Ministry of Higher Education and Scientific Research
University of Sulaimani
College of Medicine, Department of Medicine
Dermatology Unit

Serum level of tumor necrosis factor alpha (TNF-α) variation among patients with generalized vitiligo in Sulaymaniyah - Iraq

A Thesis
Submitted to the Department of Medicine and to the Graduate Studies at the College of Medicine, University of Sulaimani, as a Partial Fulfillment of the Requirement for the Degree of Doctor of Philosophy (PhD) in Dermatology and Venereology

By
Ronak Saeed Ahmed
MBChB, MSc (Dermatology and Venereology)

Supervised By
Assistant Professor Dr**. Dana Ahmed Sharif** (MD, MRCP, FRCP)
Assistant Professor Dr. **Mohammad Yousif Saeed Jaf** (MD, FICMS)

2021 ميلادي **٢٧٢١ كوردى** **١٤٤٢ هجري**

بسم الله الرحمن الرحيم

وَيَسْأَلُونَكَ عَنِ الرُّوحِ ۖ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُمْ مِنَ الْعِلْمِ إِلَّا قَلِيلاً

سورة الإسراء
الآية 85

Student Declaration

I the undersigned, PhD candidate declare that this thesis is my original work and has never been presented in any other Universities in Kurdistan- Iraq and that all resources of materials have been duly acknowledged.

Ronak Saeed Ahmed
MBChB, MSc-Dermatology and Venereology

Supervisor Certification

We certify that this thesis (**Serum level of tumor necrosis factor alpha (TNF-α) variation among patients with generalized vitiligo in Sulaymaniyah - Iraq**) was prepared by PhD candidate (**Ronak Saeed Ahmed**) under our supervision at the Department of Medicine-College of Medicine as a partial fulfillment for the Degree of Doctor of Philosophy in Dermatology and Venereology.

Assistant Professor
Dr. Dana Ahmed Sharif
(MD, MRCP, FRCP)

Date:

Assistant Professor
Dr. Mohammed Yousif Saeed
(MD, FICMS)

Date:

In view of the available recommendations, we forward this thesis for debate by the examining committee

Professor

Dr. Kosar Mohamed Ali (MD, MRCP)
Dean of College of Medicine

Date:

Exam-Committee Certification

We, the examining committee certify that we have read this thesis and examined the student **(Ronak Saeed Ahmed)** in its content and that, according to our opinion it is accepted as a thesis for the **Degree of Doctor of Philosophy (PhD) in Dermatology and Venereology.**

Professor
Kawa Abdullah Mohammad Amin
(PhD Clinical Immunology)
University of Sulaimani, College of Medicine
"Member"

Professor **Khudhair Khalaf Ibrahim (MD,**
University of Dayala, College of Medicine
" chairman "

Professor **Shwan K. Rachid (PhD**
Molecular Biology
University of Charmo
"Member"

Professor
Ali Mozan El Ethawi (MD, FICMS, CABD)
Dermatology
University of Sulaimani, College of Medicine
"Member"

Assistant Professor
Dindar Sharif Qurtas (MD, PhD), Dermatology
Hawler Medical University, College of Medicine
"Member"

Assistant Professor
Dana Ahmed Sharif (MD, MRCP, FRCP)
University of Sulaimani, College of Medicine
"Member and Supervisor"

Assistant Professor
Mohammad Yousif Saeed Jaf (MD, FICMS)
University of Sulaimani, College of Medicine
"Member and Supervisor"

Approved by the council of the college of medicine (University of Sulaimani)
Professor **Kosar Mohamed Ali (MD, MRCP),**
Dean of College of Medicine

Dedication

This work is dedicated to:

The soul of my parent, to my husband for his dedicated partnership in my success and to my children; Ahmed, Sarah, and Ara

Acknowledgment

Throughout the conduct and writing of this thesis, I have received a great deal of support and assistance.

I should start my appreciation toward the **Deanery of the college of Medicine, University of Sulaimani**, the **Scientific Committee** of the College and all that support me at different stages of my research.

Thank you a lot my supervisors, **Dr.Dana Ahmed Sharif** (Assistant Professor of Medicine) and **Dr. Mohammad Jaf** (Assistant Professor of Dermatology), whose expertise were invaluable in formulating the research questions and methodology. Your insightful feedback pushed me to sharpen my thinking and brought my work to a higher level. I would like to acknowledge my colleagues, doctors and administrative staff, at the **Sulaymaniyah Dermatology Teaching Center (Shaeed Nabaz)** for their wonderful collaboration. I should extend gratitude and appreciation to all in **Shorsh General Hospital, Dermatology Outpatient Clinic** for their cooperation. In addition, I would like to thank **Dr. Ali Hattam** (Professor of Microbiology) for his valuable supervisor of ELISA technique. Finally, I could not have completed this research without the support of both of my colleagues, **Dr. Dashty Amin** and **Dr. Hersh Abdul** for providing PCR techniques.

ABSTRACT

Background: Vitiligo is a chronic acquired pigmentary disorder of the skin that results from immunological apoptosis of functioning melanocytes. The cytokine TNF-α plays a central role in the initiation of melanocyte apoptosis in vitiligo. Single nucleotide polymorphisms (SNP) in the promoter region of the gene coding for TNF-α may affect its production and elevation in serum.

Objectives: The aim of this study was to measure TNF-α in the serum among patients with generalized vitiligo and to investigate whether-308 G/A promoter polymorphism affects the serum TNF-α levels.

Materials and Methods: This case-control study was conducted at Dermatology Teaching Center and Shorsh General Hospital, Dermatology Outpatient Clinic**,** Sulaymaniyah, Iraq .Serum concentrations of TNF-α was measured via ELISA technique in 80 patients with generalized vitiligo and 40 clinically healthy controls. Amplification refractory mutation system polymerase chain reaction (ARMS-PCR) technique was used for detection of TNF-308G/A promoter polymorphism.Serum concentrations of TNF-α and TNF-308G/A promoter polymorphism analyzed in correlation with demographic and clinical characteristics of patients with generalized vitiligo.

Results: Statistically significant elevations of serum TNF-α concentrations were found between patients and controls (*p value 0.01*). Significantly higher serum TNF-α levels (*p value 0.02*) have been found among patients with active generalized vitiligo. Elevated serum levels of TNF-α were significantly associated with both TNFA1 (TNF-308G) allele (*p value 0.04*) and with TNFA2 (TNF-308 A) allele (*p value 0.03*). TNF-α -308GA polymorphism not affected by demographic features and clinical characteristics of patients with generalized vitiligo.

Conclusions: TNF-α in the serum is a biomarker for active generalized vitiligo**.**No difference was found between serum levels of TNF-α with TNF-α G/A promoter polymorphism that involves substituting the G allele for the A allele.

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List of Abbreviations

Abbreviations	Detail
ANOVA	Analysis of variance
ARMS-PCR	Amplification Refractory Mutation System Polymerase Chain Reaction
ASDB	Amelanotic macules with sharply demarcated borders
BACH2	Basic Leucine Zipper Transcription Factor 2 , Transcription Regulator Protein
CCR6	Chemokine receptor 6
CD	Cluster of differentiation
CI	Confidence Interval
CXCL1	The chemokine (C-X-C motif) ligand 1
ELISA	Enzyme linked Immunosorbent Assay
FOXP3	Forkhead box protein P3 (FOXP3), scurfin.
gp100	Glycoprotein 100
GZMB	Granzyme B
HPDB	Hypomelanotic with poorly defined borders
IFNγ	Interferon gamma
IL	Interleukin
IL2R -α	Interleukin-2 receptor alpha chain
JAK-STAT	The Janus kinase (JAK)-signal transducer and activator of transcription
kDa	Kilo Dalton
MC1-R	Melanocortin 1 receptor
MelanA/MART-1	Melanoma Antigen A , Melanoma Antigen Recognized by T cells
NOD-like receptor	Nucleotide-binding oligomerization domain-like receptors
PTPN22	Protein Tyrosine Phosphatase Non-Receptor Type 22
pg/mL	Picograms per millilitre

rpm	Round per minute
ROC Curve	Receiver Operating Characteristic
SD	Standard deviation
SNP	Single nucleotide polymorphism
TNF-α	Tumor necrosis factor alpha
VES	Vitiligo Extent Score
VIDA	Vitiligo Disease Activity Score

Introduction

Introduction

Vitiligo is a common acquired chronic depigmenting disorder of the skin, clinically characterized by the development of white macules or patches resulting from selective destruction of functioning melanocytes in the skin, hair, or both [1].

The global vitiligo prevalence varies from 0.2-1.8%, occurs equally in both sexes and all ethnicities, may develop at any age, although it most commonly presents in children and young adult [1, 2]. In a study sample in Baghdad – Iraq vitiligo represents the most common type of hypopigmented skin disorder [3].

Vitiligo is classified in to generalized vitiligo and localized vitiligo, generalized variant is the most common pigmentation disorder, occurring at a frequency of approximately 0.2-1.0% in different populations around the world [4] and includes subtypes of vitiligo vulgaris, acrofacial vitiligo, vitiligo universalis, and mixed forms. Localized vitiligo includes variants of focal, segmental, and mucosal vitiligo [5].

The exact etiology of vitiligo is poorly understood, it is considered as a multifactorial disease with many potential pathophysiological theories involving autoimmune, neural, autocytotoxic, biochemical, oxidative stress, melanocytorrhagy, and decreased melanocyte survival hypotheses. All of these proposed hypotheses result in the loss of melanocyte in genetically predisposed individuals. Neural theory is likely to underlie localized types like segmental and focal vitiligo, melanocytorrhagy explain the lesions caused by Koebner phenomenon. Autoimmune theory is more prominent in generalized vitiligo [6, 7, 8].

In respects to immunological background of generalized vitiligo, the reactions

are mediated by disorders in cellular immunity, humoral antibody- mediated immunity, and the action of cytokines [5].

Tumor necrosis factor alfa (TNF-α) or cachectin, a proinflammatory cytokine plays a central role in many autoimmune diseases, has been implicated in the depigmentation process in vitiligo, it is produced by activated T cells, activated macrophages melanocytes, keratinocytes, fibroblasts, adipocytes, and smooth muscle cells. TNF-α affects the apoptotic pathway of melanocytes, inhibits melanogenesis, and decreases melanocyte tyrosinase activity and melanocyte stem cell differentiation [9].

TNF-α gene is located within the class III region of the gene coding for the major histocompatibility complex (MHC) on chromosome 6 (6p21.3), several single nucleotide polymorphisms (SNPs) have been identified in its promoter region, the most common one occurs at position -308 (TNF-α- 308G/A), which may affect the production of TNF-α and its serum level. To date, several studies have been carried out to identify the potential role of TNF-α-308G/A polymorphism in vitiligo susceptibility; however, these studies have reported conflicting results [10].

There is higher expression of TNF-α in vitiligo skin, compared with healthy skin that indicate an imbalance of epidermal cytokines and localized TNF-α plays an active role in vitiligo pathogenesis by activating Cutaneous T-Lymphocytes (CTLs) within the skin, but the result of studies on the role of TNF-α serum levels in vitiligo are mixed [9, 11].

There is significant variation in allele frequencies among human populations.

TNF-α-308G/A) polymorphism is more frequent in European and Indian populations (10%-23%) and less frequent in Asian (2%-9%) and African populations (7%-11%)[12].

TNF-α-308G/A (rs1800629) polymorphism is associated with risk of vitiligo in various populations; up to our knowledge presently there is no literature on the status of this polymorphism among Iraqi populations, our study is the first one in this regard and it is anticipated that the discovery of potential genetic cause of vitiligo will provide a clue to new approaches of treatment and even prevention of this challenging disease.

Aims of this study:

1. To measure the serum TNF-α concentrations in different types of generalized vitiligo.

2. To investigate the association between generalized vitiligo and TNF-α -308 G/A promoter polymorphism.

3.Is the serum level of TNF-α was genetically controlled through its correlation with 308 G/A gene polymorphism?

4. To study the correlation of serum level of TNF-α and TNF-α -308 G/A promoter polymorphism with patient's demographic features and clinical characteristics of generalized vitiligo.

Chapter One:
Review of Literatures

1.1. Vitiligo Historical Background

Vitiligo is an ancient disease that has been mentioned in the tomes of every major religion, its first described in the earliest Hindu sacred writings ("Vedas") and in texts (Papyrus) from ancient Egypt. The Roman Physician Celsus first used the term Vitiligo in the 2nd Century A.D. with its characteristic white patches to those of a spotted calf ("vitelius") [13].

1.2 Epidemiology

Vitiligo is the most prevalent pigmentary disorder, occurs worldwide, with an incidence rate of 0.1% - 2%, irrespective of age, race, ethnic origin or skin color, the highest incidence has been recorded in India (8·8%), followed by Mexico (2·6-4%), and then Japan (1·68%), In almost half of patients with vitiligo, their disease starts before the age of 20, males and females are affected with approximately equal frequency [14, 15].

1.3 Classification

According to the distribution, pattern, extension, and number of white patches, vitiligo is classified in to two major types (Figure 1.1). Overall, >90% of vitiligo patients have vulgaris or acrofacial variant. In the remainder, localized vitiligo is more common than mixed or universal vitiligo [16, 17, 18].

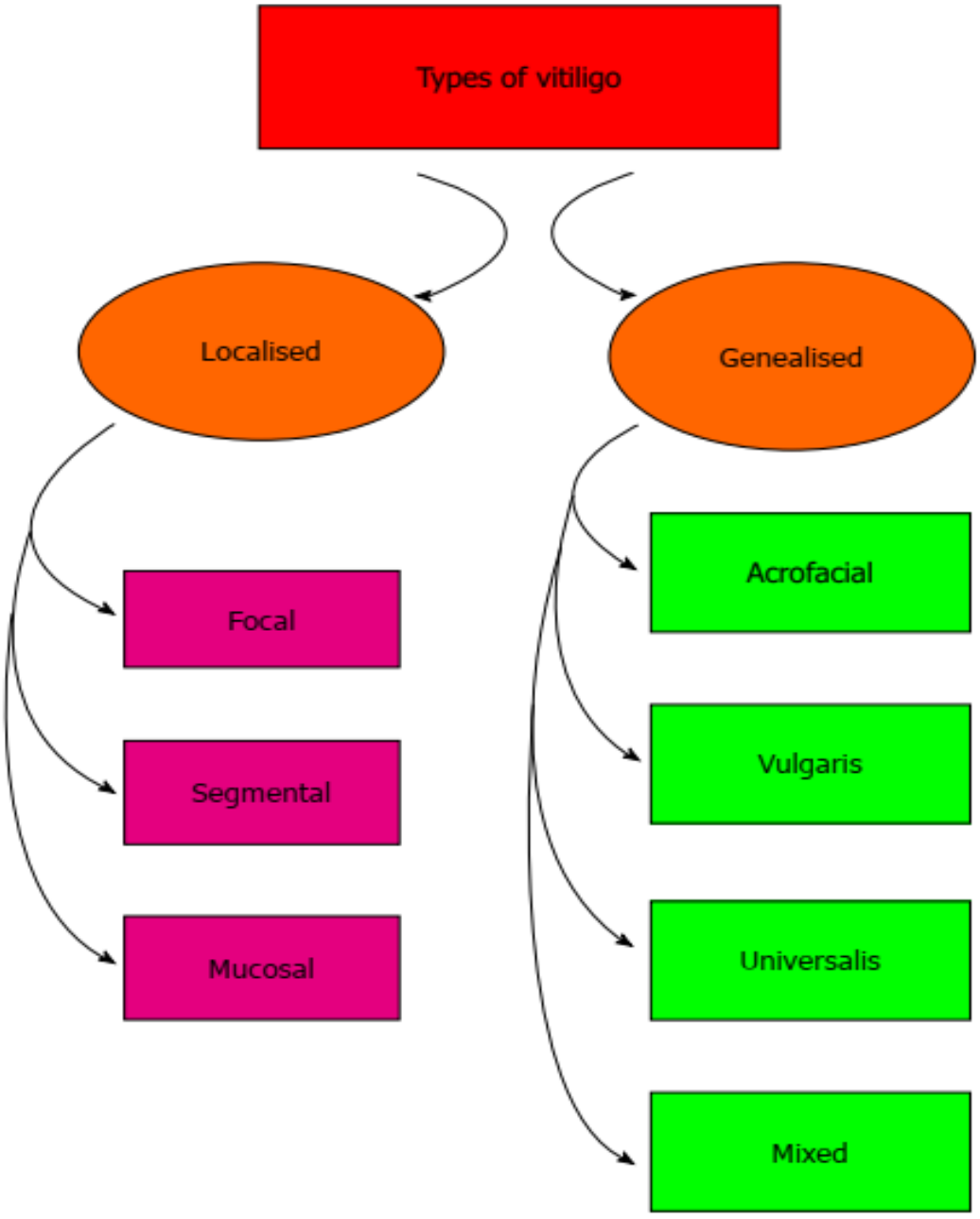


Figure (1.1) vitiligo classification [5]

1.3.1 Types of localized vitiligo

1.3.1.1. Focal vitiligo

Focal vitiligo is a localized small, isolated depigmented area without a typical segmental distribution, definitive diagnosis can be made when the lesions have not evolved in to generalized or segmental vitiligo after a period of 1-2 years, half of the cases progresses to generalized vitiligo in 2 years after disease onset. [19]

1.3.1.2. Segmental vitiligo

Segmental vitiligo accounts for 15–30% of vitiligo among pediatric age groups [16, 17], characterized by an early onset appearance, rapid progression, and unknown specific precipitating factors. The depigmentation in segmental vitiligo may follow Blaschko's lines or distributed along a dermatome with the trigeminal one most involved dermatome [20, 21]. Melanocyte mutations that occur during embryologic development, a process called somatic mosaicism is consider as one of the underling pathologies [22]. Other evidence supports neural mechanisms explaining a dermatomal distribution of the skin lesion. One unique segment is involved in most of patients and the lesion consist of one or more white macules on one side of the body, usually respecting the body midline but two or more segments on the same or opposite sides may be involved [23], the depigmentation spreads within the segment over a period of 6-24 months and then stop, further extension is rare. Involvement of body hair (leukotrichia) may be present beside rapid onset of the condition. [24, 25]

1.3.1.3. Mucosal vitiligo

Mucosal vitiligo refers to vitiligo involving lip(s) or any other mucosal structure, it's quite uncommon condition represents only 2.3% of the total cases of vitiligo, the condition can occur as an isolated phenomenon (pure mucosal vitiligo) which

is classified as undetermined vitiligo ,or it can occur in addition to cutaneous involvement in the context of generalized vitiligo .Lip involvement in vitiligo is common in pigmented races occurring in up to 50% of patients ,while in the white population underestimation of prevalence of mucosal involvement is apparently due to inability to detect hypo⁄depigmentation in the background of constitutionally pale mucosa [26, 27].

1.3.2. Types of generalized vitiligo

1.3.2.1 Vitiligo vulgaris

This type is the most common presentation of generalized vitiligo [28] characterized by milky-white macules involving multiple parts of the body, most often in a symmetrical pattern, the disease can start at any site of the body, usually increase in size with time, this corresponds with a substantial loss of functioning epidermal and, sometimes, hair follicle melanocytes, the commonest sites to be affected are the fingers and wrists, the axillae and groins and the body orifices such as the mouth, eyes, and genitalia. Skin hypopigmentation is usually asymptomatic, but a minority of patients mention preceding mild pruritus [20], Depigmentation of scars is a common manifestation of the Koebner's phenomenon in this type of vitiligo (mechanical induction of the vitiligo) that may also occur by friction or chronic pressure by clothing or daily activities [29].

