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Investigation of Tumor Necrosis Factor Alpha and Serotonin Transporter Genes Polymorphism in Recurrent Aphthous Stomatitis

A Thesis

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صدق الله العلي العظيم

(سورة الإخلاص)

Dedication

To those who are not matched by anyone in the universe, to whom God has commanded us to honor them, to those who have made a great deal, and have given what cannot be returned, my dear mother (Allah made heaven her abode) and father, they have been my best supporter throughout my academic career…

To my husband and siblings, my brothers and sisters who provide support and assistance to me…

To everyone who helped me to complete this research, my relatives and all my friends…

For the precious gifts, my beloved dear sons…

I dedicate this thesis with gratitude.

RAJAA -2022

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Summary

Recurrent aphthous stomatitis (RAS) is chronic inflammatory disease of the oral mucosa. It is characterized by painful mouth ulcers that cannot be explained by an underlying disease. The prevalence rates of RAS ranging from 5 to 60% in different series. It is slightly more common in women as well as among affluent socioeconomic classes and countries. Genetics variants inflammatory agents are associated with the risk of RAS such as serotonin transporter gene (*SCL6A4*), tumour necrosis factor alpha (TNF-α) gene and human leukocytes antigen (HLA) gene polymorphisms.

Due to the importance of these genetic variants, the present study was designed to evaluate the risk of serotonin transporter gene (SCL6A4) polymorphism G>T (rs 6354) and TNF-α gene polymorphism G>A (rs 1800629) with the related serum levels of their proteins in the development of RAS. To achief these aims, the case control study was designed and it included one hundred (100) participants, divided into two groups: 50 patients suffering from recurrent aphthous stomatitis who had been diagnosed by specialist dentist (during the period from 1/5/2018 to 20/5/2019) and age matching 50 apparently healthy individuals as control group. Mean±standard error (SE) of two groups were (32.64 ± 1.25 and 32.41 ± 1.26) respectively. Serum serotonin and TNF-α were estimated by enzyme linked immunosorbent (ELISA), while for genetic analysis, DNA was extracted from whole blood and the polymorphism of SCL6A4 gene (rs6354) and TNF-α gene (rs1800629) were examined by tri-primer amplification refractory mutation system-polymerase chain reaction (tri-primer ARMS-PCR), followed by electrophoresis on 2% agarose gel. Various statistical analyses were applied to analyse the research data.

The present results indicated that the levels of TNF-α were elevated significantly ($P<0.05$) in patients' group with mean±SE (515.05±26.49) compared to control

(421.97±33.29). Otherwise, serotonin increased insignificantly (P>0.05) in patients (41.43±2.45) in comparison with control (35.35±2.74). Furthermore, present results exhibited a highly significant (p<0.000) positive correlation between serum serotonin and TNF-α (r=0.967).

On the other hand, gene analysis of SCL6A4 revealed that the Co. dominant model explore that the genotype TT shows a significant result p-value=0.011; Odd ratio (95% CI) was 3.32(1.30 to 8.43). In the dominant model, the GT>TT genotype shows a significant effect p-value=0.009; Odd ratio (95% CI) was 2.93(1.29 to 6.64). Also, in recessive model, there was a significant result given by the genotype TT (p-value=0.036; Odd ratio (95% CI) was 2.48(1.05 to 5.85). The TNF-α genotypes with RAS under different models of inheritance did not indicate any significant results. Mean differences of serum serotonin and TNF-α was measured among different genotypes of the studied SNPs, significant results were indicted for rs1800629 only, significant difference recorded between GA with GG and AA mean differences were 184.14 and, 191.24 respectively.

From outcomes of present results, these conclusions were obtained, serum serotonin has no direct role in RAS development while serum TNF-α has an effective role, correlation between serotonin and TNF-α synergistically strengthen its relation to occurrence of RAS. Otherwise, SCL6A4 (rs 6354) polymorphism at mutant homozygous TT may be one of the underlying causes of RAS development, furthermore carriers of genotypes mutant GA and AA of rs1800629 play an important role on the elevated levels of TNF-α, that is considered as a predictive factor for developing of RAS.

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List of Abbreviations

ABBREVIATIONS	DETAILS
5-HT	Serotonin
5-HTT	Serotonin transporter
5-HTTLPR	Polymorphism of the promoter region of 5-HTT
ARMS-PCR	Amplification Refractory Mutation System
AS	Ankylosing spondylitis
AUC	Area under the curve
CNS	Central Nervous system
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
GIT	Gastrointestinal tract
HAD	Hamilton Anxiety Depression
HLA	Human leukocytes antigen
HSP	Heat shock protein
HWE	Hardy-Weinberg equilibrium
IGS	Imerslund-Grasbeck syndrome
IL-1	Interleukin-1.

MAGIC syndrome	Mouth And Genital ulcers with Inflamed Cartilage syndrome
OR	Odds ratio
OSI	Oxidative stress index
PAI-1	Platelet activator inhibitor-1
PCR	Polymerase chain reaction
PFAPA syndrome	Periodic Fever, Aphthous stomatitis, Pharyngitis syndrome
RA	Rheumatoid arthritis
RAS	Recurrent aphthous stomatitis
ROC	Receiver operating characteristic
SLC6A4	Serotonin transporter gene
SLE	Systemic lupus erythematosus
SNPs	Single nucleotide polymorphisms
SPSS	Statistical package for social sciences
TAS	Total antioxidant status
Th1	T helper 1
TLR	Toll-like receptor
TNF-Rs	TNF-α receptors
TOS	Total oxidative status

t-PA	Tissue plasminogen activator

Chapter One

Introduction

&

Literature Review

1. Introduction

Recurrent aphthous stomatitis (RAS) is a one of the most common oral mucosal diseases characterized by recurrent and painful ulcerations on the movable or nonkeratinized oral mucosae. It more commonly affects labial mucosa, buccal mucosa, and tongue. Pathologically, RAS is considered as an iodiopathic disease. The differential diagnosis for RAS is extensive and ranges from idiopathic benign causes to inherited fever syndromes, to connective tissue disease, or even inflammatory bowel diseases [1]. A thorough medical history can assist the clinician in determining whether it is related to a systemic inflammatory process or truly idiopathic. Managing aphthous stomatitis is difficult. Hence, the authors need to study the differential diagnosis and treatment scale of aphthous stomatitis [2].

The prevalence rates of RAS ranging from 5 to 60% in different series [3]. It is slightly more common in girls and women as well as among affluent socioeconomic classes and countries. Race does not appear to be a factor in the disease. Age of onset may be during childhood, but more commonly in the second and third decade of life, becoming less common with advancing age [4,5].

The etiopathogenesis of RAS remains unclear and it is considered to be multifactorial. Genetically mediated disturbances of the immunity play an important role [6]. Modification factors of the immunologic responses in RAS include microbial infections, food allergies, nutritional deficiency, hormonal imbalance, mechanical injuries, and stress [7].

