd Al Raou'f Jarrar.

Email:

a.jarrar34@student.aaup.edu

Questionnaire

Circle and fill in the answers that apply to you if you are more than 10 years old.

Num ( )

Demographic Data			
Name			**Date of birth**…./……/……
Gender	Male	Female	**Address:**
Marital status	Single	Married	**Phone No.**

Clinical history			
Type of thalassemia diagnosis:	Major	Trait	Other specify……
Date of diagnosis			
Date of first blood transfusion			
Frequency of blood transfusion	1/month	2/month	More- specify……..
Are you splenectomies?	Yes	No	
Are suffer from any immune diseases	Yes	No	If yes, specify……
Are you on Iron chelators	Yes	No	If yes, specify…

			……
History of HBV vaccine			
HBV B vaccine	Yes / No	Don't Know	Type of vaccine
Country of vaccination			

Lab test results (For researcher use only)

Do you have HCV?	Yes / No	Don't Know	
Do you have HBV?	Yes / No	Don't Know	
Level of last three Hb results			
Level of last three MCV results			
Level of last three HCT results			
Level of last three RBC's results			
Level of last three WBC's results			
Level of last three GPT results			
Level of last three GOT results			
Level of last three ALK results			
Level of last three Albumin results			
Last three IAT test results			
Level of last three ferritin results			

Molecular result

LAG-3 gene level by RT-PCR-quantitative	

By answering this questionnaire, I agree that the researcher use my answers for the purpose of conducting his research. I confirm that all the information contained herein is true and correct. Signature: ……. .

Appendix 6: English Research Questionnaire for patients less than 10 Years age.

Research questioner for " Evaluation of the level of LAG-3 gene expression among β-thalassemia major patients with and without alloantibodies in comparison with control group from patient in West Bank, Northern Palestine".

Prepared by:

Ahmed Abd Al Rou'f Jarrar.

2022-2023

Research Questionnaire

Dear brother;

We are working on research entitled "**Evaluation of the level of LAG-3 gene expression among β-thalassemia major patients with and without alloantibodies in comparison with control group from patient in West Bank, Northern Palestine"**. You client have been selected to participate in this study.

We are delighted to ask you to fill this questionnaire on behalf of your client. There is no right or wrong answer. Your responses are completely confidential

Please answer every question and may stop filling at any time if you want. The survey will take about 5 minutes to fill this questionnaire.

Please read each question carefully and show your answers by circling or filling your answers that apply to your client **if your client are less than 10 years old.**

Student name:

Ahmed Abd Al Raou'f Jarrar.

Email:

a.jarrar34@student.aaup.edu

Questionnaire

Circle and fill in the answers that apply to your client if your client are less than 10 years old.

Demographic Data			
His/ Her Name			**His/ Her Date of birth**…./……/……
His/ Her Gender	Male	Female	**His/ Her Address:**
Phone No.			

Clinical history			
Type of thalassemia diagnosis:	Major	T rait	Other specify…… ……..
Date of diagnosis			
Date of first blood transfusion			
Frequency of blood transfusion	1/month	2/ month	More- specify…… …..
Are your client splenectomies?	Yes	No	
Are your client suffer from any immune diseases	Yes	N o	If yes, specify…… ……
Are your client on Iron chelators	Yes	N o	If yes, specify…… ……
History of HBV vaccine			

HBV B vaccine	Yes	No	Don't Know	Type of vaccine
Country of vaccination				

Lab test results (For researcher use only)

Do you have HCV?	Yes	No	Don't Know	
Do you have HBV?	Yes	No	Don't Know	
Level of last three Hb results				
Level of last three MCV results				
Level of last three HCT results				
Level of last three RBC's results				
Level of last three WBC's results				
Level of last three GPT results				
Level of last three GOT results				
Level of last three ALK results				
Level of last three Albumin results				
Last three IAT test results				
Level of last three ferritin results				

Molecular result

LAG-3 gene level by RT-PCR-quantitative	

By answering this questionnaire, I agree that the researcher use my answers for the purpose of conducting his research. I confirm that all the information contained herein is true and correct. Signature: …….…… .

Appendix 7: Arabic Research Questionnaire for patients more than 10 Years age.

استبيان بحثي " تقييم معدل تواجد جين تنشيط الخلايا اللمفاوية عند مرضى التلاسيميا الحاد مقارنة بالأشخاص الطبيعيين في الضفة الغربية، شمال فلسطين".