13.2.2 Acrofacial vitiligo

Vitiligo of this type is characterized by bilateral, depigmented macules limited to the head and distal extremities particularly distal fingers and facial orifices in a circumferential pattern [30]. Another variant, known as lip-tip vitiligo, only affects the lips, fingers, and toes, and has been described most often in South Asians [31,

[32]. Acrofacial vitiligo can begin at any age occasionally in later life, as late as 70 years but also seen in young children ages 3 or 4 years, teenagers, young adults, and mature adults [33, 34].

1.3.2.3. Mixed vitiligo

This subtype is a recently described variant of vitiligo. There is a characteristic segmental involvement associated usually in a second step with the onset of bilateral vitiligo patches mostly among pediatric age group [35, 36]. In patients with segmental vitiligo presence of halo nevi and leukotrichia at onset may be risk factors for developing mixed vitiligo [37]. Cutaneous mosaicism harboring fragile melanocyte populations, which are susceptible to external as well as auto-inflammatory mechanisms is an underlying hypothesis in the causation of mixed vitiligo [38].

1.3.2.4 Vitiligo universalis

Vitiligo universalis is a rare form of widespread disease, usually seen in adults, although cases in children have been reported. [39] The term Universalis is generally used when depigmentation is virtually universal (80-90% of body surface involved) but some pigmentation may be still present, and hairs partially spared. Whereas this diagnosis is easy in dark-skinned individuals, it may be more challenging in fair-skinned individuals [40]. Classically, vitiligo universalis results from longstanding disease that steadily progresses to nearly complete whitening of the skin [18].

1.4 Clinical features of vitiligo

The main clinical feature of vitiligo is the presence of white macules of different shapes and sizes distributed in a symmetric or asymmetric pattern all over the body [41]. Considering exclusively the clinical appearance of the lesions under daylight and Wood's light, vitiligo lesions can be described as either totally amelanotic (milk or chalk-white) macules or patches with sharply demarcated borders (ASDB) or hypomelanotic with poorly defined borders (HPDB) [42, 43].

In patients with rapidly progressing vitiligo as the pigment cells are destroyed, sometimes a 'trichrome' appearance may occur consisting of a white center with an intermediate, pale area with a normal skin around is found. A biopsy of the border of these lesions reveals an inflammatory infiltrate and degeneration of the basal layer, which are signs of activity [42], vitiligo lesion may develop anywhere on the body, it is frequently localizes to sites that are normally relatively hyperpigmented, such as the face, dorsal aspect of the hands, nipples, axillae, umbilicus, sacral, inguinal and anogenital regions Typically, facial vitiligo occurs around the eyes and mouth (periorificial), and on the extremities it favors the elbows, knees, digits, flexor wrists, dorsal ankles and shins [16]. In vitiligo there is no surface change of the skin and usually there no redness. Depigmentation can affect mucosal areas such as in the mouth, this can be prominent in darkly pigmented people [12]. Lesions of vitiligo are hypersensitive to ultraviolet (UV) light and burn readily when exposed to the sun [43]. It is not unusual to note the onset of vitiligo after severe sunburn [44].

Patients with vitiligo are usually healthy, the disease is asymptomatic, but pruritus is occasionally noted, especially within active lesions. The depigmentation may affect melanocytes in the hair roots (leukotrichia) within the vitiliginous skin in 8.9- 45% cases, it represents a secondary involvement of the hair (usually vellus

hairs) following primary skin involvement. Leukotrichia can be present in any vitiligo subtype although most cases are associated with segmental vitiligo (SV) and related to a poor prognosis [45]. A rare variant vitiligo lesion presented with a rim of raised erythema and scaling at the periphery of the depigmented patches known as inflammatory vitiligo, pruritus and koebnerization are occasionally observed in this clinical variant [46]. An infiltrate of lymphocytes and macrophages with concomitant disappearance of melanocytes has been reported histologically in this form of vitiligo [47].

1.5 Etiology

The exact etiology of vitiligo is poorly understood and is often considered as a multifactorial disease with a complex pathogenesis consisting of several postulations including autoimmune, cytotoxic, biochemical, oxidant-antioxidant, and neural mechanisms for destruction of the functioning melanocyte in genetically predisposed individuals [6, 7, 8].

1.5.1 Vitiligo genetics

Vitiligo in concordant identical twin-pairs and among family members point to the importance of genetic factors in the development of vitiligo [48,49]. Inheritance has been suggested to be polygenic, approximately 30% of patients have a positive family history and first-grade relatives have a 6-8% risk of developing vitiligo. Genome-wide association studies have identified several susceptibility loci for generalized vitiligo [50]. Many of these genes are involved in melanogenesis, immune regulation, or apoptosis and have been associated with other pigmentary, autoimmune, or autoinflammatory disorders [51,52]. Several of these loci (HLA class I and II, PTPN22, IL2R α, GZMB, FOXP3, BACH2, CD80,

and CCR6) suggest a role for adaptive immunity, and some of these genes been associated with genetic susceptibility to other autoimmune diseases that are epidemiologically linked to generalized vitiligo such as type 1 diabetes, thyroid disease, and rheumatoid arthritis [53, 54]. In addition to a genetic aberration in the immune system in vitiligo genetic defect in the melanocyte itself have also been found, prior studies have demonstrated that the melanocytes in the skin of vitiligo patients appears fragile and can exhibit morphologic abnormalities including enlargement, fragmentation, extracellular granular material, and dilated rough endoplasmic reticulum [55].

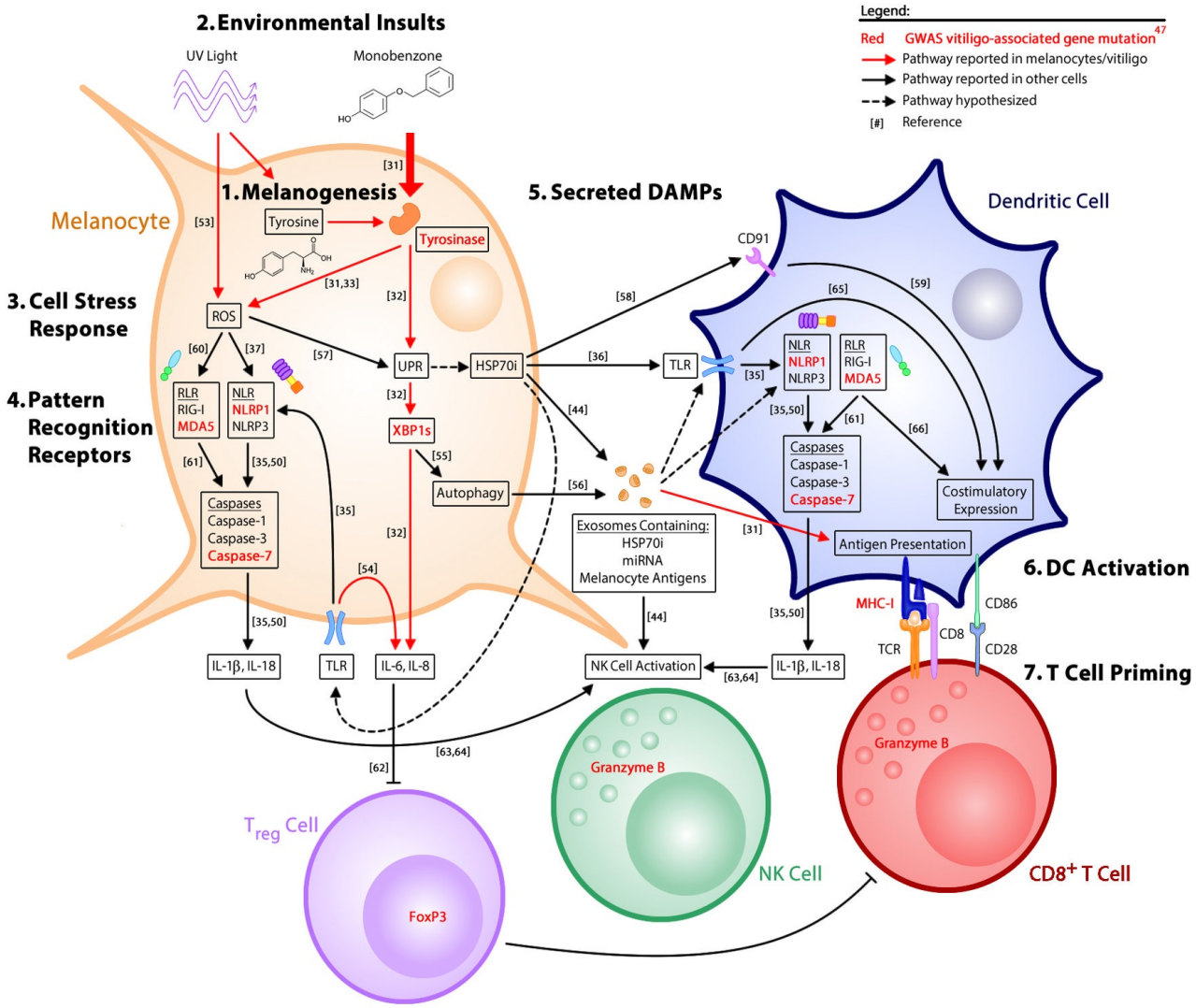
1.5.2 Autoimmune theory

Most current evidence supports an autoimmune mechanism responsible in the pathogenesis of vitiligo especially in generalized variant. Autoimmune destruction of melanocytes is mediated by innate immunity, adaptive immunity that includes cell- mediated and humoral immunity and with the action of cytokines [56].

1.5.2.1 Innate immunity

Autoimmune-mediated destruction of melanocytes and melanocyte-intrinsic abnormalities are linked to one other through innate immune mechanisms. In vitiligo, stressed melanocytes activate the innate immune system through the generation and release of damage-associated molecular patterns (DAMPs) [57 ,58], such as reactive oxygen species (ROS) and heat shock protein 70 HSP70. These molecules activate innate pattern recognition receptors (PRRs). HSP70 activate, toll-like receptors (TLR2, TLR4) [59]. NOD-like receptor (NLRP3) is activated by ROS and mitochondrial stress thus induce inflammation through innate receptors. In addition, NK-cells, macrophages, and inflammatory dendritic cells (DCs) infiltrate lesioned skin in vitiligo reflecting innate immune activation and

response during disease progression. As innate immune cells release cytokines and chemokines that promote T-cell recruitment to peripheral tissues, it may serve as a bridge between cellular stress and T cell-mediated destruction in vitiligo. S100B is also a DAMP released by damaged melanocytes. S100B levels are increased in active vitiligo and may stimulate inflammatory responses. High mobility group box 1, another DAMP, is increased in the blood of patients with vitiligo and induces melanocyte apoptosis [60] (Figure 1.2).



(Figure 1.2) Innate signaling pathways activated by cellular stress lead to adaptive autoimmune responses against melanocytes. The production of melanin results in cellular stress. Environmental insults, including UV light and chemical phenols such as monobenzone, exacerbate this response. Melanocyte stress is characterized by intracellular ROS and activation of the UPR , which are both capable of activating PRRs either directly, or through the production of HSP70i and antigen-containing exosomes .These signals function as DAMPs to activate dendritic cells and subsequent priming of $CD8^+$ T-cells for autoimmune attack of melanocytes Stressed melanocytes secrete low levels of IL-6 and IL-8, which may recruit immune populations and/or antagonize the suppressor function of regulatory T cells (Treg) [58].

1.5.2.2 Adaptive immunity

1.5.2.2.1 Cell-mediated immunity

Cell-mediated immunity in vitiligo is demonstrated by the presence of inflammatory infiltrates in perilesional vitiligo skin, there is a decrease in CD4+ to $CD8^+$ T-lymphocytes ratio in vitiligo skin compared to healthy skin. In vitiliginous skin, perilesional T-cells of the $CD8^+$ and $CD4^+$ subsets secrete predominantly type 1 cytokines, including TNF-α and IFN-γ. TNF-a plays an important role in the further development of cytotoxic T-lymphocytes (CTLs), which are implicated in disease initiation in vitiligo, and it enhances activated lymphocytic expression of interferon (IFN-γ) [61, 62, 63], that directly induces melanocyte apoptosis and inhibits melanogenesis by altering melanogenic enzyme mRNA expression [64]. $CD8^+$ T-cells specific for melanocyte antigens, such as MelanA/MART-1, gp100, and tyrosinase, have been detected in the peripheral blood and perilesional skin of patients with vitiligo. Activation of $CD8^+$ T-cells also correlates with impairment of regulatory T-cells (Tregs) and contribute to suppression of immune tolerance due to an altered proportion and/or function of effector and regulatory T-cells (Tregs). $CD4^+$ T-cells play a major role in coordinating the immune response and seem important for the generation of cytotoxic $CD8^+$ T-cells [65, 66].

Among patients with generalized vitiligo there is significantly higher serum level of IL-17 that is secreted by Th17 cells, a subset of $CD4^+$ T-cell [67], IL-17 correlate significantly positive with disease duration and with extent of body area involved compared to matching control [68, 69].

1.5.2.2. 2 Humoral immunity

Altered cellular immunity is present in vitiligo, in addition to and perhaps in

combination with a humoral immune response [70, 71]. Evidence suggests a more crucial involvement of cellular immunity in vitiligo, whereas humoral immunity probably plays a secondary part, where antibodies are produced against the fractions/antigens of melanocytes that are produced/released during the process of active melanocyte destruction by cellular immunity like tyrosinase, tyrosinase like protein 1 and 2, glycoprotein 100 (gp100) and melanoma antigen recognized by T-cells (MART-1), all are specific melanocyte antigens [72].

In active generalized vitiligo increased serum levels of certain immunologic markers including immunoglobulin G (IgG) anti-melanocyte/ vitiligo antibodies (V-IgG) are associated with augmented humoral and cellular immunity that involved in melanocyte cytotoxicity [73]. IgG anti-melanocyte antibody stimulated HLA-DR expression on melanocytes The MHC class II molecules expressed in melanocytes can present antigens to CD4 T-helper cells as antigen-presenting cells and elicit an immune response and intercellular adhesion molecule-1 expression on melanocytes was significantly induced by IgG anti-melanocyte antibodies; Intercellular adhesion molecule-1 is an important adhesion molecule involved in leukocyte and parenchymal cell interaction and plays an essential part in immunologic and inflammatory reactions and allow the antigen-specific immune effector cell attack results in melanocytotoxicity [74]. Different organ and non-organ-specific antibodies also found in the serum of patients with vitiligo including thyroid autoantibodies thyroid peroxidase antibodies (TPOAbs), thyroglobulin antibodies (TgAbs) and thyroid-stimulating hormone receptor antibodies (TSH-R Abs) and less commonly antibodies directed towards one or both thyroid hormones (THAbs), tri-iodothyronine (T3) and/or thyroxine (T4) [75].

1.5.2.3. TNF- alpha and vitiligo

Tumor necrosis factor-alpha (TNF-α) or cachectin is the prototypic member of the TNF superfamily with diverse functions in cell differentiation, inflammation, immunity, and apoptosis [76]. It is primarily secreted from activated macrophages, although it may also be secreted by other cell types including monocytes, T cells, mast cells, NK cells, keratinocytes, melanocytes, fibroblasts, and neurons TNF-α is synthesized as a transmembrane precursor protein (mTNF-α) with a molecular mass of 26 kDa [77, 78].

There are two different forms of TNF-α, soluble TNF-α (sTNF-α) and transmembrane TNF-α (mTNF-α) [79]. mTNF-α, the precursor of soluble TNF-α, can be cleaved by the action of the matrix metalloproteinase known as TNFα converting enzyme (TACE) and released as sTNF-α that circulates throughout the body and confers TNFα with its potent endocrine function, far away from the site of its synthesis [80, 81]. Various mechanisms have been postulated to explain the role of TNF-α in vitiligo pathogenesis including initiation of melanocyte apoptosis, decreased melanogenesis, inhibition of melanocyte stem cell differentiation and increased melanocyte cytotoxicity [82, 83].

TNF-α binds to, and thereby acts through, receptors TNFR1 and TNFR2, melanocytes can express both TNFR1 and TNFR2 receptors in response to stress [84]. The extracellular domains of both TNFR1 and TNFR2 are rich in cysteine and able to bind to the same TNF ligand. However, their intracellular domains are strikingly different. TNFR1 contains a cytoplasmic ‘death domain’, which is a con- served sequence of 80 amino acids that forms a distinctive fold, this death domain enables TNFR1 to recruit the adaptor molecule TNFR1-associated death domain protein (TRADD), which is a crucial component of the TNFR1 signaling complex. By contrast, TNFR2 cannot mediate apoptosis due to lack of the

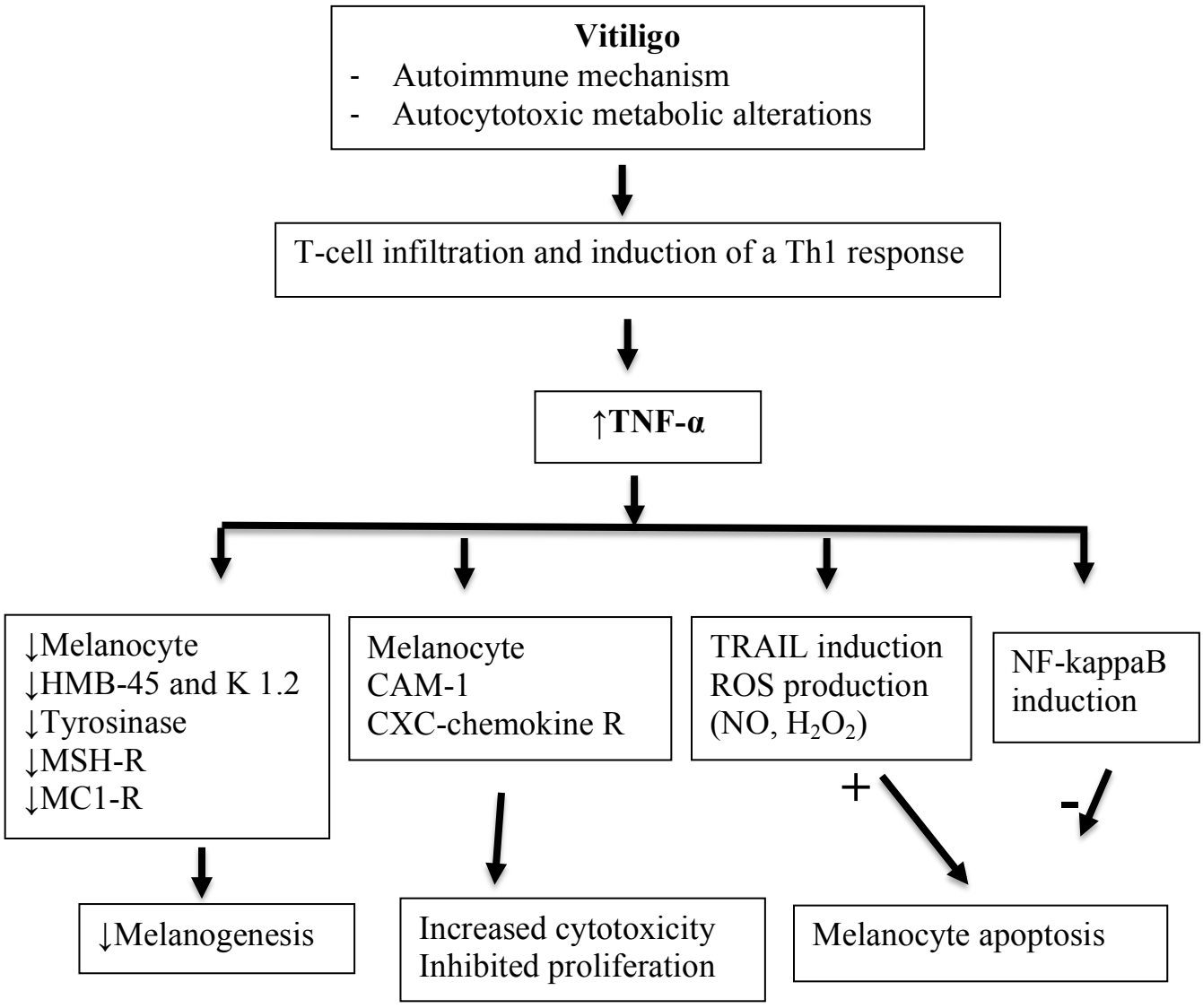
cytoplasmic death domain sequence and recruits TNFR-associated factor 1 (TRAF1) and TRAF2 rather than TRADD [85]. TNF depletion in the presence of specific TNFR2 agonists may be an ideal way to treat autoimmune disease, as TNFR2 agonism results in selective regulatory T-cell (Treg) activation [86].