Serotonin (5-HT) is a monoamine found in both the central nervous system (CNS) and in the periphery. It is synthesized from the dietary amino acid tryptophan by sequential hydroxylation and decarboxylation,

and is then stored in presynaptic vesicles in neurons [8]. Serotonergic cells are found in the cardiovascular system, red blood cells, and the central and peripheral nervous system [9].

Serotonin transporter gene (solute carrier family 6 member 4 "SLC6A4")-L/S polymorphism is associated with susceptibility to RAS. These variants could be potential predictors of RAS and could be used for the developing clinically effective genetic panel for RAS [10]. Some authors demonstrated that S allele could approximately double the risk of RAS [11].

Psychological and genetic factors have been implicated in the pathogenesis of RAS [12]. Stress is one of the important factors and there is a complex and reciprocal interrelationship between stress and brain 5-HT signalling [13]. Stress affects several aspects of serotonergic signalling in the brain and conversely the serotonergic mechanisms particularly serotonin transporter (5-HTT) may affect the risk of stress response [11]. Anxiety-related traits have been attributed to sequence variability in the genes coding for 5- HT transmission in the brain [14]. Recent findings have demonstrated that depression and stress are influenced by polymorphism of the promoter region of 5-HTT (5-HTTLPR) resulting in reduced 5- HT expression and uptake. Authors findings demonstrated that RAS patients have a tendency to show polymorphism associated with anxiety-related traits [12].

The tumor necrosis factor alpha (TNF-α) is a pleiotropic proinflammatory cytokine implicated in a wide range of cellular processes including cell proliferation, survival and death [15]. This factor plays an important role in the pathogenesis of autoimmune diseases. Several studies showed that TNF-α gene promoter polymorphisms affect the susceptibility and/or severity of autoimmune diseases. The −308 G/A

TNF-α promotor polymorphism seems to be highly associated with the development of these diseases, however some discrepant results have been recorded [16]. Some authors indicated the possible role of mucosal immune system in pathogenesis of RAS [17].

Some authors investigated the clinical significance of serum interleukin-6, interleukin-17A, and TNF-α in RAS development and they found that the levels of serum interleukin-6 and tumour necrosis factor-alpha in RAS patients were significantly increased [18]. Genetics variants inflammatory agents are associated with the risk of RAS. Most of TNF-α gene polymorphisms are thought to affect the susceptibility and/or severity of different human diseases like autoimmune diseases, however some discrepant results have been recorded [11]. Some study suggested that the TNF-α gene polymorphism is an indicator for the susceptibility of RAS [19].

1.1 Recurrent aphthous stomatitis

Recurrent aphthous stomatitis is chronic inflammatory disease of the oral mucosa. It is characterized by painful mouth ulcers that cannot be explained by an underlying disease [20].

This condition has a wide spectrum of severity and frequency of recurrence. Clinically, RAS consists of solitary or multiple nonspecific ulcers, usually on nonkeratinized oral mucosa [21] Oral aphthous ulcers are benign lesions and they are found in all ethnic groups and geographic locations worldwide [22]. Figure (1-1) shows aphthous ulcers.

Figure (1-1): Aphthous ulcers on the labial mucosa are revealed (when the lower lip is retracted) with erythematous "halo" surrounding ulcers [20].

It has been proposed that aphthous ulcerations represent an autoimmune reaction and may be precipitated by stress or hormonal changes. They can be associated with various systemic conditions such

as vitamin deficiencies, iron deficiency, and inflammatory bowel diseases [23].

Recurrent oral mucosal ulcers require a proper differential diagnosis to rule out other possible causes before RAS is diagnosed. Its pathogenesis is unknown, but multiple factors are considered to play a part. There are no standardized treatments for this condition and none of the treatments are curative. The goal of any treatment should be to alleviate pain, reduce the duration of ulcers, and prevent recurrence [24].

1.1.1. Classification of Recurrent Aphthous Stomatitis

The RAS can be classified, according to the morphology and characteristics of the ulcerative tissue, into three types [6]:

1.1.1.1 Minor Aphthous Ulcer

Minor aphthous ulcer is the most prevalent form and typically occurs in patients who are (5-19) years old. It is characterized by a few, superficial, round ulcerations (< 10mm in size) and is accompanied by a gray pseudomembrane and erythematous halo [25]. Minor aphthae are usually confined to the lips, tongue, and buccal mucosa [26].

According to the sex ratio, men and women are equally affected. The number of ulcers is usually (1-5). Scarring does not occur, and the ulcer heals within (10-14) days. If recurrence occurs, it will occur between 4-14 months [27]. Figure (1-2) shows this type below.

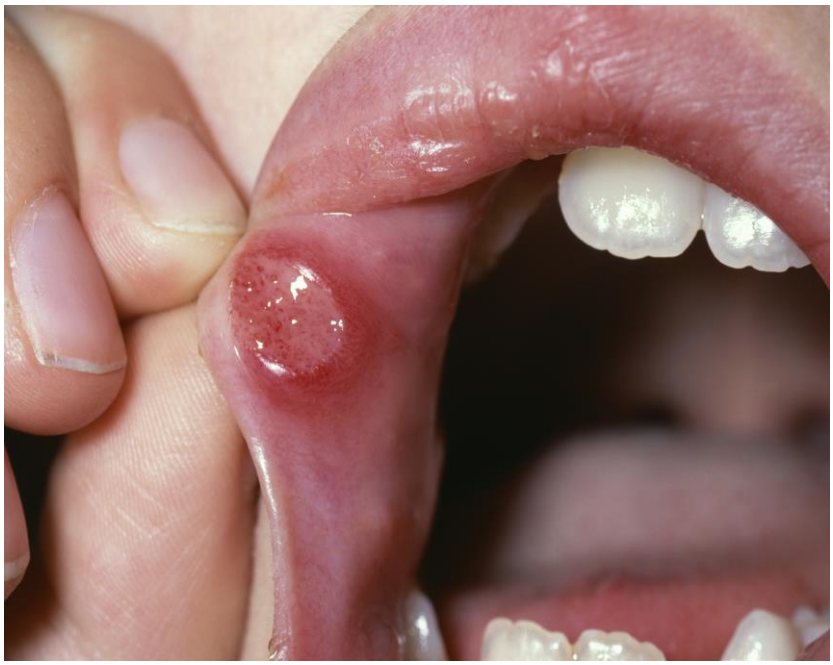


Figure (1-2): A minor aphthous ulcer (canker sore) on the inside of the lip. The ulcer depicted is larger than a typical minor aphthous ulcer [28].

1.1.1.2 Major Aphthous Ulcer

Major RAS is a severe form of RAS, also known as periadenitis mucosa necrotica. There are 10% of the affected patients present with this complaint. These ulcers usually occur on the lips, cheeks. tongue, palate, and pharynx. It just like a minor aphthous ulcer, the sex ratio in men and women is equal [2].

The age of onset is approximately between (10-19) years. The number of ulcers is usually (1-10) and the size is greater than 10 mm. If recurrence occurs, it will occur in less than a month. They persist for up to 6 weeks and scarring may or may not occur [29].