إعداد:

أحمد عبد الرؤوف أحمد جرار

2022-2023

استبيان البحث

أخي العزيز ،

نحن نعمل على بحث بعنوان " **تقييم معدل تواجد جين تنشيط الخلايا اللمفاوية عند مرضى التلاسيميا الحاد مقارنة بالأشخاص الطبيعيين في الضفة الغربية، شمال فلسطين".** لقد تم اختيارك للمشاركة في هذه الدراسة.

يسعدنا أن نطلب منك ملء هذا الاستبيان بنفسك. ليس هناك جواب صحيح أو خاطئ. ردودكم سرية تماما.

الرجاء الإجابة على كل سؤال في الجزء الأول والجزء الثاني في هذا الاستبيان وقد تتوقف عن الملء في أي وقت إذا كنت ترغب في ذلك. سيستغرق حوالي 5 دقائق لملء هذا الاستبيان.

يرجى قراءة كل سؤال بعناية وإظهار إجابتك من خلال تدوير الرقم او تعبئة مكان الإجابة **اذا كان عمرك اكثر من 10 سنوات** .

الأسم :

أحمد عبد الرؤوف احمد جرار.

البريد الالكتروني:

a.jarrar34@student.aaup.edu

الاستبيان

ضع دائرة حول الإجابات التي تنطبق عليك او قم بملء الفراغ في الأسئلة التي تحتاج لذلك اذا كان عمرك اكثر من 10 سنوات.

المعلومات الديموغرافية			
الاسم:			**تاريخ الميلاد:**
الجنس:	1) ذكر	2) أنثى	**العنوان:**
الحالة الاجتماعية	1) أعزب	2) متزوج	**رقم الهاتف:**

التاريخ الطبي			
نوع التلاسيميا:	1) حادة	2) حامل تلاسيميا	أخرى، اذكر النوع .....
تاريخ التشخيص:			
تاريخ اول مرة تأخذ بها دم:			
كل متى يتم نقل الدم اليك؟	1) مرة في الشهر	2) مرتين في الشهر	3) أكثر، كم مرة في الشهر .....
هل قمت باستئصال الطحال؟	1) نعم	2) لا	
هل تعاني من امراض مناعية؟	1) نعم	2) لا	إذا نعم اذكرها: .....
هل تستخدم مستخلبات الحديد؟	1) نعم	2) لا	إذا نعم اذكرها: .....
تاريخ طعم التهاب الكبد الوبائي ب			
هل اخذت الطعم؟	1) نعم	2) لا	3) لا اعرف / إذا نعم اذكر النوع: .....
البلد الذي اخذت بها الطعم:			

Lab test results (لاستخدام الباحث فقط)

Do you have HCV?	Yes	No	Don't Know	
Do you have HBV?	Yes	No	Don't Know	
Level of last three Hb results				
Level of last three MCV results				
Level of last three HCT results				
Level of last three RBC's results				
Level of last three WBC's results				
Level of last three GPT results				
Level of last three GOT results				
Level of last three ALK results				
Level of last three Albumin results				
Last three IAT test results				
Level of last three ferritin results				

Molecular result (لاستخدام الباحث فقط)

LAG-3 gene level by RT-PCR-quantitative	

من خلال الإجابة على هذا الاستبيان، أوافق على أن يستخدم الباحث إجاباتي لغرض إجراء بحثه. أؤكد أن جميع المعلومات الواردة هنا صحيحة.

التوقيع:

.....

Appendix 8: Arabic Research Questionnaire for patients less than 10 Years age.

استبيان بحثي " تقييم معدل تواجد جين تنشيط الخلايا اللمفاوية عند مرضى التلاسيميا الحاد مقارنة بالأشخاص الطبيعيين في الضفة الغربية، شمال فلسطين".

إعداد:

أحمد عبد الرؤوف أحمد جرار

2022-2023

استبيان البحث

أخي العزيز ،

نحن نعمل على بحث بعنوان " **تقييم معدل تواجد جين تنشيط الخلايا اللمفاوية عند مرضى التلاسيميا الحاد مقارنة بالأشخاص الطبيعيين في الضفة الغربية، شمال فلسطين".** لقد تم اختيار موكلك للمشاركة في هذه الدراسة.