1.5.2.3.1.Mechanism of TNF- α induced melanocyte apoptosis

Melanocyte function, including proliferation, differentiation and immunologic susceptibility to cytotoxicity can be altered by TNF-α [87]. Adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) are overexpressed in melanocytes from vitiligo lesions, and cytokines such as TNF-α can induce their expression on the surface of epidermal melanocytes [88, 89]. This pathway could influence melanocyte target recognition by T-cells and mediate immunologic cytotoxic damage. TNF-α can inhibit melanogenesis by decreasing the intracellular levels of tyrosinase and tyrosinase-related protein 1, an abundant melanosomal glycoprotein involved in both melanogenesis and prevention of melanocyte death [90, 91].

There is evidence as TNF-α -mediated inhibition of tyrosinase activity and melanogenesis is dependent on activation of nuclear factor (NF-κB) [92], TNF-α treated melanocytes show marked cellular shrinking and reduced melanin production in vitro, as well as down regulation of MITF, a transcription factor essential in the regulation of melanocyte development, proliferation, death, and melanogenesis [93]. TNF-α lead to a dose-dependent inhibition of melanocyte proliferation, partly through increased expression of the CXC-chemokine receptor II [94]. TNF-α also leads to reduced expression of the pigment-associated antigens Human Melanoma Black-45 (HMB-45) and K.1.2 in normal cultured melanocytes as well as to altered immunological phenotypes. Melanocyte-stimulating hormone receptor (MSH-R) and melanocortin-1 receptor (MC1-R) are known inducers of

melanogenesis, capable of inducing the expression of melanin synthase modulating pigmentation and melanocyte survival in normal and pathological conditions [95]. In vitro studies of normal human melanocytes have shown that TNF- α down regulates MSH- R binding activity and reduces the expression of MC1-R mRNA [96, 97]. TNF-α could stimulate the melanoma mitogens IL-8 and CXCL1 (melanoma growth-stimulatory activity/growth-regulated protein α), inhibit pigmentation-related signaling and melanin production, and increase the production β-defensin-3 (βD-3) an antagonist for MC1-R [98], apoptosis-sensitive melanocytes in turn show reduced melanogenesis. This suggests that melanocytes with impaired melanogenesis have altered of nuclear factor-kappa (BNF-κB) signaling that leads to susceptibility to TNF-α induced apoptosis. Impaired phosphatidylinositol 3-kinase/serine/threonine protein kinase activation followed by reduced of NF-κB not activation under increased TNF-α levels was demonstrated as a mechanism for keratinocyte apoptosis in vitiligo as well [99]. Indeed, human vitiliginous keratinocytes treated with TNF a show increased apoptosis due to an impaired phosphatidylinositol 3-kinase/protein kinase B-signaling pathway [100, 82]. (figure 1.3)



(Figure 1.2) Mechanisms of TNF-α mediated alterations in melanocyte function in vitiligo.

CXC-chemokine RII CXC- chemokine receptor II, H_2O_2 hydrogen peroxide, ICAM-1 intercellular adhesion molecule- 1, MC1-R melanocortin-1 receptor, MSH-R melanocyte-stimulating hormone receptor, NF-kappaB nuclear factor kappa B, NO nitric oxide, ROS reactive oxygen species, Th1 T-helper-1 cell, TNF-alpha tumor necrosis factor-a, TRAIL TNF-related apoptosis-inducing ligand [82].

1.5.2.3.2.TNF-alpha -308 G/A (rs1800629) promoter polymorphism

The gene encoding TNF-α is in the short arm of chromosome 6 in the major histocompatibility complex class III region. Most of the TNF-α gene polymorphisms are located in its promoter region and they are thought to affect the susceptibility and/or severity of different human diseases [(101)]. The role of TNF-α in the pathogenesis of human diseases seem to be contradictory and this was related to the genetic polymorphisms in the genes regulating its production and effect and influencing its in-vivo level [(102)]. Several polymorphisms have been identified inside the TNF-α promoter region positioned at relative to the transcription start site: −1031 (T→C), −863 (C→A), −857 (C→A), −851 (C→T), −419 (G→C), −376 (G→A), −308 (G→A), −238 (G→A), −162 (G→A), and −49 (G → A) [(103)], these genetic variations affect TNF-α protein expression. Polymorphism at -308 position guanine been reported in different autoimmune diseases resulting in the presence of two allelic features in which G is defines as the common variant, and the presence of adenine (A) define as less common. Alleles [(104)].

1.5 .3.Melanocytorragy (Adhesion defect theory)

Chronic detachment of melanocytes provoked by trauma, mainly a mechanical rubbing of healthy skin could be the cause of vitiligo, this concept is now known as “melanocytorrhagy theory”, for this reason, koebnerization in vitiligo is now considered as a migration of melanocytes through the epidermal basal cell layer of skin. Adhesion defects are involved in the disappearance of melanocytes in vitiligo lesions among patients with generalized vitiligo. Tenascin, an extracellular matrix molecule involved in adhesion, was increased in vitiligo patients, thus reducing melanocyte adhesion. Discoidin domain receptor-1

(DDR1), which is a domain implicated in the adhesion of melanocytes to the basal skin, has also been demonstrated to be reduced in vitiligo lesioned skin. In addition, vitiligo is a disease that affects not only melanocytes, but also the whole epidermis. Indeed, DDR1 expression was found to be reduced in all lesional epidermis [105, 106]. In generalized vitiligo Melanocytorragy may be the primary disorder that leads to acute loss of melanocyte (as most patients have a sudden onset of lesions), with an altered response of melanocytes to friction and possibly other types of stress, which would induce cell detachment and subsequent transepidermal loss and an autoimmune phenomenon might be triggered by antigen release and recognition of affected melanocytes by dendritic cells or memory T-cells during trans-epidermal migration, thereby exacerbating the detachment and loss of more melanocyte [107].

1.5.4. Biochemical Theory

The biochemical theory states that there is dysregulation of biopterin pathways that predisposes to melanocyte cytotoxicity and vitiligo, increased production of Pteridines (6R)-L-erythro 5,6,7,8 tetrahydrobiopterin (6BH4) and (7R)-L- erythro 5,6,7,8 tetrahydrobiopterin (7BH4) are observed in the epidermis of affected patients with vitiligo. 6BH4 is an essential cofactor for phenylalanine hydroxylase, the enzyme that converts dietary L-phenylalanine to L-tyrosine [108], its elevated level leads inhibition of phenylalanine hydroxylase leading to low levels of tyrosine and therefore, a defect in melanin synthesis. Adrenergic and cholinergic systems involved in vitiligo pathogenesis, acetylcholinesterase (AChE) is an important enzyme in promoting and maintaining oxidative stress, being inactivated in vitiligo and its activity decreased in vitiligo skin lesions during depigmentation, but it returns to normal when the damaged skin starts

Repigmentation, this could explain as mental stress facilitates the appearance of vitiligo macules by activation of the hypothalamic-pituitary-adrenal axis [109, 110].

1.5.5. Decreased melanocyte survival hypothesis

An abnormality in melanocytes or in surrounding keratinocytes in producing factors or their receptors, which are necessary for the survival or function of melanocytes within the epidermis leads to deficiency in the survival signals for melanocytes and its apoptosis. Keratinocytes contribute to melanocyte homeostasis, and keratinocyte alteration may play a role in melanocyte dysfunction in vitiligo. In particular, the release of melanogenic mediators and the level of functioning keratinocytes may affect melanocyte dysfunction in vitiligo epidermis Keratinocyte-derived stem cell factor (SCF) regulate melanocyte growth and survival by binding to membrane tyrosine kinase receptor c-Kit. The significantly decreased number of c-Kit receptors in perilesional melanocytes [111, 112] and the lower expression of stem cell factor from surrounding and in vitiliginous keratinocytes which result from keratinocyte apoptosis, might be responsible for passive melanocyte death and may explain the Koebner phenomenon [113].

1.5.6 Oxidative stress theory

In vitiliginous skin there is an imbalance in the redox (reduction-oxidation) state that results in the dramatic production of reactive oxygen species (ROS) such as Hydrogen Peroxide (H_2O_2), hydroxyl ion, superoxide, and singlet oxygen. (ROS) oxidize components of the cell leading to melanocytes destruction and creating the depigmented macules. [114] Factors involved in the redox status are selenium, malondialdehyde (MDA), vitamins A and E, glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD). Selenium is required for GPx

activity and vitamins A and E are important in antioxidant activity. Serum selenium, SOD and MDA are all prominent in both unstable and inactive types. In patients with vitiligo as a response to oxidative stress; SOD activity increased which transforms O_2- to H_2O_2 and O_2, thus H_2O_2 accumulates [115, 116]. GPx detoxifies H_2O_2 (downstream enzyme) and catalase metabolize the ROS, expression and level of these enzymes are reduced in generalized vitiligo leading to increase melanocyte sensitivity to oxidative stress is enhanced [117].

1.5.7.Neural Theory

Dysfunction of sympathetic nervous system's activity affect melanin production and lead to depigmentation [118], this explained by the role of neuropeptide and neuronal markers such as Neuropeptide Y (NPY), calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP), and polyclonal general neuronal marker (PGP). Precipitating factors, as, stress, produce significant level of NPY that found to be increased in the marginal areas of vitiligionus skin lesions in half of the patients compared to normal skin, and associated with noradrenaline release with exerting a local autonomic effect [119, 120].

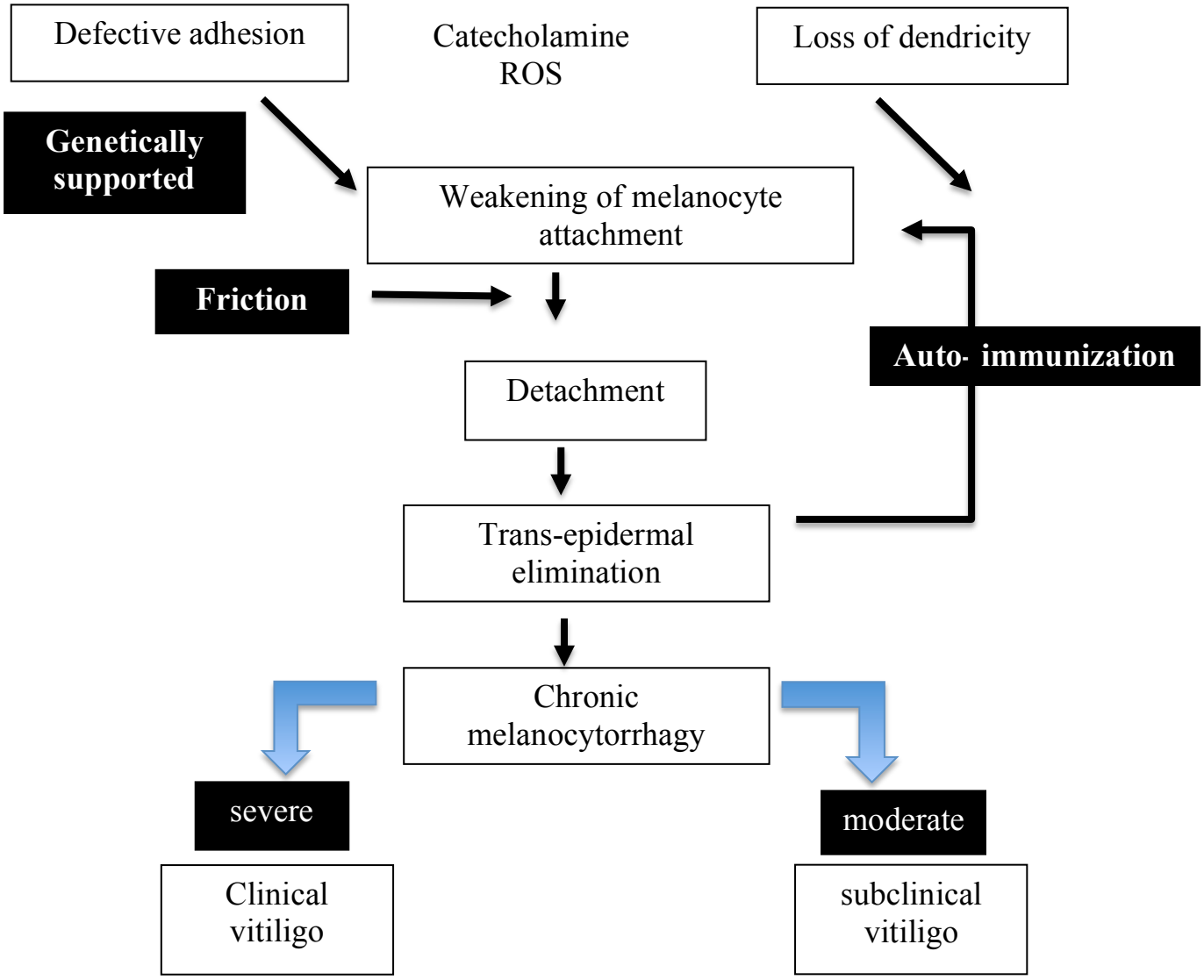
1.5.8.Autocytotoxic theory

Intracellular toxic metabolites such as those formed during melanin synthesis accumulate and bringing about autocytotoxic injury to the melanocytes in genetically susceptible individuals. Tyrosine upon entering the melanogenic pathways produces certain electrically unstable by-products, which have the potential to damage other cellular substrates resulting in death of the melanocytes. [121] High mobility group box 1 (HMGB1) is a non-histone deoxyribonucleic acid (DNA)-binding protein that is secreted from the keratinocytes following exposure to reactive oxygen species (ROS) or ultraviolet B (UVB) light. Patients with

active vitiligo showed greater expression of High mobility group box 1 (HMGB1) in vitiliginous skin and significantly higher blood levels of HMGB1 compared to healthy controls, HMGB1 could initiate autocytotoxic injury to the melanocytes by increasing the expression of cleaved caspase 3 and decreased melanin production as well as the expression of melanogenesis- related molecules leading to apoptosis and disappearance of the melanocyte [122].

1.5.9.Convergence theory

This theory postulate that vitiligo may represent a syndrome with a multi-factorial etiology rather than a single entity (123) and it is the result of the convergence of all the mentioned theories, namely genetic, neurohumoral, autocytoxic, auto-immunity, melanocytorrhagy, altered cellular environment and impaired melanocyte migration, each contribute to the pathogenesis of vitiligo and none are mutually exclusive [124, 125, 126]. (figure 1.4)



(Figure 1.4): Proposal of a new integrated theory. Neural and impaired redox status theories are compatible with soluble factor release in the epidermal melanin unit microenvironment increasing the detachment of melanocytes . [107].

1.6.Role of environmental factors in vitiligo

Several triggering factors may be involved in the onset of vitiligo and appearance of the first symptom of the disease or aggravating the preexisting symptoms, so far, the specific environmental factor causing vitiligo among genetically predisposed patients remains unknown [(127)]. Recognition of these triggering factors would be fundamental to limit the incidence and progression of vitiligo [(128)]. Physical trauma and emotional stress lead to the production of cytokines, nerve growth factor, neurotrophils, adrenocorticotrophic hormone "ACTH" and endorphins. Major illness, surgical operations, accidents, puberty, estrogens upregulation during pregnancy, malnutrition, major infections, dietary imbalance, psycho-social insecurity/shocks, intercurrent infections and repeated antibiotic-intake, UVR overexposure and sunburns. Chemical factors such as thiols, phenols, catechol, mercaptoamines, quinones and their derivatives, all these factors trigger facultative melanin synthesis by melanocytes and enhanced facultative melanization could put undue intolerable stress on the vitiligo melanocyte resulting from an elevation in the cytotoxic oxidative melanin intermediates [(129)].

1.7.Assessment methods in vitiligo

1.7.1.Measurement of extent of vitiligo

There is no standardized method for measuring vitiligo lesions [(130)]. A quantitative parametric score, named the Vitiligo Area Scoring Index (VASI) was introduced in 2004, VASI score is conceptually derived from the Psoriasis Area and Severity Index score used in psoriasis. The patient's body is divided into five separate regions. the hands, upper extremities (excluding the hands), trunk, lower extremities (excluding the feet) and the feet. The axillary and inguinal regions are

included in the upper and lower extremities, respectively, whereas the buttocks are included in the lower extremities. The face and neck areas can be assessed separately but are not included in the overall evaluation. For each body region, the VASI is determined as the product of the vitiligo area in hands units (One hand unit of the patient which encompasses the palm plus the volar surface of all the digits, is approximately 1% of the total body surface area is used as a guide to estimate the baseline percentage of vitiligo involvement of each body region set at 1% per unit) and the extent of depigmentation within each hand unit-measured patch (possible values of 0, 10, 25, 50, 75, 90 or 100%, which are illustrated with a descriptive atlas of patient photographs). The VASI, applied to the whole body, is calculated using the following formula, which considers the contributions of all body regions (possible range: 0–100):

$$VASI = \Sigma_{(\text{all body size})} (\text{hand units}) \cdot (\text{depigmentation}).$$

VASI has a strong subjective component, because it involves the physician deciding both the amount of pigmentation and the area of involvement [131].

Then in 2007 The Vitiligo European Task Force score (VETF) has been introduced that assesses three dimensions of the disease in five areas, namely extent of vitiligo (the rule of nines), depigmentation severity grading (“staging”), and disease progression (“spreading”). The ‘rule of nine’ assumes that the head/ neck, each arm, each leg and the four trunk quadrants each compose 9% of the total body surface area, leaving 1% for the genitalia.

In the ‘flat hand = 1%’ method, a flat hand represents 1% of the total body surface area. Both methods are subjective and are based on visual assessments. Staging is based on cutaneous and hair pigmentation in vitiligo patches, and the disease is

staged from 0 to 3 (revised version) based on the largest macule in each body region except the hands and feet, which are assessed separately and globally as one unique area. The assessment of spreading is based on Wood's lamp examination of the largest macule in each body area. Wood's lamp is useful for the combined assessment of staging and spreading in a selected area.

With VETF score there is a moderate correlation for staging and spreading. An explanation could be that assessment of spreading and staging is based on the examination of a target lesion, the largest one in each graded body area. This could cause a discrepancy among observers if different lesions on the same body area do not show similar changes in spreading and staging [(132)].