Large ulcers may take a longer time to resolve and mostly heal without scarring. They can be mistaken as malignant lesions due to their

clinical appearance [30]. Major RAS usually appears after puberty. It is chronic and persists for up to 30 years [31]. This type is shown in figure (3-3).

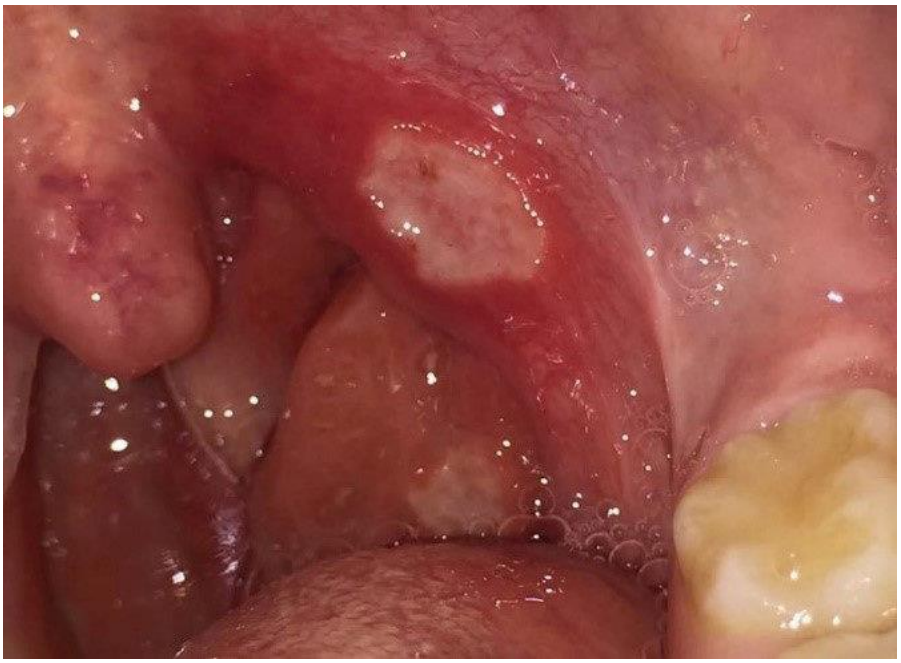


Figure (1-3): Major aphthous ulcers on palatoglossal arch prior to treatment (28)

1.1.1.3 Herpetiform Aphthous Ulcer

This is a rare form (<5% of cases). The aphthae tend to occur in clusters or crops consisting of 10-100 ulcers and the size is greater than 10 mm [32,33]. Clusters may be small and localized, or they may be distributed throughout the soft mucosa of the oral cavity. These clusters occur predominantly on non-keratinized mucosa (32). Scarring can occur following the fusion of ulcers. If recurrence occurs, it will occur in less than a month. Despite the name, there is no association with herpes viruses (33). It is important to differentiate these ulcers from herpes simplex virus (HSV), which also may appear as recurrent crops (32). Figure (3-4) shows this type.

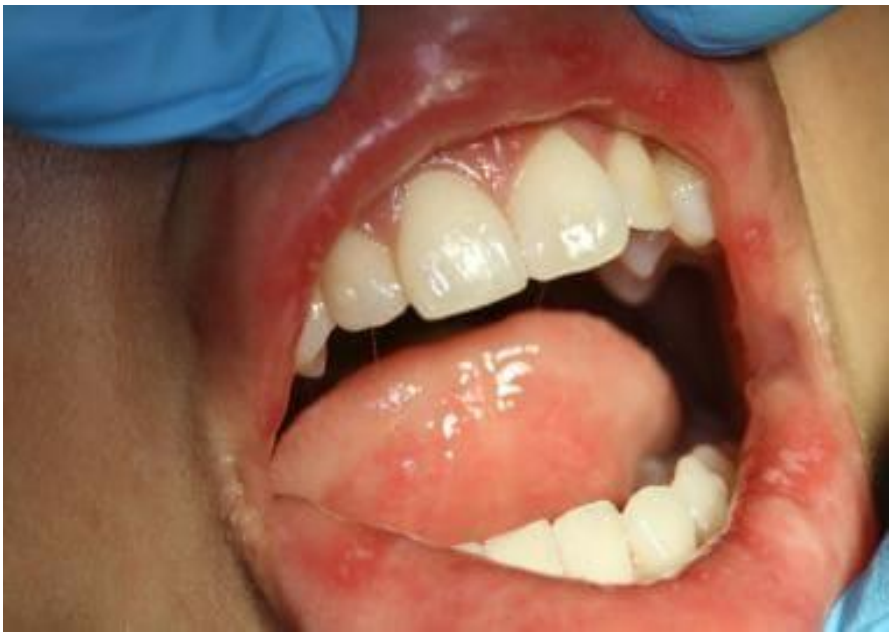


Figure (1-4): Herpetiform aphthous ulcer: grouped and single tiny white to yellow ulcers scattered on the labial mucosa and on the ventral aspect of the tongue (28).

1.1.2 Epidemiology of Recurrent Aphthous Stomatitis

Approximately 20% of the general population is affected by RAS, but incidence varies from 5% to 50% depending on the ethnic and socioeconomic groups studied. Children with RAS-positive parents have a 90% chance of developing RAS compared with 20% in those with RAS-negative parents (1972, 1997) [34,35].

Epidemiologic studies had shown that the prevalence of RAS is influenced by the population studied, diagnostic criteria, and environmental factors. In children, the prevalence of RAS may be as high as 39% and is influenced by the presence of RAS in one or both parents (Miller MF *et al,* 1980) [36]. In children of high socioeconomic status,

RAS is five times more prevalent and represents 50% of oral mucosal lesions as recorded in previous cohort study (1988, 2009) [37,38]

Kleinman *et al* (1994) reported that the point prevalence of RAS was 1.23% while lifetime prevalence was 36.5% on the basis of the results of oral mucosal examinations on a probability sample of 40 693 USA schoolchildren performed as part of the National Survey of Oral Health in USA Schoolchildren, 1986–1987 (OHSC) [39]. The prevalence of RAS is influenced by the population studied, diagnostic criteria, and environmental factors (1998) [40].

Epidemiologic studies indicated that the prevalence of RAS is between 2% and 50% in general population; most estimates fall between 5% and 25%. While in selected groups, such as medical and dental students, it has been observed with a frequency as high as 50% to 60%. The onset of RAS seems to peak between the ages of 10 and 19 years before becoming less frequent with advancing age. This finding led some investigators to theorize that stress during student life is a major factor in RAS, although the differences due to age changes should also be considered (Ship JA *et al* 2000) [41].

Activities of daily living affect the prevalence of RAS. RAS prevalence was higher (male, 48.3%; female, 57.2%) among professional-school students than in the same subjects 12 years later when they had become practicing professionals. This finding led some investigators to theorize that stress during student life is a major factor in RAS, although the difference in age groups should also be considered (Akintoye SO *et al*, 2005) [42].