يسعدنا أن نطلب منك ملء هذا الاستبيان بالنيابة عن موكلك. ليس هناك جواب صحيح أو خاطئ. ردودكم سرية تماما.

الرجاء الإجابة على كل سؤال في الجزء الأول والجزء الثاني في هذا الاستبيان وقد تتوقف عن الملء في أي وقت إذا كنت ترغب في ذلك. سيستغرق حوالي 5 دقائق لملء هذا الاستبيان.

يرجى قراءة كل سؤال بعناية وإظهار إجابتك من خلال تدوير الرقم او تعبئة مكان الإجابة اذا **كان عمر موكلك اقل من 10 سنوات** .

الأسم :

أحمد عبد الرؤوف احمد جرار.

البريد الالكتروني:

a.jarrar34@student.aaup.edu

الاستبيان

ضع دائرة حول الإجابات التي تنطبق على موكلك او قم بملء الفراغ في الأسئلة التي تحتاج لذلك اذا كان عمر موكلك اقل من 10 سنوات.

المعلومات الديموغرافية			
الاسم:			**تاريخ الميلاد:**
الجنس:	1) ذكر	2) أنثى	**العنوان:**
رقم الهاتف:			

التاريخ الطبي				
نوع التلاسيميا:	1) حادة	2) حامل تلاسيميا	أخرى، اذكر النوع	
تاريخ التشخيص:				
تاريخ اول مرة يأخذ بها دم:				
كل متى يتم نقل الدم اليه؟	1) مرة في الشهر	2) مرتين في الشهر	3) أكثر، كم مرة في الشهر	
هل قام باستئصال الطحال؟	1) نعم	2) لا		
هل يعاني من امراض مناعية؟	1) نعم	2) لا	إذا نعم اذكرها:	
هل يستخدم مستخلبات الحديد؟	1) نعم	2) لا	إذا نعم اذكرها:	
تاريخ طعم التهاب الكبد الوبائي ب				
هل اخذ الطعم؟	1) نعم	2) لا	3) لا اعرف	إذا نعم اذكر النوع: .....
البلد الذي اخذ بها الطعم:				

Lab test results (لاستخدام الباحث فقط)				
Do you have HCV?	Yes	No	Don't Know	
Do you have HBV?	Yes	No	Don't Know	
Level of last three Hb results				
Level of last three MCV results				
Level of last three HCT results				
Level of last three RBC's results				
Level of last three WBC's results				
Level of last three GPT results				
Level of last three GOT results				
Level of last three ALK results				
Level of last three Albumin results				
Last three IAT test results				
Level of last three ferritin results				

Molecular result (لاستخدام الباحث فقط)

LAG-3 gene level by RT-PCR-quantitative	

من خلال الإجابة على هذا الاستبيان، أوافق على أن يستخدم الباحث إجاباتي لغرض إجراء بحثه. أؤكد أن جميع المعلومات الواردة هنا صحيحة.

التوقيع: .....

Appendix 9: Approval to allow genetic testing within the facilities of the Palestinian Ministry of Health:

Arab American University
Faculty of Graduate Studies

الجامعة العربية الأمريكية
كلية الدراسات العليا

2024/7/22

حضرة الدكتور أسامة النجار المحترم

الوكيل المساعد للمهن الطبية المساندة وبنوك الدم

وارد دولة فلسطين
وزارة الصحة
مكتب الوكيل المساعد للمهن الطبية المساندة وبنوك الدم
وارد رقم: 2024-855
التاريخ: 30/7/2024

تسهيل مهمة بحثية

تحية طيبة وبعد،

تُهديكم كلية الدراسات العُليا في الجامعة العربية الأمريكية أطيب التحيات، وبالإشارة الى الموضوع أعلاه، تشهد كلية الدراسات العُليا في الجامعة أن الطالب احمد عبد الرؤف احمد جرار والذي يحمل الرقم الجامعي 202113045 هو طالب ماجستير في برنامج علم الدم والمناعة ويعمل على رسالة الماجستير الخاصة به بعنوان:

"Evaluation of the level of LAG-3 gene expression among β-thalassemia major patients with and without alloantibodies in comparison with control group from patient in West Bank, Northern Palestine"

تحت إشراف الدكتور كمال ضميدي، وذلك في دائرة المختبرات المركزية التخصصية لمحافظة جنين حيث سيقوم الطالب فقط باستخدام جهاز ال PCR الموجود في الدائرة علماً بان الطالب يمتلك جميع المواد اللازمة لعمل الفحوصات. نأمل من حضرتكم الإيعاز لمن يلزم لمساعدته للحصول على المعلومات اللازمة للدراسة، علماً أن المعلومات ستستخدم لغاية البحث فقط وسيتم التعامل معها بغاية السرية، وقد أعطي هذه الرسالة بناءً على طلبه.