Vitiligo Extent Score (VES) is another tool that used to measure the global extent of vitiligo, this is an objective, noninvasive method for measuring the extent of the disease and monitoring hyper- or hypopigmented skin compared with the normal skin. The Vitiligo Extent Score is a reliable tool to assess the global extent of vitiligo. This measurement instrument shows the important advantage of being fast, intuitive, and user-friendly. The first aim of this score is to assess the extent of vitiligo. The group drew clinical pictures for different body areas that reflect different degrees of involvement. The validated VES instrument is based on clinical pictures that mimic the natural distribution of vitiligo (nonsegmental) including 19 different body areas reflecting 6 different degrees of involvement (1%, 5%, 10%, 25%, 50%, 75%). The purpose of this tool is to select the most representing pictures from the scoring sheet Users choose the pictures that best represent the patient's skin lesions, getting finally the total extent of the disease. If a patient's lesions differ from the pictures, users can adjust the score, using different options, introducing a certain amount of subjectivity [(133)]. The score was strongly validated in different sessions involving assessors with different levels of

experience in vitiligo management, patients (during live scoring session), and clinical pictures. The reliability of this tool was compared with the VASI score. First, the affected body surface area values were better estimated with the VES than the VASI. this score seems to show a better capacity to detect small clinical changes compared with the VASI, which will be of great utility in clinic. Finally, the VES offers the advantage of being user-friendly, intuitive, and fast to use for clinicians in everyday practice [134]. In association with the international Vitiligo Score Working Group a simplified version of the validated VES tool was developed as a self-administered tool for the patients called (SA-VES). In the SA-VES the number of body areas to score is reduced to 12 and no sub-scoring options are included, as in the physician's version. The total and final SA-VES score is the sum of measurements from all areas, which can be calculated by using a converting table, like the VES [133].

1.7.2.Measurement of activity of vitiligo

The evolution of vitiligo is not easy to predict; however, two states can be described, the active state and the stable state [135], determining the status of vitiligo includes history taking on the onset of new lesions and on the extension of pre-existing lesions from 1-2 years [136,137] presence or absence of Koebner phenomenon and high Vitiligo Disease Activity Score (VIDA score) [138].

Vitiligo disease activity score (VIDA Score) is a six-point scale for assessing vitiligo stability. It depends on patient's own opinion of the present disease activity over time. Active vitiligo involves either the expansion of existing lesions or the appearance of new lesions. Grading is as follows:

VIDA Score +4 – activity lasting 6 weeks or less; +3 – activity lasting 6 weeks to 3 months; +2 – activity lasting 3–6 months; +1 – activity lasting 6–12 months; 0 – stable for 1 year or more; and – 1 – stable with spontaneous repigmentation for 1

year or more. A low VIDA score indicates less vitiligo activity [139].

Clinical and Wood's light examination of the individual lesion describe stable vitiligo as amelanotic macule with sharply demarcated borders (ASDB) active lesions as hypomelanotic with poorly defined borders (HPDB).ASDB clinical type corresponds to weak or absent inflammatory reaction at the histologic level, whereas active vitiligo lesions were significantly characterized with the HPDB type along with the presence of an evident lymphocyte infiltration at the edge both in the dermis and epidermis. Immunohistochemical staining identified clustered $CD8^+$ T-lymphocytes (CD8) in epidermis and dermis in active vitiligo with rare or isolated $CD8^+$ T-lymphocytes (CD8) in dermis and epidermis as in normal appearing skin in stable vitiligo lesions [42, 43].

1.8. Management of vitiligo

1.8.1 Diagnosis

Diagnostic criteria are mainly clinical, based on the findings of acquired, well-demarcated white lesions on the skin, with no associated inflammation that tend to enlarge centrifugally [140], often the diagnosis aided by a thorough history and examination. Wood's lamp examination of vitiligo reveals a chalky white enhancement of depigmented skin and helps to confirm the diagnosis and extent of disease particularly in fair-skinned individuals, rarely histopathologic examination may be necessary to confirm the diagnosis [141].

1.8.2.Differential diagnoses for vitiligo

Vitiligo should be differentiated form other skin hypopigmentation (Table 1.1) [142]

Table 1.1 Differential diagnoses for vitiligo

Pityriasis versicolor
Idiopathic guttate hypomelanosis
Tuberous sclerosis
Piebaldism
Discoid or systemic lupus erythematosus
Sarcoidosis
Scleroderma
Post-inflammatory hypopigmentation/depigmentation: e.g., atopicdermatitis, psoriasis
Post-traumatic depigmentation (e.g. scars, burns)
Progressive macular hypomelanosis
Cutaneous T-cell lymphoma Leprosy
Pityriasis alba
Melasma
Naevus anemicus, naevus depigmentosus, halo naevus
Leukoderma of variable causes: for example post-traumatic, melanoma associated leukoderma
Amelanotic melanoma
Lichen sclerosus et atrophicus
Topical or drug-induced depigmentation.

1.8.3.General measures

1.8.3.1.Avoiding koebnerizing factors

Friction and trauma can help to limit the triggering factors that develop new depigmentations. A good sun protection with sunscreens or clothing prevents sunburn on depigmented areas and help to diminish the bothersome enhanced contrast between depigmented and tanned pigmented skin, which occurs during summer, however, both spontaneous and treatment-induced repigmentation are mostly observed during summer periods on ultraviolet (UV)-exposed areas. This indicates that a moderate UV exposure could be a useful strategy to induce repigmentation in vitiligo [(143)].

1.8.3.2.Camouflage

Camouflage does not treat the underlying disease and will not induce repigmentation but it is offered as conservative therapy for patients who do not wish to have treatment. Cosmetic and tanning products hides the patches of depigmented skin, improve the patients' self-confidence and quality of life [(144)].

1.8.3.3.Psychotherapy

Vitiligo can be very distressing because of its chronic course, cosmetic disfigurement, and perceived stigma, treating psychological manifestations of the disease might not only help patients' overall sense of well-being, but may improve clinical outcomes. A one hour of weekly cognitive behavior therapy and coping skills training profoundly improved quality of life, self-esteem, and perceived body image. In addition, the evaluation of photographs taken pre- and post-treatment suggested that it may affect disease progression. While these results are preliminary and need to be validated, they do support the impact of psychological stress on physical disease [(145)].

1.8.4.Treatment strategies

Treatment of vitiligo based on the clinical classification and disease characteristics; it comprises of two strategies. The first involves arresting the progression of active disease (to provide stability) to limit the area involved by depigmentation. The second strategy aims at repigmentation of the depigmented area. It is also important to maintain the disease in a stable phase and to prevent relapse [146], treatment options include: (1) Stabilization Treatments (2) Stabilizing and Repigmenting Treatments (3) Repigmenting Treatments. (4). Preventing Vitiligo Relapse (5) Depigmentation

1.8.4.1.Stabilization Treatments

The initial therapy must be directed to arrest disease progression, stabilization therapies help in halting the progression of ongoing active disease and are usually accompanied by minimal or significant degrees of repigmentation [146,147,148].

1.8.4.1.1.Topical corticosteroids

Topical corticosteroids are the mainstay of treatment for localized disease and have been shown to have a reservoir effect that lasts for around five days [149] the anti-inflammatory effects decrease the disease progression and repigmentation can be observed also specially within the recent lesions and lesions in the face and neck while the trunk, extremities, and especially the hands display usually only limited repigmentation [150, 151]. The efficacy between potent and ultra-potent corticosteroids seems to be similar. Side effects include skin atrophy, telangiectasia, and striae, which are rare if a discontinuous treatment scheme is used (e.g., 15 days of application per month). In the case of the occurrence of acneiform eruptions (especially in the face), a switch to topical immunomodulators may be advisable. Treatment with potent topical

corticosteroids is often continued for at least 6 months to monitor its efficacy [152].

1.8.4.1.2.Topical immunomodulators

Tacrolimus and pimecrolimus are calcineurin inhibitors attenuating T-cell activity and decreasing the production of proinflammatory cytokines. Overall, results of topical immunomodulators are comparable to corticosteroids with the most repigmentation in the face also, while the results are moderate at other sites of the body [153]. Twice-daily application of tacrolimus has been shown to be more effective compared with once-daily application [154]. Most common side effects are burning sensation, pruritus, and erythema, Other adverse events, including verruca, dysesthesia, and contact dermatitis, were rarely reported. All the events were transient and did not require additional treatment or discontinuation of treatment [155].

1.8.4.1.3.Systemic steroids

Low‑dose oral prednisolone (0.3 mg /kg) led to stability as well as repigmentation in active vitiligo patients, with minimal side effects [156], treatment administered at 0.3 mg /kg for two months, half of the initial dose for the third month, and again halved for the fourth and fifth months. To minimize the side effects with daily steroid intake, oral mini pulse (OMP) therapy is used, an effective treatment modality to arrest the progression of unstable vitiligo vulgaris and induce repigmentation. Extensive and/or fast- spreading vitiligo were treated with 5 mg betamethasone/dexamethasone as a single oral dose after breakfast on 2 consecutive days per week. The response to treatment was evaluated by photographs taken every 2-4 months with recording of the side effects [146].

1.8.4.1.4.Methotrexate (MTX)

In a recent study, MTX at a dose of 10 mg per week with folic acid 2.5 mg a day

prior to and on the day after the MTX was found to be as effective as low- dose dexamethasone OMP, and can be used in patients with active vitiligo, whenever corticosteroids are contraindicated [157].

1.8.4.1.5. Minocycline

Minocycline acts as anti-inflammatory, immunomodulatory, and free radical scavenging in addition to their antimicrobial effects. Minocycline has been shown to protect melanocytes against hydrogen peroxide-induced death in vitro, in patient with gradually progressive vitiligo Minocycline 100mg once daily for 4 weeks arrest further depigmentation [158].

1.8.4.1.6.Treatment with biologics

In patients with vitiligo increased levels of TNF-α in lesioned and peri- lesioned skin, has been demonstrated, blocking TNF-α may reduce melanocyte destruction and promote melanocyte stem cell differentiation [159]. Infliximab, adalimumab and etanercept are all used in vitiligo with conflicting results [144].

1.8.4.2.Stabilizing and repigmenting treatments

There are few therapies that both stabilize the disease and cause successful repigmentation. The foremost of these include phototherapeutic modalities ultra-violet radiation has maximum potential to cause melanocyte differentiation [160].

1.8.4.2.1.Phototherapy

Phototherapy has been used as mainstay of vitiligo therapy for several decades that can both stabilize vitiligo and cause successful repigmentation. Variety of wave lengths and modalities are available; including psoralen plus long-wave (320-340nm) UVA radiation (Photochemotherapy or PUVA), khellin plus long-wave (320-340nm) UVA radiation (KUVA), L-phenylalanine (L-phe) combines

the use L-phe with UVA (PAUVA), 311nm narrow band UVB (NB-UVB) phototherapy, and laser therapy (targeted phototherapy) including: 308 nm excimer laser [161]. Both PUVA and narrow- band UV-B (NBUVB) therapy, constitutes the principle treatment modality for generalized vitiligo, to induce stability it takes around 3.5 months as per vitiligo disease activity (VIDA) scoring (VIDA score 0) [162], NB-UVB is also considered an effective modality for repigmentation that maintained stable even after 4 years of phototherapy [163].

1.8.4.3.Regimentation therapies

1.8.4.3.1.Laser therapy (targeted phototherapy)

Excimer laser therapy monochromatic excimer laser (MEL) 308 nm is the most popular targeted phototherapy used for treatment of localized vitiligo, MEL is used 1-3 times weekly for an initial course of 12 weeks [164] with less total body irradiation, and less side effects on normal skin. The 308-nm excimer laser has similar biological and clinical effects with NB-UVB; and has the advantage of enabling the treatment of small, non-accessible or resistant areas when compared to ordinary phototherapies [165].

1.8.4.3.2.Surgical treatments

Surgical treatment should be reserved for stable recalcitrant lesions, special care must be taken to evaluate for koebnerization, which could worsen the disease after the surgery, it is an excellent option for patients who are unable to achieve cosmetically pleasing results with nonsurgical methods. Surgical treatment options consist of tissue grafts (full-thickness punch, split-thickness, and suction-blister grafts) and cellular grafts (cultured melanocytes and non-cultured epidermal cellular grafts), adverse outcomes include scarring, graft failure,

koebnerization, infection, cobble- stoning, and variegated pigmentation [166, 167]. Various other techniques, including dermabrasion, fractional laser surgery, 5-fluorouracil application, and chemical peels have been found to improve melanocyte migration, which in turn hastens repigmentation; however, these techniques are not commonly used except in rare circumstances [168, 169].

1.8.4.4.Preventing vitiligo relapse

Maintenance treatment indicated in patients who already had relapses after having achieved repigmentation and in active vitiligo, twice-weekly applications of 0.1% of tacrolimus are effective for decreasing vitiligo relapse and according to the data available in atopic dermatitis and the comparable efficacy of topical steroids and tacrolimus for treating vitiligo topical steroids could also be effective for preventing vitiligo relapse [170].

1.8.4.5.Depigmentation

In patients with extensive vitiligo body surface area (50-60%) or disfiguring refractory vitiligo on the face or the hands, depigmentation of the remaining islands of normal skin can be considered with bleaching creams (monobenzone ethyl ester) such as monobenzyl ether of hydroquinone (MBEH), monomethyl ether of hydroquinone, or with the use of 88% phenol, laser, and cryotherapy.

Monobenzone ethyl ester requires a relatively long duration of treatment and a 5- to 12-month period can be necessary to achieve satisfactory depigmentation which is almost always causes irreversible, cryotherapy and lasers such as Q-switch ruby laser that has been extensively used for depigmentation in vitiligo universalis and Q-switch Alexandrite multiple sessions are required. [171] Repigmentation of previously depigmented areas can occur regardless of the used method. Therefore, strict sun protection during and after depigmentation

procedures is advised to limit the chance of repigmentation [172].

1.8.5.Future Perspectives

1.8.5.1.The Janus kinase signal transducer and activator of transcription (JAK-STAT) pathway inhibitors

Melanogenesis directly inhibited by IFN--ϒ signaling through JAK-STAT pathway, recent studies shown that JAK-STAT inhibitors have promising results in the treatment of vitiligo, including successful repigmentation outcomes, these molecules have been tried in both topical and systemic formulations. Pan JAK inhibitor, Tofacitinib and JAK-1,2 inhibitor, Ruxolitinib, have been found successful in causing repigmentation in vitiligo [173].

1.8.5.2.Topical prostaglandin E2

Prostaglandin E2 can stimulate the proliferation of melanocytes and melanogenesis, twice-daily application of topical prostaglandin E2 used in the treatment of localized and stable vitiligo, the results are potentially interesting because the mechanism of action of prostaglandins probably differs from the current therapeutic approaches and may be combined with them to enhance the repigmentation rate [160].

1.8.5.3.Afamelanotide

Afamelanotide is a melanocortin-1 receptor agonist, monthly administration of afamelanotide, by subcutaneous implants, was combined with UVB (repigmentation compared with UVB alone provides a promising results .The most frequent side effects were nausea and fatigue and better results were obtained, in dark-skinned patients. The potent tanning of the non-lesional skin is also a limitation in fair- skinned patients because it increases the contrast between

healthy and lesional skin [160].

1.8.5.4.Gene therapy

Gene therapy is a potential treatment in the future, with research into the pathophysiology of vitiligo and the methods and effectiveness of gene therapy and stem cell treatment [174] and in the future, vaccines targeting specific components of the immune system such as heat shock proteins may be effective in preventing or halting disease [175]

Chapter Two:
Materials and Methods

2. Materials and methods

2.1 study design and setting

This study is a case-control study, includes 80 patients with different types of generalized vitiligo and 40 clinically healthy controls. Study groups were collected from **Dermatology Teaching Center** in Sulaymaniyah city (this center is the sole teaching center dedicated to managing all dermatological venereological disorders and receives patients refereed from all hospitals and health centers), another set of patients was from the **Dermatology out-patient clinic at Shorsh General Hospital**. Cases that later enrolled into this study starts from 1st of April 2018 until 30th November 2019.

2.2.1 Patient inclusion criteria

1. The current study included all patients presented with different types of generalized vitiligo that are:

(a). Vitiligo Vulgaris (b). Acrofacial vitiligo (c). Mixed type of vitiligo

(d). Vitiligo universalis.

Both sexes and all age groups were included.

2.2.2 Patient exclusion criteria

1. Subtypes of localized vitiligo excluded from the study, such as:

(a). Focal vitiligo (b). Segmental vitiligo (c). Mucosal vitiligo.

2. Patients with generalized vitiligo on Aspirin treatment [176].

3. Patients with generalized vitiligo in associated with thyroid dysfunction [177].

4. Patients with generalized vitiligo in association with diabetes mellitus, autoimmune thyroiditis, pernicious anemia, and Addison's disease [7].

2.3 Sampling collection method

In the current study eighty patients with different types of generalized vitiligo, with forty healthy controls were enrolled. Both, lingual and written informed consent have been taken from every participant. A designed questionnaire was used for study groups. Detail history was taken from each patients, thorough physical examination and laboratory investigation were carried out (Appendix 2) The study was approved by the Research Ethics Committee of the College of Medicine, University of Sulaimani.

Diagnosis of the subtypes of generalized vitiligo was made clinically, with the aid of Wood's light (Kuangta, 1013, Taiwan) extent and clinical phenotypes of vitiligo lesions were detected. Vitiligo Extent Score (VES) was used to measure skin surface involvements, for each patient a group of clinical pictures that mimic the natural distribution of vitiligo including 19 different body areas reflecting 6 different degrees of involvement (1%, 5%, 10%, 25%, 50%, 75%) was used [133]. (appendix 3)

Vitiligo Disease Activity Score, a six-point scale for assessing vitiligo stability over time was used, depending on patient's own reports of disease activity. active vitiligo involves either the expansion of existing lesions or the appearance of new lesions.

VIDA Score +4 (activity lasting 6 weeks or less); +3 (activity lasting 6 weeks to 3 months); +2 (activity lasting 3 to 6 months); +1 (activity lasting 6 to 12 months); 0 (stable for 1 year or more); and –1 (stable with spontaneous repigmentation for 1 year or more). A low VIDA score indicates less vitiligo activity [139]. Based of history and clinical examination kobner phenomenon was detected,40 healthy controls were selected among whom attended Dermatology teaching center for other skin disorders. None of the controls or their first-degree

relatives had vitiligo and any other autoimmune diseases.

A baseline 10 milliliters of venous blood were collected from both study groups under complete sterile condition. Five milliliters of the venous blood centrifuged at 400 rpm to obtain the serum and stored at -80°C until the time of cytokine estimation. The remaining 5 milliliters of the blood stored in the anticoagulant Ethylene Diamine Tetra Acetic acid (EDTA) tubes and used for Deoxyribonucleic Acid (DNA) extraction.

In this study the following laboratory tests were done for both patients and control groups:

1. Estimation of serum level of TNF-α.
2. Detection of TNF-α -308G /A gene polymorphism

2.3.1.Estimation of serum level of TNF-α.

Serum levels of TNF-α in patients with generalized vitiligo and healthy controls were measured by Enzyme Linked Immunosorbent Assay (ELISA) technique (Biotek ELx800, Agilent Technologies, USA). Enzyme Immunoassay for the quantitative determination of Human Tumor Necrosis Factor α (TNF-α) kit in serum (DRG Instruments GmbH, Germany) was used as per the following protocol:

The DRG TNF-ELISA is a solid phase Enzyme Amplified Sensitivity Immunoassay performed on microtiterplate; the assay uses monoclonal antibodies (MAbs) directed against distinct epitopes of TNF. Standards and samples react with the capture monoclonal antibody (MAb 1) coated on microtiter well and with a monoclonal antibody (MAb 2) labelled with horseradish peroxidase (HRP). After an incubation period allowing the formation of a sandwich: coated MAb1-human TNF-MAb2-HRP, the microtiterplate was washed to remove unbound enzyme labelled antibody.