1.1.3 Etiology of Recurrent Aphthous Stomatitis

There are many hypotheses that are put forth for the etiology of RAS. There is no conclusive evidence regarding the exact etiopathogenesis. Its etiological factors could be considered as host or environmental factors [43].

1.1.3.1 Host Factors: These are including:

A. Genetic Factors

There is a genetic predisposition for RAS and more than 40% of affected individuals have first degree relatives with RAS [44]. The likelihood of RAS is 90% when both parents are affected, but only 20 % when neither parent has RAS. A family history is evident in some patients. RAS are highly correlated in identical twins [45].

Human leukocytes antigen (HLA) subtypes were found to be closely associated with RAS [46]. Psychological factors such as stress and anger play a role in its manifestation. The serotonergic mechanisms particularly the *SLC6A4* gene may affect the risk of psychological alterations and stress response [11].

Most of the TNF-α gene polymorphisms are thought to affect the susceptibility and/or severity of different human diseases like the association with the development of autoimmune diseases [47]. There was certain study that showed that inheritance of specific gene polymorphisms for TNF-*α* does not appear to be a significant factor in determining susceptibility to minor RAS [48].

B. Food Allergy

Foods such as chocolate, coffee, peanuts, cereals, almonds, strawberries, cheese, tomatoes (even the skin of the tomatoes) and wheat flour (containing gluten) may be implicated in some patients (43). In one study of patients with RAS who previously were diagnosed in patch tests as reactive to agents such as benzoic acid, 50% showed clinical improvement when certain foods were excluded from the diet [49].

C. Vitamin Deficiency

Hematinic (iron, folic acid, vitamins B-6 and B-12) deficiencies were twice as common in patients with RAS [50]. As many as 20% patients with RAS had a hematinic deficiency. Lower dietary intake of folate and vitamin B-12 is more common among persons with aphthous ulcers and treatment with 1000 µg/d has shown benefit in individuals regardless of serum vitamin B-12 levels [51].

D. Immune Dysregulations

The immune dysregulations may play a significant role but no conclusive evidence has been noted [52]. Cytotoxic action of lymphocytes and monocytes on the oral epithelium seems to cause the ulceration, but the trigger remains unclear. Upon histologic analysis, RAS consists of mucosal ulcerations with mixed inflammatory cell infiltrates. T-helper cells predominate in the pre-ulcerative and healing phases, whereas T-suppressor cells predominate in the ulcerative phase [53].

There is reduced response of patients' lymphocytes to mitogens. There may be alterations in the activity of natural killer cells in various stages of disease [54]. Increased adherence of neutrophils and reduced quantities and functionality of regulatory T cells in tissue with lesions and release of TNF-α is seen [55].

There is significant involvement of mast cells in the pathogenesis of RAS. Reduced cellular expression of heat shock protein 27 (HSP) and interleukin 10 is seen in aphthous lesions [56,57]. There is an increase in the Toll-like receptor (TLR) activity in RAS [58].

Oxidative stress markers (glutathione and malondialdehyde) show altered levels and impaired balance [59]. Serum IgE levels were found to be increased in RAS patients by several investigators (52). It was reported that increased IgE concentrations might be related to cell-mediated phenomena in the immunopathogenesis of RAS. The expression of protein C, protein S and D-dimer were increased, while tissue plasminogen activator (t-PA) was reduced in patients with RAS. Remarkably, the expression of Platelet activator inhibitor-1 (PAI-1) was significantly elevated in RAS patients compared with that in healthy controls [60]. The previous results suggested the abnormal fibrinolytic activity is due to increased inhibition of t-PA [61].

E. Psychological stress:

Psychological stress may play a role in the manifestation of RAS as a trigger or a modifying factor [62]. A study found a high correlation between anxiety, depression, and psychological stress with symptoms of RAS [63].

The previous studies have suggested that psychological disturbances such as stress and anxiety could play a role in the onset and recurrence of RAS lesions. However, the obtained results were rather varied and conflicting [64].

1.1.3.2 Environmental Factors: These factors include:

A. Trauma

Local trauma may play a role in initiating the mucosal injury which leads to ulcers in RAS patients. It was suspected that mechanical injury would lead to ulcers in patients prone to RAS when compared with normal controls and experimental biopsies failed to disclose any histological differences between mechanically induced and spontaneous ulcers [65].

B. Infections

The possible immunopathological destruction of oral mucosa by *viridian streptococci* was under consideration until 1986 but was disproved [66]. L-form of *Streptococcus sanguinis* has been implicated, as has autoimmunity to the oral mucosal homogenate. A common or cross-reactive antigen between streptococci and oral epithelium has been suggested and demonstrated between the streptococcal 60–65 kD HSP and oral mucosal tissue. Significant increase in serum antibodies to HSP has been detected in patients with RAS [67].

1.1.4 Pathogenesis of Recurrent Aphthous Stomatitis

Several theories describing the etiopathogenesis of RAS have been described in several studies. The pathogenesis of RAS is multifaceted with significant physiological interplay between the immune system, genetics, and environmental factors. Similar to other chronic inflammatory conditions, deoxyribonucleic acid (DNA) damage secondary to oxidative stress is thought to play a large role in recurrent ulcerations. In a case-control study, total oxidative status (TOS), total

antioxidant status (TAS), and the TOS:TAS ratio (oxidative stress index, OSI) were used as parameters to assess oxidative damage in RAS patients against unaffected controls. The results strongly suggested that RAS patients have a systemic imbalance in the oxidant-to-antioxidant ratio favouring oxidative damage [68].

The cause for this imbalance is likely multifactorial. Evidence also suggests an immunological basis for the chronic inflammation in RAS patients. It is currently thought that an unknown antigen stimulates keratinocytes, resulting in cytokine secretion and leukocyte chemotaxis as in figure (1-5). TNF-α has been found to be significantly increased in the saliva of RAS patients. A recent study explored the significance of single nucleotide polymorphisms (SNPs) in the genes for proinflammatory cytokines IL-1 and IL-6 in RAS. The average frequency of IL-6 C-174C haplotype, which is associated with an increase in IL-6 secretion, was detected in higher amounts in affected patients than in controls [69].

This suggests a genetic component to the immunopathogenesis of RAS. Further implicating a genetic component, there is evidence that RAS may be associated with a specific HLA haplotype [70].