وتفضلوا بقبول فائق الاحترام

عميد كلية الدراسات العليا

د. نوار قطب

كلية الدراسات العليا
FACULTY OF GRADUATE STUDIES

Page 1 of 1

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Ramallah Tel: +970-2-2941999 Fax: +970-2-2941979 Abu Qash - Near Alrehan
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الملخص باللغة العربية

الخلفية: يحافظ الجهاز المناعي على توازنه الداخلي، ويحمي نفسه من العدوى، بينما يُقلّم الخلايا التي قد تتصرف بعدوانية. ومن بين البروتينات التي تُحدد مدى قوة تأثيره على هذه العناصر، برز مؤخرًا بروتين LAG-3 كجزيء ذي أهمية في كل من المناعة الذاتية والتخلص من خلايا الأورام. عادةً ما يتلقى مرضى بيتا ثلاسيميا الكبرى عمليات نقل دم مدى الحياة، وهي ممارسة تجعلهم عرضة لتكوين الأجسام المضادة الخيفية والانحراف المناعي غير المتوقع. ومن المثير للدهشة أن المساهمة الدقيقة لتعبير LAG-3 ضمن هذه المجموعة تحديدًا لا تزال غير مفهومة جيدًا.

الأهداف: تبحث هذه الدراسة تقييم دور تعبير LAG-3 لدى المرضى الذين يعتمدون على علاج نقل الدم المنتظم - بيتا ثلاسيميا الكبرى ومرض فقر الدم المنجلي - في محاولة لرسم خريطة لأي تحول مناعي ناتج. ومن خلال ربط هذه النتائج بالمتغيرات الديموغرافية والسريرية، يهدف هذا العمل إلى توضيح دور LAG-3 في ضبط المناعة المرتبط بنقل الدم.

المنهجية: شملت الدراسة 100 من المرضى المعرضين لعمليات نقل دم متكررة: 80 مصابًا بثلاسيمية بيتا الكبرى، و20 مصابًا بفقر الدم المنجلي، وعددًا مماثلًا من الأصحاء، متطابقين في العمر والجنس. استُخلص الحمض النووي الريبوزي (RNA) من خلايا الدم، وقُيست وفرة LAG-3 باستخدام تقنية (quantitative real-time PCR) باستخدام طريقة SYBR Green. حُوّلت القيم إلى مستويات تعبير نسبية باستخدام معادلة $2^{-\Delta\Delta CT}$ بالاستناد على جين بيتا أكتين (β-actin) كجين مرجعي للمرضى والأشخاص السليمين (housekeeping gene). أُدخلت البيانات وحُسِّبت إحصائيًا باستخدام برنامج IBM SPSS Statistics.

النتائج: أظهر المرضى المعتمدون على اخذ الدم بشكل متكرر مستويات أعلى بكثير لظهور جين LAG-3 مقارنة بالمجموعة الضابطة من الأصحاء. لم يكن هناك فرق كبير في تعبير LAG-3 بين المرضى الذين لديهم أجسام مضادة (alloantibodies) والذين ليس لديهم هذه الأجسام. ومع ذلك، لم تؤثر مستويات مخزون الحديد في الدم واستئصال الطحال على تعبير LAG-3، بينما كان لعدد كريات الدم البيضاء وكمية وحدات الدم المنقولة تأثير إحصائي بارز.

الخلاصة: يبدو أن التعرض المزمن لنقل الدم المتكرر يُغيّر تعبير LAG-3 عن المعدل الطبيعي، مما يُشير إلى أن الجهاز المناعي قد يُعيد ضبط نفسه تحت الضغط. ومن المفارقات أن تكوين الأجسام المضادة التباينية بحد ذاته لا يرتبط بارتفاع إضافي في مؤشر نقطة التفتيش. وتظل الدراسات الطولية ضرورية إذا كان الباحثون يأملون في توضيح ما إذا كان LAG-3 مجرد عامل مساعد في نقل الدم أم أنه عامل مُسبب لاضطراب مناعي مستمر.