Bound enzyme-labelled antibody was measured through a chromogenic reaction. Chromogenic solution (TMB) is added and incubated.

The reaction was stopped with the addition of Stop Solution and the microtiterplate is then read at the appropriate wavelength. The amount of substrate turnover was determined colourimetrically by measuring the absorbance, which is proportional to the TNF-α concentration. A calibration curve was plotted and TNF-α concentration in samples was determined by interpolation from the calibration curve and expressed as picograms per millilitre (pg/mL).

2.3.2 Detection of TNF-α - 308G /A promoter polymorphism

A single base polymorphism within the promoter of the gene for TNF-α (SNP -308G>A) (rs1800629) results in 2 Allelic forms, in one Guanine defines the common allele (TNFA1) and other Guanine is substituted by Adenosine forms the rarer allele (TNFA 2) at position -308. This Polymorphism results in differential binding of nuclear factors and increase inducible level of TNF-α gene transcription that stimulate TNF-α production in in vitro and vivo [178].

In the current study TNF-308G /A gene polymorphism has been detected based on Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS–PCR) technique, according to the following steps:

Step one: Deoxyribonucleic Acid (DNA) extraction.

Step two:Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS–PCR)

2.3.2.1 Step one: deoxyribonucleic Acid (DNA) extraction.

Genomic DNA extraction kit (**RIBO-prep; AmpliSens, Moscow, Russia**) was used to extract DNA from fresh whole blood samples, according to the manufacturer protocol: From the blood samples 100µl was added to 1.5ml disposable tube that contains 300µl solution for lysis (contains a chaotropic agent guanidine thiocyanate which lyses cells and denatures cell proteins after warming at temperature 60-65°C until the crystals disappear). All tubes tightly closed and properly labeled. The contents of the tubes mixed thoroughly by vortexing, then centrifuged for 5min to assure no drops on the cap, incubation was done at 65°C for 5min. 400µl of Solution for Precipitation that contain isopropanol added with mixing by vortexing and Centrifuging for 5min at 13,000 rpm were done. Careful removal of the supernatant done without disturbing the pellet using a vacuum aspirator using 200µl tips. Later, 500µl of (washing solution 3) added to each tube, tightly closed, and turn them carefully upside down 3-5 times to wash the pellet followed by Centrifuging at 13,000 rpm for 1-2min then the supernatant carefully removed using vacuum aspirator and 10µl tips. 200µl of (washing solution 4) added to each tube that were tightly closed and turned upside down and back 3-5 times to wash the pellet followed by centrifugation 13,000 rpm for 1-2min and removal of the supernatant without disturbing the pellet using a vacuum aspirator and 10-µl tips. All the tubes were incubated with open caps at 65°C for 5min (to dry the pellet). Finally, 50µl of RNA buffer was added into each tube and Mixed by vortexing, then incubation done at 65 °C for 5min with centrifuging the tubes at 13,000 rpm for 1min. Supernatant carefully removed contains purified DNA. Isolated DNA checked by UV spectrophotometry absorbency at 260 and 280 nm (**Eppendorf Biophotometer, Germany**). Isolated DNA stored at 4°C until use.

2.3.2.2 Step two: ARMS–PCR

2.3.2.2.1 Polymerase Chain Reaction (PCR)

PCR was developed in 1984 by the American biochemist, Kary Mullis, it is a scientific technique in molecular biology used to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence [179]. PCR is a sensitive assay, can be performed using only a trace amounts of DNA source from a variety of tissues and organisms, including peripheral blood, skin, hair, saliva, and microbes to generate enough copies DNA to be analyzed using conventional laboratory methods.

PCR is carried out in a reaction mixture which comprises:

(i) The DNA extract (Template DNA) that is to be detected and amplified.

(ii) Taq polymerase, the key enzyme that links individual nucleotides together to form the PCR product, these enzymes are thermostable, they are resistant to denaturation by heat treatment.

(iii) Primers: these are short DNA fragments with a defined sequence complementary to the target DNA, they specify the exact DNA fragment to be amplified and serve as an extension point for the DNA polymerase to build on.

(iv) Four-deoxyribonucleoside triphosphates (dNTPs) or Nucleotides: includes the four bases – Adenine, Thymine, Cytosine, and Guanine (A, T, C, G) – that are found in DNA. These act as the building blocks that are used by the DNA polymerase to create the resultant PCR product.

These components more than buffer solution are all mixed in a test tube or 96-well plate and then placed in a machine (Thermal cycler) that has thermal block with holes, into which the test tubes or plates holding the PCR reaction mixture

are inserted. The machine raises and lowers the temperature in discrete, precise, and pre-programmed steps, this allowed repeated cycles of DNA amplification to occur in three basic steps with the Initial denaturation step 95°C for 5min (done just once at the beginning of the reaction) [179]. In the first step (Denaturation step) the mixture is heated to 94°C for 30-60seconds, at which temperature the hydrogen bonds holding together the two polynucleotides of the double helix are broken, so that the target DNA becomes denatured into single-stranded molecules. The temperature is then reduced in the second step (Annealing step) to 50-60°C for 30-45seconds, that allowed the primers to attach to their annealing positions. DNA synthesis can now begin, and the temperature is raised to 74°C (Extension step) for 1-2 min. In this first cycle of the PCR, a set of 'long products' is synthesized from each strand of the target DNA.

The cycles of denaturation–annealing–synthesis is now repeated between 25 and 40 cycles then add a final extension step of 70°C for 8-10 min. The long products will denature, and the four resulting strands are copied during the DNA synthesis stage. This gives four double-stranded molecules, two of which are identical to the long products from the first cycle, and two of which are made entirely of new DNA. During the third cycle, the latter give rise to 'short products', the 5′ and 3′ ends of which are both set by the primer annealing positions. At the end of PCR technique the number of short products accumulates in an exponential fashion (doubling during each cycle), this equates to several micrograms of PCR product from a few nanograms or less of target DNA.

The PCR product is usually analyzed using an Agarose gel electrophoresis, or, alternatively, the PCR product can be examined by techniques such as DNA

sequencing [181].

2.3.2.2.2 Agarose gel Electrophoresis

Electrophoresis is a technique that employs the differences in electrical charge to separate the molecules in a mixture. DNA molecules have negative charges, and so when placed in an electric field will migrate towards the positive pole. The rate of migration of a molecule depends on its shape and its charge-to-mass ratio, because most DNA molecules are the same shape and have very similar charge-to-mass ratios, therefore the size become a factor for separation of different DNA molecules when the electrophoresis is performed in a gel. A gel, which is usually made of agarose, polyacrylamide (or a mixture of the two) comprises a complex network of pores through which the DNA molecules must travel to reach the positive electrode. Gel electrophoresis separates different-sized DNA molecules, with the smallest molecules travelling the greatest distance toward the positive electrode. A Special mixtures of DNA fragments called DNA ladders, whose sizes are multiples of 100 bp or of 1 kb, can be used as size markers, and size estimations by comparison with DNA markers can be performed. The results of a gel electrophoresis experiment can be visualized by staining the gel with either Ethidium bromide (EtBr) or non-mutagenic dyes that stain DNA green, red or blue such as SYBR Safe dye that can be included in the buffer solution in which the agarose or polyacrylamide is dissolved when the gel is prepared.

Some of these dyes require UV irradiation to make the bands visible, but others are visualized by illumination at other wavelengths such as blue light. Bands showing the positions of the different size classes of DNA fragment are clearly visible under UV irradiation. The most sensitive dyes can detect bands that contain less than 1 ng DNA [182].

2.3.2.2.2 (ARMS–PCR)

Amplification refractory mutation system– polymerase chain reaction (ARMS–PCR) is a variant of PCR based on the principle that under optimized conditions, the primers with 3′ end mismatch with the complementary template DNA will not result in the amplification of targeted DNA fragment.
For each DNA template two complementary reactions were established, in each two set of primers were used (Humanizing Genomics, Macrogen, OG181216-002), one forward primer to screen for G allele and A allele respectively and a common reverse primer [183].

Forward G allele primer: 5`-ATAGGTTTTGAGGGGCATCG-3`

Forward A allele primer: 5`-ATAGGTTTTGAGGGGCATCA-3

Common Reverse Primer: 5`-AAGAATCATTCAACCAGCGG-3`

Each PCR reaction contained {10 microliter master mix (**MyTaq™ HS Mix Bioline, USA**), 10pmol of each primer and 20ng template DNA}. PCR was performed using a three-step cycling protocol with initial denaturation for polymerase activation (95°C/5 min) 1 cycle; followed by (denaturation (95°C/ 30sec), annealing (56°C/ 30sec) (extension 72°C/30sec) 40 cycle] and final extension (72°C/5) 1 cycle (**Bio-Rad C1000 Thermal Cycler, USA**). A known positive and negative controls were included in the run with each batch of amplification.

The PCR products were analyzed in 1% Agarose gel (**Canvax Biotech**) (1g of Agarose was dissolved in 100 mL (1X TAE buffer TAE: Tris-acetate EDTA) stained with SYBR Safe Dye (**GENETBIO**). Gels were places horizontally in plastic tanks (**BioRad**) and ran at 110 Volts for appropriate time .The size of target DNA bands compared with 100 bp DNA ladders (**GENETBIO**). Gels were then documented with images taken under ultraviolet light (Ultraviolet

Transilluminator, BioDoc-It). To evaluate the efficiency and accuracy of the assay, the PCR product of 10 random samples examined for TNF-α GA promoter polymorphism by Sanger's sequencing (**Macrogen, Inc. South Korea**) and the results obtained were comparable with those determined by ARMS-PCR.

2.4 Statistical analysis

Data entry performed via using an excel spreadsheet then the statistical analysis was performed by SPSS program, version 24 (**IBM SPSS Statistical Package for the Social Sciences**). The data presented in tabular forms showing the frequency and relative frequency distribution of different variables.

Chi-square tests were used to compare the categorical data. Compliance of quantitative random variables with Gaussian curve (normal distribution) was analyzed using *Kolmogorov-Simonov test*. TNF-α concentration was not normally distributed. Quantitative continuous variables and described by mean, standard deviation and median. The statistical significance of difference in mean in TNF-α between two groups (cases and control or male and female etc.) was assessed using independent sample *t-test*, between more than 2 groups ANOVA test was used. The difference in the mean rank between 2 groups was assessed by non-parametric test (Mann- Whitney), while between 3 and more groups Kruskal- Wallis tests were used. The correlation coefficient tests between variables were done by using *Spearman's Rank linear correlation coefficient*.

P-values of 0.05 were used as a cut off point for significance of statistical tests.

Chapter Three:
Results

Results

3.1. Demographic features

3.1.1. Age distribution

In the current study the mean age of patients with generalized vitiligo and controls were 29.93 ± 11.24 and 28.8 ± 12.42 years, respectively. In vitiligo, the age of the patient varied from 7 to 50 years and in healthy volunteers from 9 to 49 years. According to our data 5 different age groups were recorded in this study. (Table 3.1)

Table 3.1 Age distribution groups for patients and controls.

Age groups (year)	Participant		Total	*P-value*
	Patients	Controls		
Mean ± SD	29.93 ± 11.24	28.8 ± 12.42	29.15 ± 12.01	0.62
1 - 9	6	1	7	0.62
10 - 19	16	7	23	
20 - 29	18	12	30	
30 - 39	23	9	32	
40 – 50	17	11	28	
Total	**80**	**40**	**120**	

P-value ≤ 0.05 statistically considered significant

3.1.2. Gender

Female patients with subtypes of generalized vitiligo were 57 (71.2%) out of 80 patients and 23(28.8%) were males compared to 18(45%) females with 22(55%) males among control group. Male to female ratio for patient group was 0.4:1 and 1.2:1 for control groups. (Figure 3.1)

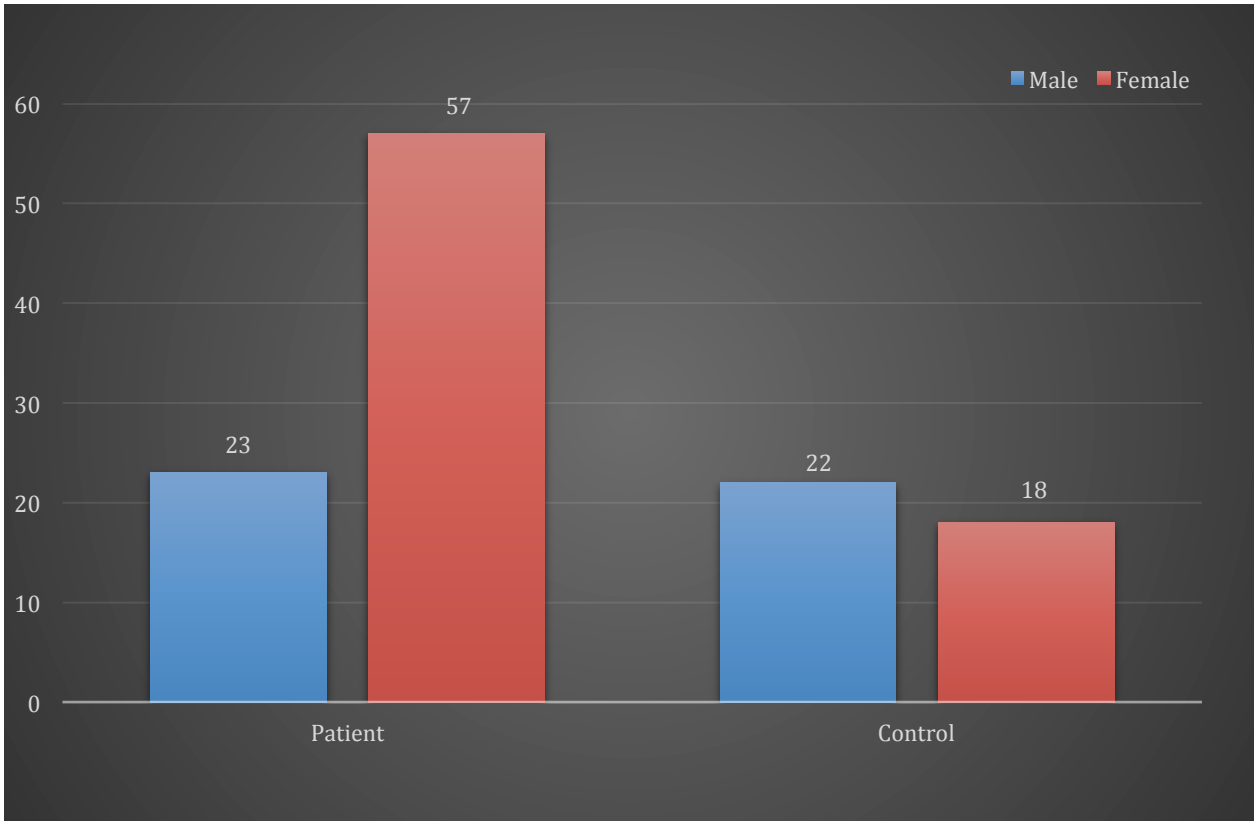


Figure 3.1 Gender distributions of patient and control groups

3.2. Clinical characteristics of patients with generalized vitiligo

3.2.1. Age of onset of generalized vitiligo

Age of onset among patients with generalized vitiligo categorized in to 5 groups with the Mean ±SD (21.53± 12.77) years, 23 patient had generalized vitiligo appeared between the age of 10-19 years , this represent the most frequent age of onset group in the present study , followed by the second most frequent group between the age of 30-39 years that include 18 patients , and the third group of 17 patients their age of onset of generalized vitiligo were between 20-29 years .(Table 3.2)

Table 3.2 Age of onset of generalized vitiligo

	Age of onset of generalized vitiligo (year)					Total
	1-9	10-19	20 - 29	30 - 39	40 - 50	
Frequency	16	23	17	18	6	80
Percent	20	28.75	21.25	22.5	7.5	100

3.2.2. Fitzpatrick's skin phototypes:

Fitzpatrick's skin phototypes IV constitute the most frequent types for enrolled patients with generalized vitiligo, followed by 20 patients with Fitzpatrick's skin phototype III, patients with Fitzpatrick's skin phototype I not recorded in the current study. (Table 3.3)

3.2.3. Clinical variants of generalized vitiligo

Vitiligo vulgaris was the most frequent variant of generalized vitiligo accounts for 71(88.8%) patients followed by acrofacial vitiligo 5(6.35%), vitiligo universalis 3(3.8%), and mixed vitiligo recorded in only 1 (1.3%) patient (Table 3.3). Clinical examination with the aid of Wood's light examination of the white macules found two common types of lesions, amelanotic with sharply demarcated borders and hypomelanotic with poorly defined borders, trichrome lesion, and confetti-like pigmentation each found in one patient. Face, forearms, dorsum of the hands, fingers and feet, chest and genital areas were the most common sites of involvement.

3.2.4. Chief complaint and duration of presentation

Cosmetic appearance or disability was the most frequent chief complaint of enrolled patients in this study account for 67 (83.7%) of patients, cosmetic appearance associated with pruritus recorded in 10 (12.5%) of patients and only 1 (1.3%) sunburn was the chief complaint.