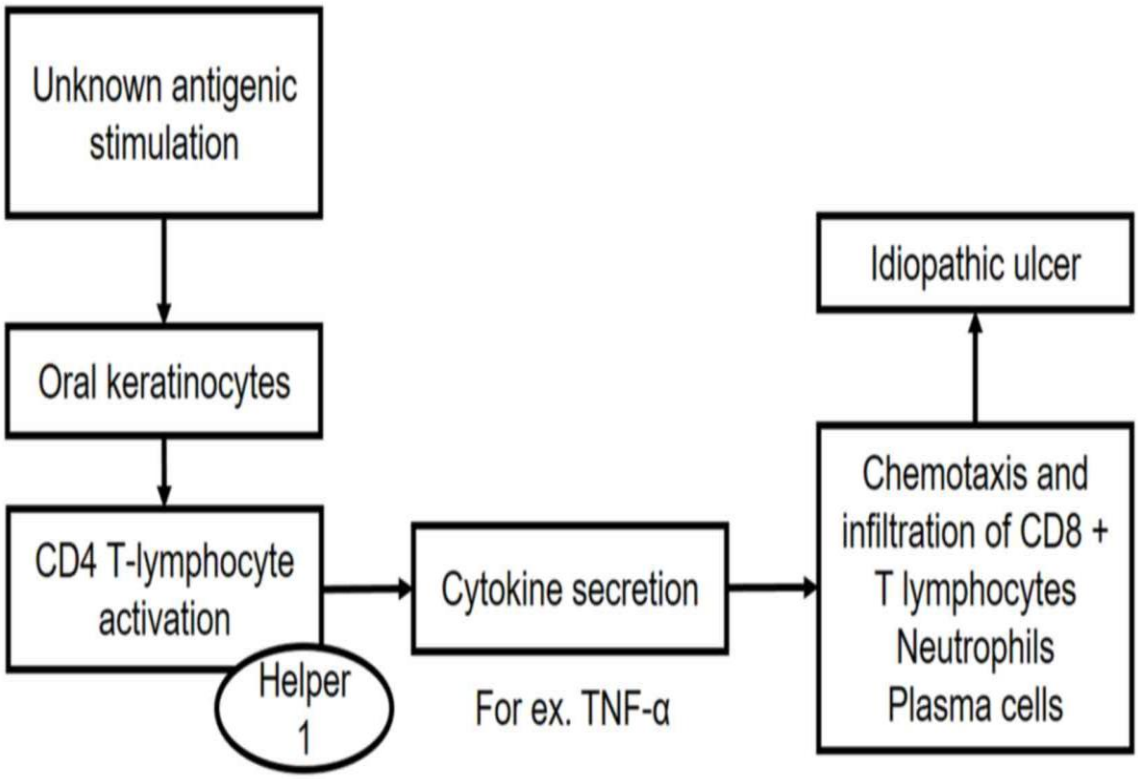


Figure (1-5) Cell-mediated immunity in the pathogenesis of recurrent aphthous stomatitis [71].

Lymphocytic cells infiltrate the oral epithelium and edema develops as a result of inflammatory stimuli. Keratinocyte vacuolization and localized vasculitis cause a papular swelling. The papule ulcerates and is infiltrated by neutrophils, lymphocytes and plasma cells, followed by healing and regeneration of the epithelium [71].

1.1.5 Oral Aphthous-Associated Syndromes

i. Behçet's disease: It is a multisystemic, chronic, relapsing vasculitis that affects nearly all organs and systems. It is associated with multiple

oral and genital ulcers, arthritis, hematemesis, melena, and epigastric pain as predominant manifestations [60].

ii. PFAPA syndrome [**P**eriodic Fever, **A**phthous stomatitis, **P**haryngitis (or called sore throat) and cervical **A**denitis (represent inflammation of the lymph nodes in the neck)]. RAS is a part of PFAPA syndrome which is regarded as a nonhereditary disease of unknown etiology [72].

iii. MAGIC syndrome (Mouth And Genital ulcers with Inflamed Cartilage syndrome). This syndrome is a cutaneous condition [73].

iv. Imerslund-Grasbeck syndrome (IGS): It is characterized by Juvenile megaloblastic anaemia due to vitamin B12 deficiency and proteinuria. All the three cases of IGS were associated with RAS. The cause of RAS in IGS was inconclusive [74].

v. Sweet's syndrome: It is also known as acute febrile neutrophilic dermatosis and is characterized by fever, neutrophil leukocytosis, erythematous skin plaques or nodules and often; classical RAS (73).

vi. Celiac disease: This disease is caused by gluten sensitivity of the small intestines. Celiac disease prevalence (40%) in patients with RAS is higher than in the normal population [75].

vii. Crohn's disease: The intraoral involvement in Crohn's disease is observed in approximately 9% of cases and oral inflammation precedes intestinal symptoms in about 60% of these patients [76].

1.1.6 Signs and Symptoms of Recurrent Aphthous Stomatitis

The signs and symptoms of RAS include:

- Discomfort and prodromal sensations such as burning, itching, or stinging, sometimes preceding lesion appearance with pain which is worsened by physical contact and certain acidic or abrasive foods and drinks [77].
- The ulcers are typically well-circumscribed and commonly localized to the inner lips, cheeks, and tongue. No fever, rash, headache, or lymphadenopathy [78].
- Uncomfortable speaking and chewing when there are lesions on the tongue and painful swallowing when ulcers are found on the soft palate, back of the throat, or oesophagus [77] with no detectable systemic symptoms or signs [79].
- Episodes usually occur about 3–6 times per year [80]. However, severe disease is characterized by virtually constant ulceration and may cause debilitating chronic pain interfering with comfortable eating [77].
- The ulcers typically begin as erythematous macules (reddened flat area of mucosa) over the course of 1-2 days, then gradually expands to its maximal size and develop into ulcers which covered with a yellow-grey fibrinous membrane. The size, number, location, healing time, and periodicity between episodes of ulcer formation are all dependent upon the subtype of RAS [81].

1.1.7 Diagnosis of Recurrent Aphthous Stomatitis

The correct diagnosis of RAS is dependent on a detailed and accurate clinical history and examination of the ulcers. Furthermore, it is necessary to carry out an external examination including palpation of the cervical lymph nodes. The important features to be noted when examining a patient with oral ulceration include family history, frequency of ulceration, duration of ulceration, number of ulcers, site of ulcers (non-keratinized or keratinized), size and shape of ulcers, associated medical conditions, genital ulceration, skin problems, gastrointestinal disturbances, drug history, edge of ulcer, base of ulcer, and surrounding tissue. Furthermore, the investigation tests for patients with persistent RAS including hemoglobin and full blood count, erythrocyte sedimentation rate/C-reactive protein, serum B12, serum/red cell folate, anti-gliadin, and anti-endomysial autoantibodies. Clinical assessment of an ulcer includes inspection and palpation, which complement each other. The base of the ulcer can be necrotic, granular purulent, or covered with mucus [82].

1.2 Recurrent Aphthous Stomatitis-Associated Biochemical and Molecular markers

1.2.1 Serotonin

Serotonin is a monoamine neurotransmitter involved in the regulation and control of various complex biological, physiological and psychological processes [83].

1.2.1.1 Structure of Serotonin

The molecular formula of 5-HT is [$C_{10}H_{12}N_2O$] with molecular weight of [176.21 g/mol]. Its common abbreviation (5-HT) came from chemical name (5-hydroxytryptamine). The attribution for the discovery of 5-HT is that it occurred serendipitously by Vittorio Erspmarer during his attempt to purify extract from enterochromaffin cells in 1935 [84]. The structure of 5-HT is demonstrated in figure (1-6).