According to the duration of presentation we classify our included patients to those who presented with long standing disease (vitiligo > 2 years duration) and those with recent onset presentation (vitiligo ≤ 2 years). Majority of our patients (70%) hade vitiligo > 2 years duration. (Table 3.3)

3.2.5.History of clinical erythema preceding vitiligo and hypersensitivity to sunlight with sunburn

Erythema before the appearance of white macules of vitiligo only recorded among 5 patients (6.3%) and in 75 patients (93.8%) acquired white macule not preceded by erythema, 12 patients (15.0%) had history of sunburn and had history of hypersensitivity to sunlight exposure in the involved skin with vitiligo and 68 patients (85.0%) tolerate sun exposure with out sunburn and hypersensitivity. (Table 3.3)

3.2.6.Precipitating (aggravating) factors for generalized vitiligo

Psychological stress was the most frequent (28.8%) aggravating factor for vitiligo in the current study (Table 3 .3)

3.2.7. Associated leukotrichia & premature hair greying

Associated leukotrichia or white hair within the vitiliginous patches recorded in 24 (30%) of patients with generalized vitiligo and premature hair greying recorded in 15 patients (18.8%). (Table 3.3)

3.2.8.Family History of vitiligo and family history of premature hair greying

Positive family history for vitiligo among first-degree relatives of patients was 22.5%, 20% of patients had positive family history of vitiligo among their second-degree relatives and 5.0% of patient positive family history of vitiligo recorded among both first- and second-degree relatives. Family history of premature hair graying was positive in 44 patients (55.0%), while 36 patients (45.0%) record negative family history of premature hair greying. (Table 3.3)

Table 3.3 Clinical characteristics of patients with generalized vitiligo

Patient s clinical Characteristics	Frequency	Percent
Fitzpatrick's skin phototypes		
I (pale white skin, blue/green eyes, blond/red hair)	0	0.0%
II (Fair skin blue eyes)	1	1.3
III (Darker white skin)	20	25.0
IV (light brown skin	43	53.8
V (Brown skin)	16	20.0
VI (dark brown or black skin	0	0.0%
Clinical variants of generalized vitiligo		
Vitiligo vulgaris	71	88.75
Acrofacial vitiligo	5	6.3
Mixed vitiligo	1	1.25
Vitiligo universalis	3	3.8
Chief complaint		
Cosmetic disability	67	83.7
Cosmetic disability and pruritus	10	12.5
Pruritus	2	2.5
Sun burn	1	1.3
Duration of presentation.		
Vitiligo > 2 years	56	70
Vitiligo ≤ 2 years	24	30
Precipitating factor		
None	42	52.5
Sun burn	12	15.0
Psychological stress	23	28.8
Pregnancy	1	1.3
Contraceptive	1	1.3
Sun burns and psychological stress	1	1.3
Associated clinical features		
None	39	48.8
Leukotrachia	24	30.0
Premature hair greying	15	18.8
Leukotrachia and premature hair greying	2	2.5
Family history		
Negative	42	52.5
Positive for first degree	18	22.5
Positive for second degree	16	20.0
Positive for first and second degree	4	5.0
Family history of premature hair greying		
Positive	44	55.0
Negative	36	45.0

3.3. Measurement of vitiligo activity

Activity of vitiligo is determined in the current study based on:

3.3.1. Vitiligo Disease Activity Score

Vitiligo Disease Activity Score (VIDA) score a six-point scale for assessing vitiligo stability over time, low VIDA score indicates less disease activity, depending on patient's own reports active vitiligo involves either the expansion of existing lesions that is reported in 24 patients (30%) or the appearance of new lesions recorded in 56 patients (70%), VIDA score of 1 was most frequent grading of vitiligo activity that is recorded in 35 patients (43.8%). The lowest VIDA score is -1 recorded in 7 patients only (8.8%) and highest score which is 4 is recorded in 9 patients (11.3%). (Table 3.4)

3.3.2.Koebner phenomenon

Positive Koebner phenomenon was found in 22 patients (27.5%) (Table 3.4)

Table 3.4 Predictors of vitiligo activity

Grades of VIDA score	Frequency	Percent
+4 Activity of 6 weeks or less duration	9	11.3
+3 Activity of 6 weeks to 3 months	4	5.0
+2 Activity of 3 - 6 months	14	17.5
+1 Activity of 6 - 12 months	35	43.8
0 Stable for 1 year or more	11	13.8
-1 Stable with spontaneous regimentation (≥1 year)	7	8.8
Koebner phenomenon		
Positive	22	27.5
Negative	58	72.5

3.4. Measurement of skin surface involvements

For the first time in Iraq, we used Vitiligo Extent Score (VES) for determination of extent of skin surface involvement in patients with generalized vitiligo and according to our results we classify patients to those with VES to less than 2, 2-10, 10.1-20 and more than 20. 31 patients (38.8%) record VES less than 2 and 8 patients (10.0%) had VES more than 20.0. (Table 3.5).

Table 3.5 skin surface involvements based on VES

Vitiligo Extent score (VES)	Frequency	Percent (%)
< 2	31	38.8
2 - 10	30	37.5
10.1 - 20	11	13.7
> 20	8	10.0

3.5. Serum level of TNF-α

Serum TNF-α concentration was measured in patients with generalized vitiligo and control group using ELISA technique. Range value of serum TNF-α for patients was (3.06 - 80.07 pg/mL) and for controls was (2.64-22.51 pg/mL). Among patient group Mean ±SD of serum TNF-α concentration was significantly higher than control group (*p-value* of 0.01). (Table 3.6). Odds ratio for High level of TNF-α (more than cutoff point which is found by ROC curve as 5.7) is 2.81 with 95% CI of {1.285 to 6.143}

Table 3.6 Comparison of serum TNF -α level of patient and control groups

Study groups (n=number)	Serum TNF-α concentration (pg/mL)		*p-value*
	Range values	**Mean ±SD**	
Patients (n=80)	3.06 - 80.07	12.92±14.40	
Controls (n=40)	2.64-22.51	6.43±4.24	0.01

P-value ≤ 0.05 statistically considered significant

3.5.1.Measurement of serum TNF‑α concentrations in correlation with demographic features

Elevated serum TNF-α concentrations were analyzed with participant's demographic features:

3.5.1.1. Age groups

Serum TNF‑α concentrations has been measured among different age groups, statistically no significant different were recorded between different age groups of patients and controls. (Table 3.7)

Table 3.7 Measurement of serum TNF-α level among different age groups

Age groups (year)(n=number of participants)	Serum TNF-α concentration Mean ± SD (pg/mL)		*P-value*
	Patients	**Controls**	
1 – 9 (n= 7)	21.7 ± 20.99	7.63 ± 2.3	0.56
10 – 19 (n=23)	9.27 ± 8.65	7.83 ± 5.81	0.69
20 – 29 (n=30)	11.96 ± 12.11	4.75 ± 2.46	0.053
30 – 39 (n=32)	14.82 ± 19.42	5.43 ± 2.61	0.16
40 – 50 (n=28)	11.68 ± 9.52	8.09 ± 5.40	0.27

P-values ≤ 0.05 statistically considered significant

3.5.1.2. Comparison of serum TNF-α concentration with gender

In the current study, statistically no significant difference was found in the serum TNF-α concentrations between male and female of the patient group and between male and female of the control group. Comparing serum TNF-α concentrations between patients with controls in correlation with gender statistically no significant difference was found with the *p-value* of 0.13. (Table 3.8).

Table 3.8 Comparison of serum TNF-α level with gender

Study groups	Serum TNF-α concentration Mean ± SD (pg/mL)		*P-value*
	Male (n=number of participants)	**Female**	
Patient	11.40 ± 10.49 (n=23)	13.53 ± 15.74 (n=57)	0.55
Control	5.61 ± 2.86 (n=22)	7.44 ± 5.41 (n=18)	0.097
Total	8.57 ± 8.22 (n=45)	12.07 ± 14.18 (n=75)	0.13

P-values ≤ 0.05 statistically considered significant

3.5.2.Measurement of serum level of TNF-α in correlation with clinical characteristics of generalized vitiligo

In the present study serum TNF α-levels were analyzed with clinical characteristics of patients with generalized vitiligo:

3.5. 2.1. Measurement of serum TNF‑α concentrations among different groups of age of onset of generalized vitiligo

Among patients groups serum TNF‑α concentrations has been analyzed with age onset of generalized vitiligo, statistically no significant difference was found for elevated serum concentration of TNF‑α between different groups of age of onset of generalized vitiligo (Table 3.9).

Table 3.9 Measurement of serum TNF‑α concentrations among different groups of age of onset of generalized vitiligo

Age of onset of generalized vitiligo (year) (n=number of patients)	Serum level TNF-α concentration Mean ± SD (pg/mL)	*P-value*
1-9 (n=16)	13.38 ± 14.49	0.47
10-19 (n=23)	13.43 ±15.45	
20 - 29 (n=17)	8.58 ± 8.46	
30 - 39 (n=18)	17.23 ± 18.81	
40 - 50 (n=6)	9.04 ± 3.35	

P-values ≤ 0.05 statistically considered significant

3.5.2.2. Measurement of serum TNF-α concentrations with duration of presentation of generalized vitiligo

Mean serum TNF-α concentrations among patients presented with generalized vitiligo ≤2 years duration is different from those presented with generalized vitiligo >2 years, the difference is statistically not significant (*p-value* = 0.27). (Table 3.10)

Table 3.10 Serum TNF-α concentrations with duration of generalized vitiligo

Duration of presentation of generalized vitiligo) (n=number of patients)	Serum TNF-α concentration Mean ± SD **(pg/mL)**	*P-value*
≤ 2 years (n=24)	15.62 ± 18.99	0.27
> 2 years (n=56)	11.76 ± 11.93	

P-values ≤ 0.05 statistically considered significant

3.5 .2.3. Measurement of serum level of TNF‑α concentration with family history of generalized vitiligo

Family history of vitiligo analyzed with elevated serum level of TNF-α concentration, *p-value* was 0.78 for serum level of TNF□α among patients with +ve family history of vitiligo compared to those with -ve family history of vitiligo. (Table 3.11)

Table 3.11. Serum level of TNF‑α concentration with family history of vitiligo

Family history of vitiligo (n=number of patients)	Serum TNF-α concentration Mean ± SD **(pg/mL)**	*p-value*
Positive (n=38)	13.39 ± 13.49	0.78
Negative (n=42)	12.49 ± 15.32	

P-value ≤ 0.05 statistically considered significant

3.5.2.4 Measurement of serum level of TNF-α with VES

Skin surface involvement with generalized vitiligo not affected by the elevated serum TNF-α concentrations and statistically no significant differences were found among the four groups of VES. (*p*-value= 0.98). (Table 3.12)

Table 3.12 Measurement of serum level of TNF-α with VES

VES groups (n=number of patients)	Serum TNF-α concentration Mean ± SD **(pg/mL)**	*p-value*
< 2 (n = 31)	12.37 ± 16.90	0.98
2 – 10 (n = 30)	13.77± 14.20	
10.1 – 20 (n = 11)	12.79 ± 10.97	
> 20 (n = 8)	12.02 ± 10.12	
Total (n = 80)	12.92±14.40	

P-values ≤ 0.05 statistically considered significant

3.5.2.5. Measurement of serum level of TNF-α concentration with vitiligo activity

In the present study serum TNF-α concentrations were analyzed with grades of VIDA score and kobner phenomenon

3.5.2.5.1 Measurement of serum level of TNF-α with grades of VIDA score

Patients with active generalized vitiligo have significantly higher TNF-α levels based on grades of VIDA score. (*p-value= 0.01*). (Table 3.13)

Table 3.13 Measurement of serum level of TNF-α with grades of VIDA score

VIDA Score (n= number of patient)	**TNF- α concentration Mean ± SD (pg/mL)**	***P- value***
4 (n = 9)	13.2±10.89	0.02
3 (n = 4)	12.51±4.99	
2 (n = 14)	19.98 ± 23.42	
1 (n = 35)	8.27 ±6.22	
0 (n =11)	22.98 ±16.91	
-1 (n = 7)	6.1± 4.63	
Total (n = 80)	13.2±7.17	

P-values ≤ 0.05 statistically considered significant

3.5.2.5.2 Measurement of serum level of TNF-α with kobner phenomenon

Statistically no significant difference in the serum TNF-α concentration was recorded in correlation with kobner phenomenon. (Table 3.14)

Table 3.14 Measurement of serum level of TNF-α with kobner phenomenon

kobner phenomenon (n=number of patients)	Serum TNF-α concentration Mean ± SD **(pg/mL)**	*P-value*
Positive (n=22)	14.71±14.09	0.79
Negative (n=58)	12.25 ±14.7	

P-values ≤ 0.05 statistically considered significant

3.6. TNF-α -308 G/A promoter polymorphism

In the current study detection of TNF-α -308 G/A promoter polymorphism were done among both patients and control groups using ARMS-PCR technique. (Figure 3.2)

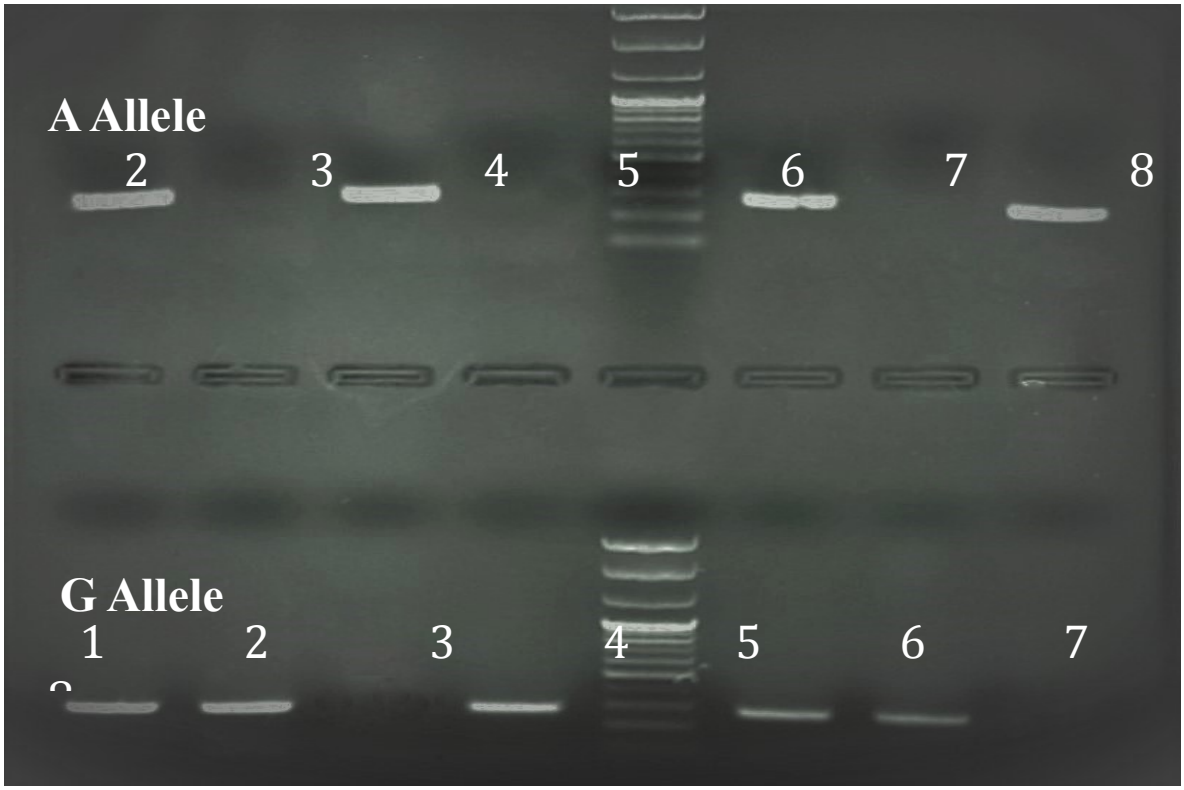


Figure 3.2 Agarose gel Electrophoresis: Denaturing gradient gel electrophoresis of the denaturing gradient gel electrophoresis of the ARMS-PCR proving the specificity of the two primers were used for typing TNF-α -308 promoter polymorphism. Lanes 1, 2, 3, 4, 6, 7, 8 in the upper panel represents A allele. Similarly, lanes in the lower panel represent G allele. Lane 5 is 100 bp DNA ladder.

Among patient group, 46(57.5%) patients were homozygous G/G (TNFA1, common or wild allele, 20(25%) of patients were Heterozygous G/A (TNFA 2, rare allele) and 14(17.5%) patients were homozygous A/A, among control group 27(67.5%) were homozygous G/G, 11(27.5) were Heterozygous G/A and 2 (5%) of controls were homozygous A/A and. Statistically there was no difference in the distribution of TNF –α genotypes and allele frequencies between patients and control groups (Table 3.15)

Table 3.15 Genotype & Allele distribution of TNF -α (-308G/A) in study groups.

Genotype and Alleles	Patients	Controls	Total	*p-value*
Homozygous G/G	46	27	73	0.21
Heterozygous G/A	20	11	31	
Homozygous A/A	14	2	16	
Total	80	40	120	
Alleles				
G (Wild)	66	38	104	0.31
A (Rare)	34	13	47	

P-values ≤ 0.05 statistically considered significant

3.6.1. Genotype distribution of TNF -α -308G/A promoter polymorphism in correlation with demographic features

3.6.1.1.Genotype distribution of TNF -α -308G/A promoter polymorphism among different age groups

Statistically no significant different was recorded between different age groups with distribution of TNF -α -308G/A promoter polymorphism (Table 3.16)

Table 3.16 Genotype distribution of TNF -α -308G/A promoter polymorphism among different age groups

Age groups (year)(n=number of participants)	Homozygous G/G	Heterozygous G/A	Homozygous A/A	p-value
1 – 9 (n=7)	5	1	1	0.82
10 – 19 (n=23)	13	5	5	
20 – 29 (n=30)	15	11	4	
30 – 39 (n=32)	20	8	4	
40 – 50 (n=28)	20	6	2	
Total (n=120)	73	31	16	

P-values ≤ 0.05 statistically considered significant

3.6.1.Genotype distribution of TNF -α -308 G/A promoter polymorphism in relation with gender

In the current study genotype distribution of TNF -α -308 G/A promoter polymorphism between male and female of patient group was statistically not significant (P-values=0.97) and between male and female of control groups (P-value =0.36). Comparing genotype distribution of TNF -α -308 G/A promoter polymorphism between patient and controls in relation with gender was statistically not significant (*p-value*= 0.95). (Table 3.17)

Table 3.17 Genotype distribution of TNF -α -308 G/A promoter polymorphism in relation with gender

Gender (n=number of participants)		**TNF -α -308G/A** polymorphism			***p-value***
		Homozygous G/G	Heterozygous G/A	Homozygous A/A	
Patients	Male (n=23)	13	6	4	0.97
	Female (n=57)	34	14	9	
Controls	Male (n=22)	15	5	2	0.36
	Female (n=18)	11	5	0	
Total	Male (n=45)	28	11	6	0.95
	Female (n=75)	46	20	9	

P-values ≤ 0.05 statistically considered significant

3.6.2 .Genotype distribution of TNF -α -308G/A promoter polymorphism in relation with clinical characteristics of generalized vitiligo

Genotype distribution of TNF -α -308G/A promoter polymorphism was statistically not significant among different groups of age of onset of generalized vitiligo (*p-value* =0.74). (Table 3.18). Duration of presentation of generalized vitiligo, family history of vitiligo, VIDA score and VES score were not affected by TNF -α -308G/A promoter polymorphism with the p values of 0.82, 0.23, 0.66, and 0.76, respectively.

Table 3.18 Genotype distribution of TNF -α -308G/A promoter polymorphism among groups of age of onset of generalized vitiligo

Age of onset of generalized vitiligo (in year), (n=number of patients)	**TNF -α -308G/A Promoter Polymorphism**				*p-value*
	G/G	G/A	A/A	Total	
1-9 (n = 16)	10	4	2	16	0.74
10-19 (n =23)	11	6	6	23	
20 - 29 (n = 17)	11	4	3	18	
30 - 39 (n = 18)	12	5	1	17	
40 - 50 (n = 6)	2	1	2	5	
Total	46	20	14	80	

P-values ≤ 0.05 statistically considered significant

3.6. 3.Correlation of TNF -α -308G/A promoter polymorphism with serum level of TNF-α.

Elevation of serum level of TNF -α could be genetically controlled due to polymorphism at the position of -308 in which Guanine a common allele substituted with Adenine. In this study both patient and control groups with Homozygous G/G allele have significant elevation of serum level of TNF- α, those with Heterozygous G/A allele also have significant elevation of serum level of TNF-α (Table 3.19). Odds ratio for genotype G/A to be risky in comparing with Genotype G/G is 1.12 with 95% CI of {0.469 to 2.676} which indicates it is not significant (*p-value*=0.798)

Table. 3.19: Correlation TNF -α -308G/A promoter polymorphism with serum level of TNF- α

TNF-α/-308GA polymorphism (n=number of participants	**Serum TNF-α concentration** Mean ±SD (pg/mL)		***p-value***
	Patients	**Controls**	
Homozygous G/G (n=73)	13.51±16.56	6.85±4.79	0.046
Heterozygous G/A (n=31)	11.50±7.81	5.84±2.93	0.03
Homozygous A/A (n=16)	12.96±14.53	4.06±0.62	0.42

P-values ≤ 0.05 statistically considered significant

Chapter Four:
Discussions

Discussions

4.1.Patients demographic features

4.1.1.Age distribution

In our study mean age of patients with SD was (29.93±11.24) years and for controls was (28.8 ± 12.42), 22 patients (27.5%) were younger than 20 years old, earlier study by Tariq *et al* [184] records higher percentage 38 (63%) out of 60 enrolled patients under the age of 20. Mean age of our patients was lower than that by Sushama *et al* [185] that include 30 patients with generalized vitiligo (Mean± SD was 37.07±16.208 in years) and in a study by Rajendiran *et al* [186] with total of 264 vitiligo patients mean age at the time of enrollment was 40.34 ±14.27 years. 23(28.75%) of our patients were among the age group of 30-39 years, similarly in the Rajendiran *et al* [186] study 157(60%) of patients were older than 30 years. Sample size difference explain the differences in the result of comparing studies, our study result confirms that generalized vitiligo is more common in the third decade of life.