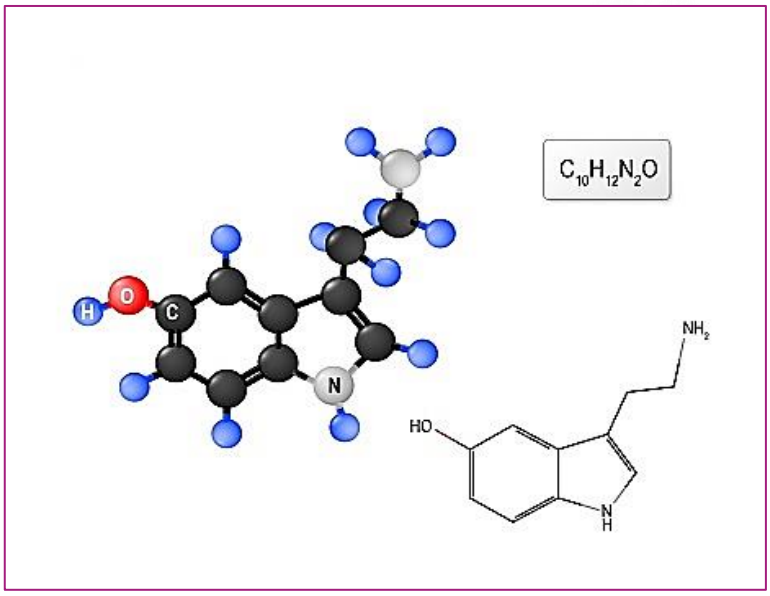


Figure (1-6): Structure of Serotonin [84]

1.2.1.2 Serotonin Biosynthesis

Biochemically, the indole amine molecule derives from the amino acid tryptophan, via the (rate-limiting) hydroxylation of the 5- position on the ring (forming the intermediate 5-hydroxytryptophan), and then decarboxylation to produce 5-HT [85], as shown in figure (1-7).

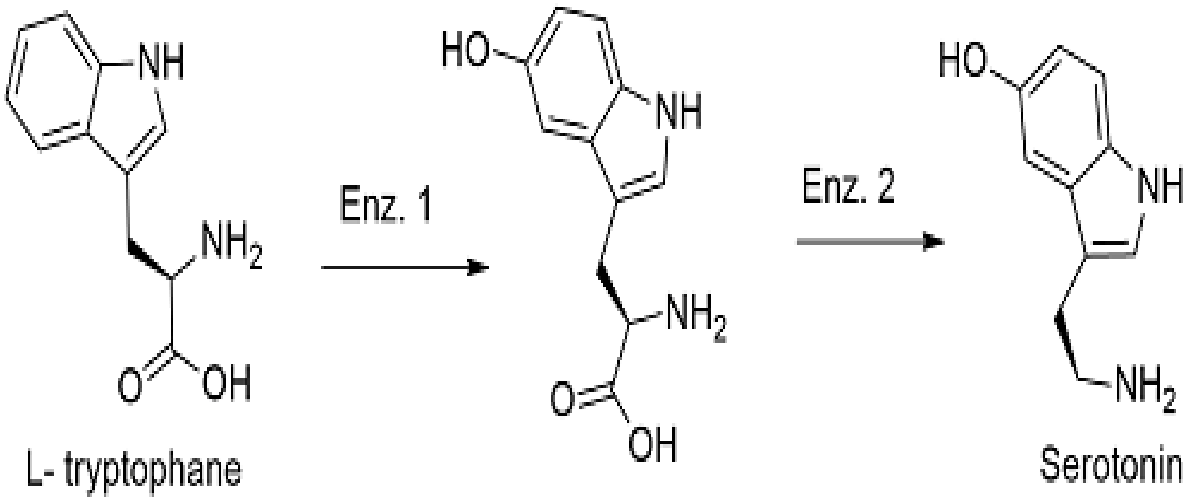


Figure (1-7): Biosynthesis of Serotonin: Enz. 1=Tryptophan hydroxylase, Enz. 2=Aromatic amino acid decarboxylase [86]

Early studies have shown that hydroxylation and decarboxylation occur almost instantaneously in the presence of tryptophan [84].

The 5-HT is primarily found in the enteric nervous system located in the gastrointestinal tract (GIT). However, it is also produced in the CNS, specifically in the raphe nuclei located in the brainstem, Merkel cells located in the skin, pulmonary neuroendocrine cells and taste receptor cells in the tongue. Additionally, 5-HT is stored in blood platelets and is released during agitation and vasoconstriction, where it then acts as an agonist to other platelets [86].

1.2.1.3 Mechanism of Action of Serotonin

The various functions of 5-HT in the CNS relate to the action of the serotonergic system on the forebrain, brainstem, and cerebellum. 5-HT, is a direct-acting neurotransmitter, is commonly stored in presynaptic vesicles. Upon activation of the nerve by adjacent nerve impulses, 5-HT

is released into the synaptic cleft, where it can bind to postsynaptic receptors [87].

These postsynaptic 5-HT receptors, also known as 5-hydroxytryptamine receptors, either act as G-couple protein receptors or ligand-gated ion channels. This activation ultimately allows activation of a second intracellular messenger cascade producing either an excitatory or inhibitory response. There are seven subtypes of 5-HT receptors present in the body [88].

Most subtypes exhibit heterogeneity and further subdivide into 5-HT1A, 5-HT2B, 5-HT3, etc. Six of these subtypes involve G-protein-coupled receptors. The 5-HT receptor is unique in that it involves a ligand-gated Na/K ion channel similar to gamma-aminobutyric acid (GABA), and *N*-methyl-d-aspartic acid [89].

The 5-HT1 and 5-HT5 receptors negatively couple with adenylyl cyclase; the activation of these receptors downregulates cyclic AMP. 5-HT receptor upregulates the inositol triphosphate and diacylglycerol pathways, resulting in intracellular Ca release. A combination of 5-HT4, 5-HT6, and 5-HT7 receptors activate adenylyl cyclase, increasing cAMP activity [90].

The Na/K cation channel associates with 5-HT results in plasma membrane depolarization. The termination of serotonergic activity is facilitated by the reuptake of 5-HT from the cellular synapse [84].

The 5-HT is synthesized and stored within the CNS in the presynaptic neurons (serotonergic neurons, pineal gland, and catecholaminergic neurons). The 5-HT is present in nine groups of cell bodies isolated to the pons and midbrain. The raphe nuclei are the major nuclei, possessing both ascending serotonergic fibres that project to the

forebrain as well as descending fibres extending to the medulla and spinal cord. A small number of serotonergic nuclei also reside in reticular formation with fibres that remain within the medulla [91], as in figure (1-8)

The 5-HT binding to the autoreceptors acts as negative feedback against the further release of 5-HT into the synaptic cleft. The highly selective 5-HTT, which is located on the presynaptic membrane, functions to remove 5-HT from the synaptic cleft [92].