4.1.2.Gender distribution

Although vitiligo can be found in both sexes, in some studies it was reported to be more common in women due to cosmetic reasons [187,188]. In our study 57 (71.2%) of patients were female compared to 23(28.8%) males, male to female ratio was 0.4:1, similar result recorded in a studies by Tariq et al [184] that found 37 (61.67%) females among 60 patients with generalized vitiligo and 23 (38.33%) males patients with male to female ratio 1:1.6, similarly a study by Akay *et al* [189] records female predominate participant and accounts for 50 (62.5%) of patients compared to 30(37.5%) of male patients, in contrary to the study by Mraisl *et al* [190] that reports males predominating enrolled patients .In

two other studies on in Turkey by Gönül *et al* [191] and other in China by Lu T *et al* [192] with a sizable number of participant male and female ratios were quite similar. Difference in the sample size and female patients seeking treatment more than males explain the difference in the result between these studies.

4.2.Clinical characteristics of patients with generalized vitiligo

4.2.1.Age of onset of generalized vitiligo

Mean age of onset for generalized vitiligo ± SD in our study was 21.53±12.77, this is consistent with a study by al Harithi [193] that reports mean age of onset ±SD of 22.57±15.42, but lower than that reported by Rajendiran et al [186] that found 33.58 ± 15.80 years age of onset of enrolled patients with generalized vitiligo. Differences in the sample size explain the differences in the age of onset of appearance of generalized vitiligo and different populations has different groups of age of onset of vitiligo.

4.2.2.Clinical variants of vitiligo

In our study vitiligo vulgaris was the most frequent variant of generalized vitiligo accounts for 71(88.8%) patients followed by acrofacial vitiligo 5(6.35%), vitiligo universalis 3(3.8%), and mixed vitiligo recorded in only one (1.3%) patient, similar results also found in studies by Mraisl et al [190], and earlier studies by Oguz et al [194], Shah H *et al* [195] and Mahajan [196].

4.2.3.Chief complaint and duration of presentation

Cosmetic appearance or disability was the most frequent chief complaint of enrolled patients in this study account for 67(83.7%) of patients, cosmetic appearance associated with pruritus recorded in 10(12.5%) of patients and only

1(1.3%) sunburn was the chief complaint, in comparison to a study done by Kanwar *et al* [147] that reports significant photosensitivity in 8.1% patients experienced and another 2.4% patients had associated itching. Duration of presentation ranges from one month to 20 years, 56 (70%) our patients had vitiligo >2 years duration similar to Al Harithi [193] result that reports duration of generalized vitiligo ranging from 1-19 years, Sushama *et al* [185] reports 171.07 month and Rajendiran *et al* [186] reports 63.60±86.82 month mean duration of presentation. These results indicate chronicity of generalized vitiligo.

4.2.4.Precipitating (aggravating) factors for vitiligo

In vitiligo physical or environmental stressors are reported both in the onset of the disease and in its progression, events such as death of a family member, work and financial problems have been associated as preceding factors to the onset of vitiligo [197, 198, 199]. In our study, psychological stress was the most frequent (28.8%) aggravating factor for vitiligo, in a study by Kanwar *et al* [147] with 1,416 participants, trauma was the most common factor for vitiligo exacerbation followed by stress such as pregnancy, surgery, febrile illness and drugs, including traditional medications, similar finding also reported by Shah *et al* [195].These finding support the role of environmental factors in the exacerbation of vitiligo .

4.2.5.Associated leukotrichia and premature hair greying

Associated leukotrichia or white hair within the vitiliginous patches recorded in 24(30%) of patients with generalized vitiligo, a high percentage 77(61.1%) of patients had amelanotic hairs in a study by Namian *et al* [200] and Sushame *et al* [185] reports associated leukotrichia in 63.3% of patients with generalized vitiligo.

Patients with vitiligo as well as other members of their family frequently have prematurely grey hair [201]. In our study premature hair greying recorded in 15 (18.8%) patients with generalized vitiligo, a study by Mahajan *et al* [196] reports premature hair greying only in 6 patients out of 945 and explained canities to represent a form of vitiligo and favors involvement, at least partly, of an autoimmune pathway of disease etiology. Family history of premature hair graying was positive in 44 patients (55.0%), while 36 patients (45.0%) record negative family history of premature hair greying, a study done by Fatani *et al* [202] report family history of premature graying of scalp hair in 7.27% among 135 patients with vitiligo.

4.2.6.Family History of vitiligo

Familial occurrence in vitiligo has been reported to be in the range of 6.25% to 30% and positive family history is a poor prognostic factor for vitiligo. [203]

In the current study positive family history for vitiligo among first-degree relatives of patients was 22.5%, 20% of patients had positive family history of vitiligo among their second-degree relatives and 5% of patients had positive family history of vitiliog among their first and second degree relatives, this result is contrary to earlier study by Tariq *et al* [184] that report most of participant were sporadic cases of vitiligo and family history was positive only in 23(38.33%) patients out of 60. In a study by Shah *et al* [195] that include 365 patients, family history was positive in 50 patients (13.7%) and considered to be a poor prognostic factor for vitiligo.

4.2.7.Vitiligo activity

There is no universal consensus on the optimum duration of non-progression vitiligo to be labeled as stable clinically [204].Clinical criteria used for the categorization of stages of vitiligo are:

1.Active vitiligo – Recent development of new lesions, extension of the old lesions in size and shape, ill-defined borders, and presence of the Koebner phenomenon was taken as active stage [205]

2. Stable vitiligo – No development of new lesions during the past 6 months, lesions stationary in size during the past 6 months, hyperpigmented and well-defined borders and absence of the Koebner phenomenon was taken as stable vitiligo. [206]

The prevalence of Koebner phenomenon in vitiligo differs widely, and it is reported to occur in 21% to 62% of patients [207] Koebner phenomenon as a sign of activity in the present study was positive in 22 patients (27.5%), among 30 patients Susahme *et al* [185] reports koebnerization in 26.7%, and a study by Mahajan *et al* [196] found kobnerization in 260 (27.5%) patients while Shah *et al* [195] reports Koebner phenomenon in 6% of the enrolled patients. Pathogenesis of Koebner phenomenon has not been elucidated to date [207]. It may support new theory of melanocytorrhagy that proposes generalized vitiligo is a primary melanocytorrhagic disorder with altered melanocyte responses to friction or following minor trauma and possibly other types of stress inducing their detachment and subsequent transepidermal loss .It could be speculated that the Koebner phenomenon appears only when melanocyte loss reaches a certain

threshold value, variable for each patient and disappearance of melanocytes is certainly not balanced by an influx of melanocytes from the follicular reservoir, where melanocytic stem cells are situated.[208]

4.3.Serum level of TNF-α

Cytokines are important mediators in the immune attack on melanocytes. Death of melanocytes in vitiligo occurs either due to release of TNF-α or IFNγ by immune cells leading to cell apoptosis, or through the release of cytotoxic proteins as perforin and granzymes by the infiltrating CD8+ cytotoxic T cells leading to direct killing of melanocytes [209].Relevant to tissue level TNF-α plays a central role in melanocyte disappearance and contribute to the depigmentation process particularly in patients with active vitiligo [210, 211] while only few studies determine the role of serum TNF-α in vitiligo pathogenesis and data for TNF‑α serum levels are mixed.

In the present study serum TNF-α concentrations were measured in both patients with generalized vitiligo and control groups using ELISA technique. Among patient group serum TNF-α concentrations were significantly higher than that of control group with the p value of 0.01. For the first time elevated serum level of TNF‑α among patients with vitiligo has been reported in a study by Laddha *et al* [212], similar results found by Tariq *et al* [184] Sushama *et al* [185], Rajendiran *et al* [186] and Singh *et al* [213], although in the later study the result was not significant statistically, in contrary no difference in the serum level of TNF-α in patients with vitiligo compared healthy control was reported in a study by Tu CX *et al* [214]

4.3.1.Measurement of serum TNF-α concentrations in correlation with demographic features

In the current study statistically no significant difference was found between serum level of TNF-α among different age groups (*p-value*=0.53) and with gender (p-value=0.55), in study by Laddha *et al* [212] female patients with vitiligo showed significantly higher TNF-α expression as compared to male patients (*p-value* = 0.0066). Differences in the sample size and larger number of female patients enrolled in Laddha *et al* study explain the contradictory results.

4.3.2.Measurement of serum level of TNF-α in correlation with clinical characteristics of generalized vitiligo

Serum TNF-α concentrations were statistically not different among different age of onset groups (*p-value*=0.33) and family history of generalized vitiligo (*p-value*=0.78). There was no statistically significant correlation between serum TNF-α concentrations with the mean of the duration of presentation of generalized (*p-value*=0.27), our result is similar to Sushama *et al* [185] result that report for the first-time lack of correlation of serum level of TNF-α with duration of vitiligo.

4.3.3.Measurement of serum level of TNF-α with grades of VIDA score

In the present study based on VIDA score, patients with active generalized vitiligo appears to have significantly higher TNF-α levels (*p-value*=0.01), earlier studies by Laddha *et al* [213], Aydingoz *et al* [215] and Rajendiran *et al* [186] confirms patients with active vitiligo had significantly higher TNF-α serum levels compared to those with stable disease with the *p-values* of 0.0001, 0.02,

0.01 respectively. Sushama *et al* [185] reports elevated serum TNF-α levels among patients with active localized vitiligo but not in those with active generalized variants (*p-value*= 0.077).

4.3.4.Measurement of serum level of TNF-α with VES.

Serum TNF-α levels not affect the extent of skin involvement based on VES in our study (*p-value*=0.98), consistent to Sushama *et al* [185] study but contrary to Laddha *et al* [212] study that analyzed serum levels of TNF-α among patients with localized and generalized variants of vitiligo and reports significantly higher TNF-α levels among patients with generalized vitiligo (*p-values*=0.014) compared to those with localized type.

4.4.TNF-α -308 G/A promoter polymorphism

A tight control of cytokine level and their sequential expression should ideally be maintained for an effective non-harmful immune response to take place. [216] TNF-α- polymorphism at position-308 (TNF -308), which involves substituting G for A, is associated with a higher rate of TNF gene transcription than the common G allele and increased plasma levels of TNF-α.[217] In the present study statistically there was no difference in the distribution of TNF-α -308 G/A genotypes (Homozygous G/G, Homozygous A/A and Heterozygous G/A) with the *p-value*=0.21. Allele frequencies between patients and control groups (G wild Allele or TNFA1, A rare Allele or TNFA 2) was not significant (*p-value*=0.31), among patients with generalized vitiligo similar results were reported in earlier studies by Yazici *et al* [218] in Turkey that reporte for the first time lack of difference in GG and GA alleles distribution between study groups, two other studies done by Odeh *et al* [219] and Salinas-Santander M

el al [220] reports same results among Jordanian and Northeastern Mexican populations respectively, in contrary other studies by Aydıngöz *et al* [215] in Turkey, Al-Harthi *et al* [193] in Saudi Arabia , Namian *et al*[200]in Iran and Laddha *et al* [212] in North Indian populations confirms that the TNF-α -308G/A promoter polymorphism is associated with vitiligo (Table 4.1).

Table 4.1 comparison of genotype distribution of TNF-α −308 G/A in different studies

Studies (n=number of participants)	Homozygous G/G	Heterozygous G/A	Homozygous A/A	*p-value*
Present study (n=120)	73	31	16	0.21
Odeeh *et al* (n=80)	57	19	4	1
Yazici *et al* (n=184)	157	26	1	0.5
Aydıngoz *et al* (n=317)	274	39	3	0.01
Al Harithi *et al*(n=323)	117	179	27	0.0001
Salinas *et al* (n=613)	533	57	3	0.9
Namian *et al* (n=726)	622	95	9	0.0004
Laddha *et al* (n=1709)	1097	495	117	0.0001

Probable differences in the genetic background in the populations studied might be considered as source of variation in these results. Cuenca *et al* concluded that there is a gradient in the distribution of the TNF-308 promoter mutation (TNF-α -308A allele) in different population types, it is present in approximately 5% of the population of South Africa compared with 30% in white Caucasians in the United Kingdom, this makes populations bearing a higher proportion of the mutated TNF-308 allele at increased predisposition towards several chronic metabolic, degenerative, inflammatory, and autoimmune diseases[221, 222].A meta-analysis by Lee and Bae [223] showed no association between the TNF-α -308 A allele and vitiligo when all subjects were considered, while after geographical stratification by continent, the TNF-α -A allele was significantly associated with vitiligo susceptibility in Middle Eastern populations. (Table 4.2 A ,Table 4.2B). Another source of variation in the in results between these studies explained by the differences in the sample size.

Table 4.2 A. Meta-analysis of the association between the TNF-α -308 A/G polymorphism and vitiligo in different geographical areas

Study name	Odds ratio	Lower limit	Upper limit	P Value
Al-Harthi, 2013	1.771	1.275	2.461	0.001
Laddha, 2012	4.625	3.908	5.474	0.000
Salinas, 2012	0.997	0.582	1.709	0.992
Namian, 2009	1.271	0.822	1.964	0.281
Yazici, 2006	1.568	0.717	3.428	0.260
	1.787	0.894	3.573	0.101

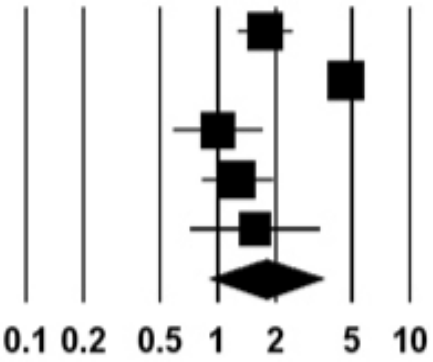
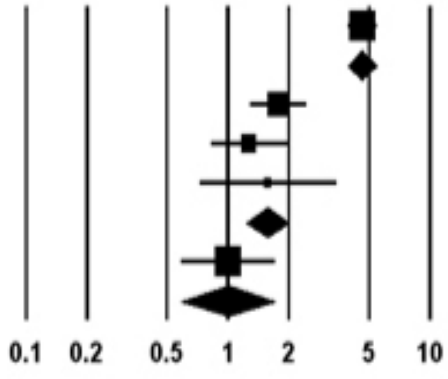


Table 4.2 B. Meta-analysis of the association between the TNF-α -308 A/G polymorphism and vitiligo after geographical stratification by continent

Group by Continent	Study name	Odds ratio	Lower limit	Upper limit	P Value
Asian	Laddha, 2012	4.625	3.908	5.474	0.000
Asian		4.625	3.908	5.474	0.000
Miiddle East	Al-Harthi, 2013	1.771	1.275	2.461	0.001
Miiddle East	Namian, 2009	1.271	0.822	1.964	0.281
Miiddle East	Yazici, 2006	1.568	0.717	3.428	0.260
Miiddle East		1.569	1.224	2.013	0.000
South American	Salinas, 2012	0.997	0.582	1.709	0.992
South American		0.997	0.582	1.709	0.992



4.4.1.Genotype distribution of TNF -α -308G/A promoter polymorphism in correlation with demographic features

There was no difference in the distribution of TNF-α -308 G/A promoter polymorphism among different age groups in our study (*p-value*=0.82). Genotype analysis with gender among patients (*p-value*=0.97) and between patients and controls (*p-value*= 0.95) were not significant, similar result reported earlier in a study by Odeh *et al* [(219)] (*p-value*=0.58). In contrary a study by Namian *et al* [(200)] in Iran that enrolled 176 patients (81 females and 95 males) found significant difference between female patients (*p-value* < 0.0004), while there was not a significant difference between males (*p-value*= 0.9) in addition to significant difference in the frequency TNF-α -308 G/A promoter polymorphism between cases and controls P = 0.0001).Differences in the number of females that enrolled in these studies explain the conflicting results.

4.4.2.Genotype distribution of TNF -α -308G/A promoter polymorphism in relation with clinical characteristics of generalized vitiligo

In the present study genotype distribution of TNF-α -308 G/A promoter polymorphism with age of onset of generalized vitiligo was not significant (*p-value*=0.74), unlikely Laddha *et al* [(212)] confirms TNF-α -308 G/A promoter polymorphism significantly affect age of onset of appearance of vitiligo. Duration of presentation of generalized vitiligo, family history of vitiligo, VIDA score and VES were not affected by TNF-α -308G/A promoter polymorphism with the *p-values* of 0.62, 0.23, 0.66 and 0.76 respectively, similarly Aydıngöz *et al* [(215)] reports no statistically significant associations between TNF-α -308G/A polymorphisms with patient's clinical characteristics and in a study by

Namian *et al* [200] there was no association between TNF- α gene polymorphism and severity or progression of the disease, in contrary a study by Salinas-Santander *et al* [220] suggested a possible association of this polymorphism only in the active form of vitiligo among Mexican population. In the present study lack of statistically significant effect of TNF-α -308G/A polymorphism on the serum level of TNF-α among patients with generalized vitiligo underlie the lack of association of TNF-α -308G/A polymorphism with clinical characteristics of generalized vitiligo

4.4.3.Correlation TNF-α -308 G/A promoter polymorphism with serum level of TNF-α

The cytokine mRNA and protein levels depend on both genetic and environmental factors. Analysis of cytokine gene polymorphisms would be able to detect a genetic abnormality of cytokine regulation that may play a role in the pathophysiology of the disease [224].In the current study elevated serum level of TNF-α was significantly associated with both TNFA1 (TNF-308G) wild allele (*p-value*= 0.046) and with TNFA2 (TNF-308 A) rare allele (*p-value*=0.03). Odds ratio for genotype G/A to be risk for generalized vitiligo in comparing with Genotype G/G was 1.12 with 95% CI of {0.469-2.676} which indicates it is not significant and the *p-value* was 0.798. In a study by Laddha *et al* [212] the -308 GA and AA genotypes showed significant increase in TNF-α levels in patients (*p-value*= 0.019 and 0.0001 respectively); whereas the levels were not differed for GG genotypes between patients and controls (*p-value*=0.082). A third study by Aydıngöz *et al* [215] shows that patients with the TNF-α -308 GA genotype had increased TNF-α levels compared with patients with GG genotype (*p-value* < 0.01). TNF-α gene locus is located within the Class III region of the

human major histocompatibility complex (MHC) on chromosome 6 (6p21.31). Several single-nucleotide polymorphisms (SNPs) have been identified in the human TNF-α gene promoter region having the potential to cause structural changes within regulatory sites that could affect the function or regulation of TNF-α production [215, 225] Elevated serum TNF- α may explain by other functional single-nucleotide polymorphisms (SNPs) in the TNF-α promoter region at positions: -238, -857, and -1031 that were not investigated in the present study. When cytokines levels are measured in serum, it is not possible to determine the origin of these cytokines. [225]. High levels of TNF-α correlated with VIDA score and thus measurements of serum levels of this cytokines may be an objective parameter for generalized vitiligo activity and clinical severity and may confirm the hypothesis that vitiligo might be considered as a true systemic disease with immunologic pathways.

Chapter Five:
Conclusions and Recommendations

Conclusions

1. Serum TNF-α concentrations were significantly higher among patients with generalized vitiligo
2. Statistically no significant association was found between TNF-α concentrations with patient's demographic features.
3. TNF-α is a biomarker for active generalized vitiligo based on VIDA score independent of duration and extent of skin surface involvements, this signifies the role of TNF-α in disease progression and supports further approach for the development of TNF-α specific immunological therapies for the treatment of this challenging disease.
4. Generalized vitiligo correlation with TNF-α polymorphism at position-308 (TNF-308), which involves substituting G for A was not significant.
5. Elevated serum levels of TNF-α not correlated with the TNF-α G/A promoter polymorphism.
6. Patient's demographic features and clinical characteristics of generalized vitiligo not affected by TNF-α G/A promoter polymorphism.

Recommendations

1. To determine the role of TNF-α - 308 G/A promoter polymorphism in the pathogenesis of generalized vitiligo, larger- scale studies on a much bigger samples are recommended.
2. To support the genetic etiology for elevated serum TNF-α concentrations detection of other functional single nucleotide polymorphisms in the TNF-α promoter region is recommended.

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Appendices

Publications

The following articles abstracted from the thesis content has been published in Clinical, Cosmetic and Investigational Dermatology and Medical Science with impact factors according to Clarivate Analytics (Thomson Reuters).

1.Ronak Ahmed, Dana Sharif, Mohammad Jaf, Dashty Mohammed Amin. Effect of TNF-α −308G/A (rs1800629) Promoter Polymorphism on the Serum Level of TNF-α Among Iraqi Patients with Generalized Vitiligo. Clin Cosmet Investig Dermatol. 2020; 13: 825–835.

Publisher: Dovepress, Journal impact factor :1.97

2.Ronak Saeed Ahmed, Dana Ahmed Sharif, Mohammad Yousif Jaf, Ali Hattem Hussain. Tumor Necrosis Factor alpha (TNF-α), as a biomarker for disease activity among Iraqi patients with generalized vitiligo, independent on disease duration and extent of skin involvement. Medical Science, 2020, 24(106), 4295-4302.

Publisher: Discovery Scientific Society.

Questionnaire

*Patient s Name: Code No. Date :

*Residency:

*Gender: 1. Male 2. Female

*Marital status:

Occupation: 1.Employee 2.unemployee 3.student

*Chief complaint:

1.Cosmetic disability 2. Pruritus 3.Sun burn

* Duration:

*Age at onset of appearance:

*Fitzpatrick's skin phototypes:

*Hx of erythema preceding vitiligo. 1.yes 2.no

*Precipitating factors. : Zero

1.Sunburn, 2. Psychological stress 3. pregnancy. 4.

Oral contraceptives. 5.physical trauma to the skin

*Hypersensitive to UV light and sunburn. 1.yes 2.No

*Family history of vitiligo: 1.yes

A. first degree relative B. second degree relative

*Family history of premature hair graying: 1.yes 2.No

- Past medical history : exclusion criteria , patients with generalized vitiligo in association with one or more of the following diseases;

  Diabetes mellitus, thyroid disorder, psoriasis, lichen planus, Alopecia areata,

  systemic lupus erythematous, pernicious anemia, and Addison's disease .

Patient's first degree relatives should not have above AID.

* Treatment history: 1. Topical 2.systemic 3.topical &systemic

*Physical examination: Clinical Variant: Generalized vitiligo

1. Vitiligo Vulgaris 2. Acrofacial vitiligo 3. Mixed vitiligo

4. Vitiligo universalis

*Estimation of skin surface involvement Wood's light with Vitiligo Extent Score

*Predictors of disease activity:

*VIDA score

+4 Activity of 6 weeks or less duration

+3Activity of 6 weeks to 3 months

+2 Activity of 3 - 6 months

.+1Activity of 6 - 12 months

0 Stable for 1 year or more

-1Stable with spontaneous regimentation since 1 year or more

*Koebner phenomenon at scar site, waist area, site of trauma.

* Associated clinical features:

1.Zero 2.Leukotrachia 3.Premature hair graying

VITILIGO EXTENT SCORE (VES)

Please select for each area 1 picture, which best represents the extent of the vitiligo lesions.

You can also choose a second option (if necessary): e.g. if the extent is half (½) or a quarter (¼) of the involved area in the picture or slightly less (-) or more (+) than the involved area in the picture. The ">75%" option can be selected if the involved area is more than shown in the last picture, but less than 100% (i.e. complete depigmentation). For the final score please use the calculator: www.vitiligo-calculator.com

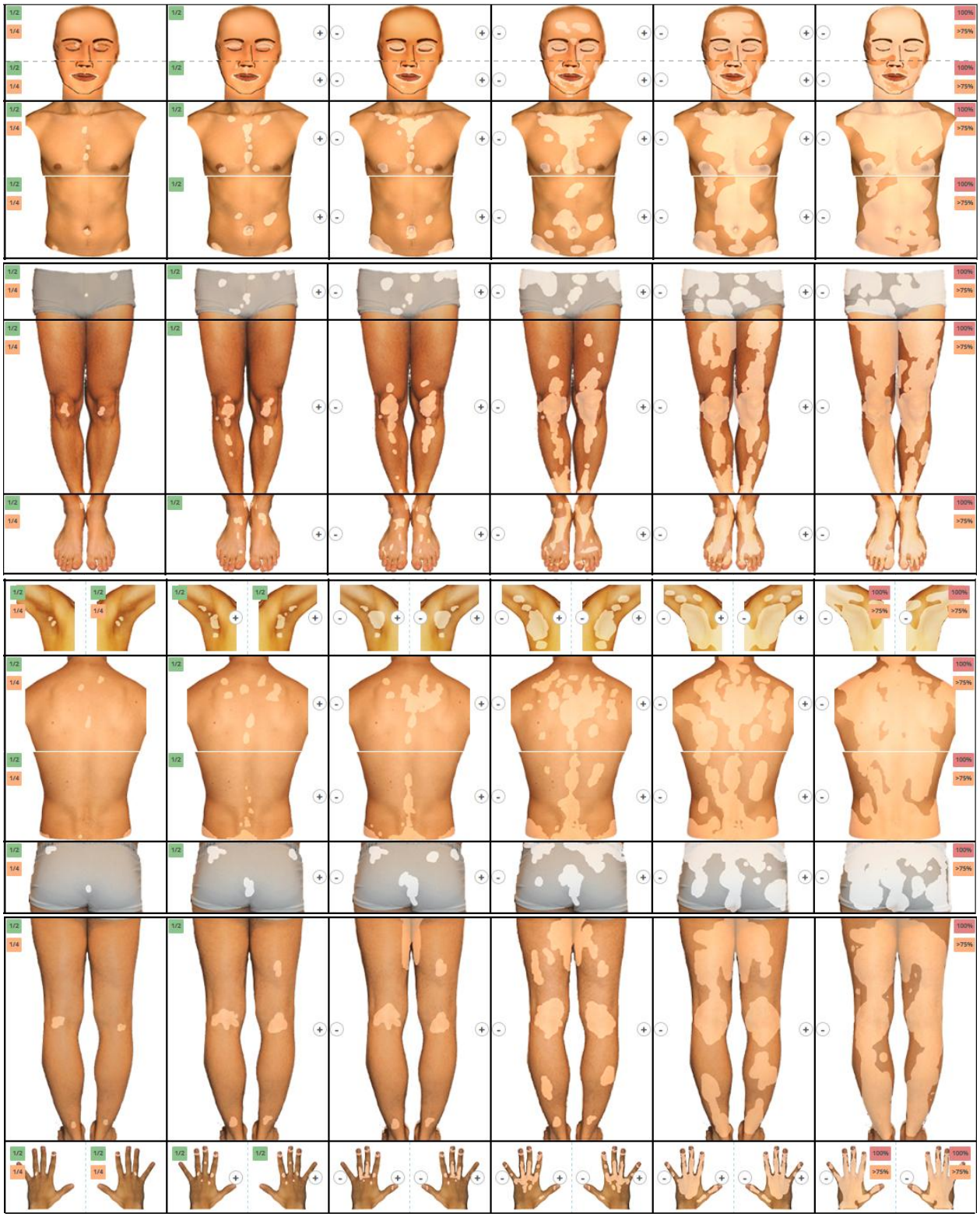
Date:.....

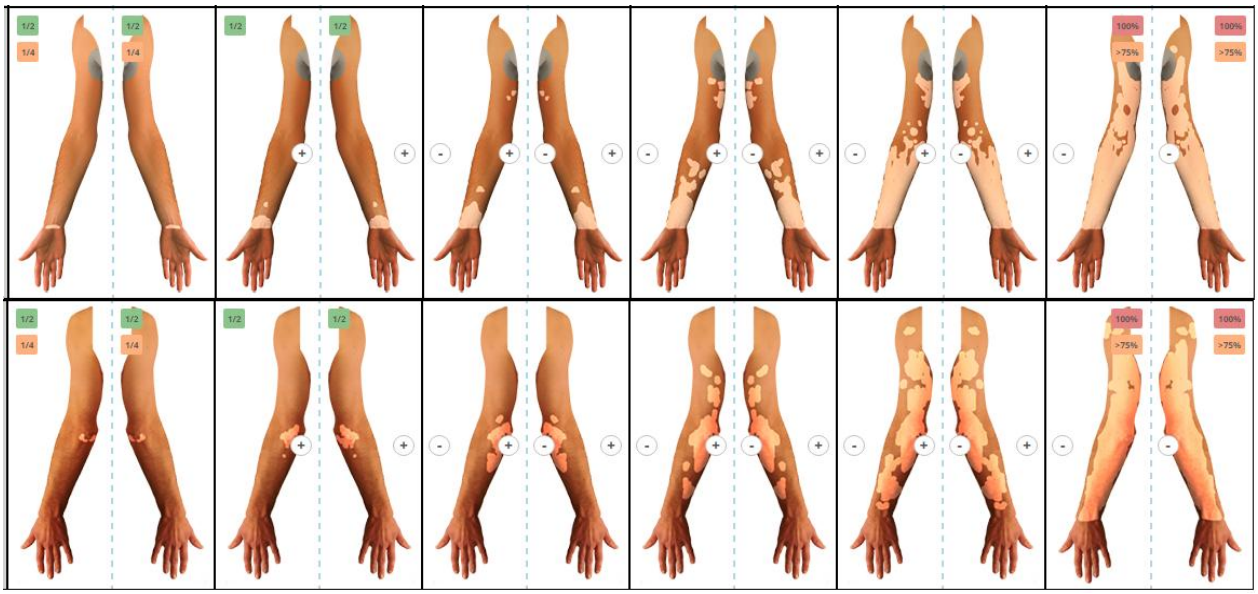
Name/Number pt:.....

Skin phototype (Fitzpatrick) I – II – III – IV – V - VI

Physician.....

Total score (www.vitiligo-calculator.com).....





وزارة التعليم العالي والبحث العلمي
جامعة السليمانية
كلية الطب ، وحدة الأمراض الجلدية

جامعة السليمانية

تباين مستوى مصل عامل نخر الورم ألفا (TNF-α) بين مرضى البهاق المعمم في السليمانية - العراق

رسالة

مقدمة لقسم الطب وللدراسات العليا في كلية الطب -جامعة السليمانية ، كجزءمن متطلبات الحصول على درجة دكتوراه الفلسفة في الأمراض الجلدية والزهرية

من قبل

روناك سعيد احمد

ماجستيرامراض الجلدية والزهرية

MBChB,MSc.D&V

باشراف

أستاذ مساعد د. دانا احمد شريف

(MD, MRCP, FRCP)

أستاذ مساعد د.محمد يوسف سعيد جاف

(MD, FICMS)

شباط 2021

الخلاصة

الخلفية: البهاق هو اضطراب صبغي مزمن مكتسب يصيب الجلد ينتج عن ارباك مناعي للخلايا الصباغية العاملة. يلعب السيتوكين TNF-α دورا مركزيا في بدء عملية الموت المنظم للخلايا الصباغية في البهاق. قد يؤثر أشكال النوكليوتيدات المفردة(SNP) في المنطقة المحفزة لتشفير الجينات لـ TNF-α على إنتاجها وارتفاعها في المصل.

اهداف البحث: الهدف من هذه الدراسة هو قياس عامل (TNF-α) في المصل بين مرضى المصابين بالبهاق المعمم واستبعاد أن تعدد الأشكال في الموضع 308- استبدال (G→A) يؤثر على مستويات TNF-α في الدم.

المواد وطرق البحث: أجريت دراسة الحالات والشواهد هذه في المركز التعليمي للأمراض الجلدية وشعبة الأمراض الجلدية في مستشفى شورش التعليمي- السليمانية- العراق. تم قياس تركيزات TNF-α في المصل عبر تقنية (ELISA) في 80مريضا يعانون من البهاق المعمم و 40شخصا يتمتعون بصحة جيدة سريريا. تم استخدام تقنية (ARMS-PCR) للكشف عن تعدد الأشكال الجين TNF-α- 308G / A. تم تحليل تركيزات مصل و TNF -α -308G /A

تعدد الأشكال الجيني بالارتباط مع السمات الديموغرافية والخصائص السريرية للمرضى الذين يعانون من البهاق المعمم.

النتائج: تم العثور على ارتفاعات ذات دلالة إحصائية لتركيزات TNF-α في المصل بين المرضى مقابل مجموعة التحكم (قيمة P= 0.01) . تم العثور على مستويات أعلى بكثير من تركيزات TNF-α (قيمة P=0.02)بين المرضى الذين يعانون من البهاق المعمم النشط. ارتبطت مستويات مصل الدم المرتفعة من TNF-α بشكل كبير مع أليل G(TNFA1) (p value=0.03) و مع أليل A (TNFA2) (p value=0.04). لم يتأثر TNF-α -308G/A تعدد الأشكال بالسمات الديموغرافية والخصائص السريرية لمرضى البهاق المعمم.

الاستنتاجات: TNF-α في المصل هو علامة حيوية للبهاق المعمم النشط لم يتم العثور على فرق بين مستويات مصل TNF- α مع تعدد الأشكال TNF-α في الموضع 308- يتضمن ذلك استبدال الأليل G بالأليل A .

وەزارەتی خوێندنی باڵا و توێژینەوەی زانستی

زانکۆی سلێمانی/ کۆلێژی پزیشکی

بەشی هەناوی/ یەکەی نەخۆشییەکانی پێست

جیاوازی ئاستی TNF-∝ لە خوێندا لە نێوان تووشبووان بە نەخۆشی بەڵەکیی گشتی لە شاری سلێمانی/ کوردستان - عێراق

دکتۆرا نامەیەکە

پێشکەشکراوە بە بەشی پزیشکی و خوێندنی باڵا لە کۆلێژی پزیشکی زانکۆی سلێمانی، وەک بەشێک لە پێداویستییەکانی بەدەستهێنانی بڕوانامەی دکتۆرا لەنەخۆشییەکانی پێست و زوهری

لە لایەن

ڕوناک سەعید ئەحمەد

ماستەر لە نەخۆشییەکانی پێست و زوهری

MBCHB,MSc.D&V

بەسەرپەرشتی

پرۆفیسۆری یاریدەدەر د. دانا أحمد شریف

(MD,MRCP, FRCP)

پرۆفیسۆری یاریدەدەر د. محمد یوسف سعید جاف

(MD,FICMS)

(ڕەشەمێ 2720)

پوختە

پاشخان: بەڵەکی ئەو تێکچوونە بەردەوامەیە کە دووچاری پێست دەبێت بەهۆی تێکچوونی بەرگری ئەو خانانەی بەرپرسی ڕەنگە لە پێسدا، سیتۆکینی TNF-∝ ڕۆڵێکی ناوەندی دەگێڕێت لە مردنی خانەکانی دروست کردنی ڕەنگ لە بەڵەکیدا. جۆرەکانی نۆکلیوتیداتی تاک لە پێگەی 308- هاندەری بەکۆدکردنی جیناتەکان بۆ TNF-∝ لەسەر بەرهەمهێنانی وبەرزکردنەوەی ڕێژەکەی لە خوێندا.

ئامانجەکان: ئامانج لەم لێکۆڵینەوەیە بریتییە لە پێوانەکردنی ڕێژەی TNF-∝ لە خوێندا لە نێوان ئەوانەی کە تووشی نەخۆشی بەڵەکیی گشتی بوون و هەروەها دوورخستنەوەی ئەوەی کە چەندێتی لە جۆرەکان لە پێگەی 308- (گۆڕینی G→A)کاریگەری ئەبێت لەسەر ڕێژەی TNF-∝ لە خوێندا.

نەخۆش و ڕێگاکانی توێژینەوەکە: لێکۆڵینەو و بینیی تووشبووەکان لە ناوەندی فێرکاری نەخۆشییەکانی پێست/بەشی ڕاوێژکاری پێست لە نەخۆشخانەی شۆڕش/سلێمانی - عێراق ئەنجامدراوە. پیوانەی چڕبوونەوەی ڕێژەی TNF-∝ لە خوێندا لەڕێگای تەکنیکی (ELISA) وە لە ٨٠ نەخۆشی تووش بوو بە بەڵەکی گشتی و (٤٠) کەسی تەندروست. تەکنیکی (ARMS – PCR) بەکارهاتووە بەمەبەستی دۆزینەوەی هێندەبوونی شێوەی جیناتەکان TNF∝-308G/A. لێرەدا دەستکراوە بە شیکردنەوەی ڕێژەی TNF-∝ و TNF-∝ 308G/A بە پێی تایبەتمەندییە دیمۆگرافییەکان و ئەو دیاردانەی لەو نەخۆشانەدا دەرکەوتوون کە دووچاری بەڵەکی گشتی بوون.

ئەنجامەکان: ئەوەی بەدیکرا بەرزبوونەوەیەک بوو بە بەڵگەی ئاماری لە ڕێژەی TNF-∝ لە خوێندا لە نەخۆشانی تووشبوو بە بەڵەکی گشتی لەبەرامبەر کۆنترۆڵ (p value=0.01)، بەدیکرا کە ڕێژەیەکی زۆر بەرزتر لە TNF-∝ (p value=0.02) لەو نەخۆشانەدا هەیە کە دووچاری بەڵەکی گشتی و چالاک بوون، بەرزبوونەوەی ڕێژەی TNF-∝ لە خوێندا بەشێوەیەکی گەورە پەیوەندی هەبووە بە TNF-∝ -308G (TNFA1) (p value=0.03) و TNF-∝ -308A (TNFA2) (p value=0.04) ، زۆر بوون جۆرەکانی TNF-∝ لە پێگەی 308- کار ناکاتە سەر تایبەتمەندییە دیمۆگرافییەکان و تایبەتمەندییەکانی نەخۆشی تووشبوو بە بەڵەکی گشتی.

دەر ئەنجامەکان: TNF-∝ لە خوێندا بەڵگەیە لەسەر بەڵەکی گشتی و چالاک، لە میانەی لێکۆڵینەوەکەدا جیاوازی ڕێژەی TNF-∝ی خوێن بەپێی جۆرەکانی TNF-∝لە پێگەی 308- بەدی نەکراوە. لە کاتێکدا کە جۆرەکانی A و G جێگۆڕکێیان پێکراوە.