Once transported into the presynaptic neuron, 5-HT is recycled back into presynaptic vesicles where it is protected from metabolism. Metabolism by monoamine oxidase (MAO) occurs within the cytosol of the neuron. An alternate pathway for 5-HT exists in the pineal gland; it converts to melatonin [88].

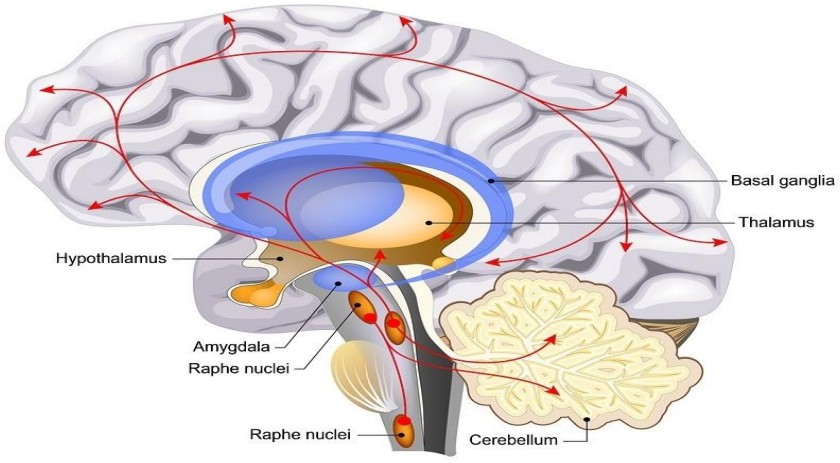


Figure (1-8): Serotonin Pathway

The 5-HT is originating from enterochromaffin cells is released into the portal circulation and undergoes rapid elimination from the plasma by way of uptake into platelets and liver metabolism. The 5-HTT on the platelet membrane and enterochromaffin cells function to uptake 5-HT into those cells. The 5-HT that escapes uptake and liver metabolism reaches the lung, where it then undergoes metabolism [92].

1.2.1.4 Biochemical Functions and Clinical Significance of Serotonin

Serotonin plays a critical role in the human body. Over the last 70 years, researches have been able to obtain a great understanding of which disease processes are influenced by this neurotransmitter, as well as its therapeutic properties in potential medical interventions. It appears to play an essential role in the CNS and the body's general functioning and, in particular, the GIT. Studies have shown links between 5-HT and bone metabolism, the production of breast milk, liver regeneration, and cell division. The 5-HT induces changes in the cell by its action on the serotonergic receptors, which are coupled to different proteins mediating intracellular changes [93]

There are several biological functions of 5-HT such as influencing learning, memory, happiness, and reward as well as physiological processes such as regulation of sleep, behaviour, and appetite [91]. Stress affects several aspects of serotonergic signalling in the brain [13].

Some authors, also showed that the social defects like social anxiety are associated with impaired serotonergic signalling, in particular, reduced receptor expression, and increased 5-HT level in a dorsal raphe projection area. These findings underscore the susceptibility of social behaviours and serotonergic pathways to early stress [94].

Stress is one of the most important causes lead to RAS development [95]. Tryptamine, an endogenous metabolite that can induce the release of 5-HT, was significantly increased in the saliva of patients with RAS, suggesting that imbalanced tryptophan metabolism may be associated with the incidence of oral ulcers [96]. Recent studies have shown that elevated salivary 5-HT is positively correlated with detrimental psychological factors including depression and stress [97].

Additionally, depression is reportedly an important psychological factor in the pathogenesis of RAS, and an increased tryptamine level may play a role in occurrence of RAS caused by negative emotions. However, the exact molecular mechanism of how the salivary tryptamine and tryptophan metabolism pathways are involved in the onset of RAS remains unclear and requires further study. The study had indicated that the serotonergic mechanisms particularly 5-HTT may be associated with RAS [98]. The 5-HT has various important functions in the body including:

A. Bowel function:

Most of 5-HT in the body is in the GI tract, where it regulates bowel function and movements. The human digestive tract is composed of several layers of enterochromaffin cells. These cells sense food in the stomach and release 5-HT as a response. Increased 5-HT levels in the gut cause digestive processes to increase in speed, which often occurs as a result of digesting toxin or noxious substances. It also plays a part in reducing appetite while eating [93].

B. Mood:

Serotonin plays a significant role in the nervous system and, therefore, largely affects mood. In the brain, 5-HT changes mood,

anxiety, and happiness by increasing nerve stimulation and electrical impulses. Drugs like ecstasy and LSD increase the levels of 5-HT in the brain to produce effects like increased appetite, increased sexual drive, euphoria, and even hallucinations [92].

C. Clotting:

When 5-HT is released into the blood, it is often absorbed by platelets rather than remaining free 5-HT. The effect of 5-HT has on the platelets is similar to those produced by the interaction of platelet factor 2 and platelets. The 5-HT accelerates the metabolism of fibrinogen to fibrin. This action causes platelet aggregation leading to vasoconstriction, and the result is a reduction in the blood flow and an increase in clot formation; this is one of the earliest defined functions of 5-HT [99].

D. Nausea:

When 5-HT is released into the gut faster than it can be digested, it is oven reabsorbed into the bloodstream. In the bloodstream, the neurotransmitter can interact with 5-HT3 receptors, which in turn activate chemoreceptor trigger zones. The activation of these sites causes stimulation of the brain to cause expulsion of the substance eaten; this is perceived as nausea by us [91].

E. Bone density:

Several research studies have shown that it may have links to a decrease in bone density, but the relationship lacks sufficient proof. This correlation has been hypothesized by an early study that measured the changes in mice that lacked brain 5-HT. The researchers found that these mice have severe osteopenia, while mice that only lack intestinal 5-HT have regular bone density. Humans with increased levels of blood 5-HT

have linked to increased or regular bone density. The belief is that the 5-HT1B receptor is the link between blood 5-HT and bone density [100].

1.2.1.5 Serotonin Transporter Gene

Serotonin is transported by the 5-HTT which is a type of monoamine transporter protein. It transports 5-HT from the synaptic cleft back to the presynaptic neuron [101]. It is also known as the sodium-dependent 5-HTT [102].

This protein, 5-HTT, is encoded by SLC6A4 gene which is found on chromosome 17 on location 17q11.1–q12 [103]. The 5-HTT, the SLC6A4 gene product is located on chromosome 17q11.2 [104], (figure 1-9). The Human SLC6A4 gene contains 15 exons spanning ~40 kb, while the human 5-HTT protein contains 630 amino acids with 12 transmembrane domains. It has been seen that both normal and pathological association of the *SLC6A4* gene variants was identified with human behaviours [105].

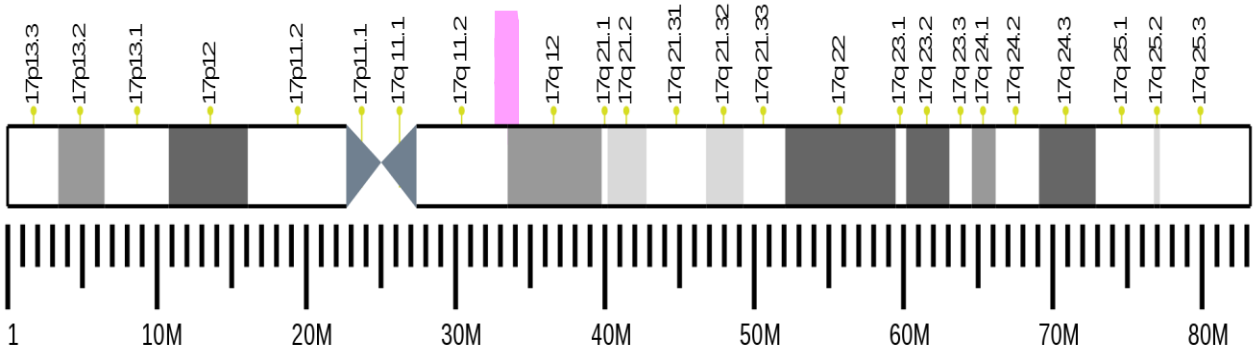


Figure (1-9): SLC6A4 Gene Location on Chromosome 17 [104]

The mutations that associated with SLC6A4 gene may result in changes in 5-HTT function, and experiments with mice have identified more than 50 different phenotypic changes as a result of genetic variation. These phenotypic changes may increase e.g., anxiety and gut dysfunction [106].

The promoter region of the SLC6A4 gene contains a polymorphism with "short" and "long" repeats in a region: 5-HTT-linked polymorphic region (5-HTTLPR) [107]. The short variation has 14 repeats of a sequence while the long variation has 16 repeats [108].

Another study stated that the short variation leads to less transcription for SLC6A4, and it has been found that it can partly account for anxiety-related personality traits [109]. This polymorphism has been extensively investigated in previous scientific studies [110].

The RAS is not recognized as a single disease, but a clinical manifestation of several pathologic conditions such as hematologic and immunologic diseases, stress, and psychological disorders [111-113]. Other authors recorded that the levels of depression, anger, and stress in the patients suffering from RAS were determined using Hamilton Anxiety Depression (HAD) scale, and the psychological factors were demonstrated to be highly associated with RAS pathogenesis [114,115].

Approximately, 46% of the patients with RAS have a positive family history 15. Local trauma, iron deficiency anaemia, folic acid deficiency, vitamin B12 resorption defect [116], neutropenia [117] and psychological factors such as stress and anger can play a role in RAS etiology [115].

The SLC6A4 gene modulates the intensity and duration of serotonergic neurotransmission, thus, this gene polymorphism can

influence the anxiety related behaviours [118,119]. It has been recently reported that the L and S variants of the promoter polymorphism modulate SLC6A4 differently. The S allele is associated with reduced transcription of SLC6A4 and consequently a reduction in 5-HT reuptake [119].

In addition to altering the expression of 5-HTT protein and concentrations of extracellular 5-HT in the brain, the 5-HTTLPR variation is associated with changes in brain structure. One study found less grey matter in perigenual anterior cingulate cortex and amygdala for short allele carriers of the 5-HTTLPR polymorphism compared to subjects with the long/long genotype [120].

In contrast, another study found no significant overall association between the 5-HTTLPR polymorphism and autism [121].

A hypothesized gene–environment interaction between the short/short allele of the 5-HTTLPR and life stress as predictor for major depression has suffered a similar fate: after an influential [122], initial report in 2003 [123], there were mixed results in replication in 2008 [124], and a 2009 meta-analysis was negative [125].

1.2.2 Tumor Necrosis Factor Alpha

Tumor necrosis factor alpha (TNF-α) is a cytokine that has pleiotropic effects on various cell types. It has been identified as a major regulator of inflammatory responses and is known to be involved in the pathogenesis of some inflammatory and autoimmune diseases. Structurally, TNF-α is a homotrimer protein mainly generated by activated macrophages, T-lymphocytes, and natural killer cells. It is

functionally known to trigger a series of various inflammatory molecules, including other cytokines and chemokines [126].

1.2.2.1 Structure of Tumor Necrosis Factor Alpha

The TNF-α is primarily produced as a 233-amino acid-long type II transmembrane protein arranged in stable homotrimers [127, 128]. From this membrane-integrated form the soluble homotrimeric cytokine is released via proteolytic cleavage by the metalloprotease TNF-α converting enzyme (TACE) [129].

The soluble 51-kDa trimeric soluble TNF tends to dissociate at concentrations below the nanomolar range, thereby losing its bioactivity. The secreted form of human TNF-α takes on a triangular pyramid shape, and weighs around 17-kDa. Both the secreted and the membrane bound forms are biologically active, although the specific functions of each is controversial. But both forms do have overlapping and distinct biological activities [130]. Crystal structure of TNF-α is in the figure (1-10).

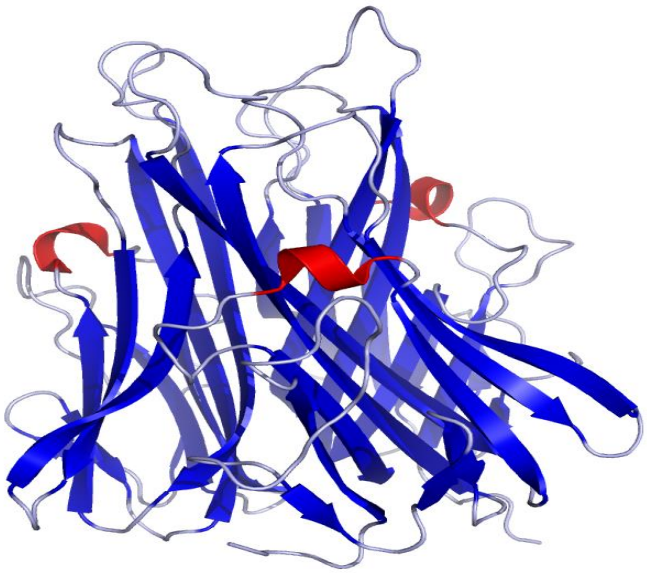


Figure (1-10): Crystal structure of TNF-α [130]

1.2.2.2 Mechanism of Action of Tumor Necrosis Factor-Alpha

The TNF-α has been widely studied and reviewed in the scientific literature regarding its participation in mechanisms involving inflammation-related cell pathways [131].

The TNF-α is produced by several cell types; however, the main producers are monocytic lineage cells, such as macrophages. This cytokine plays a key role during stationary or pathological conditions, for example, infections, lesions, inflammation, and tumor development [132]. Once released from macrophages, which constitute the first line of defence, TNF-α activates other immune cells and mediates the production of additional proinflammatory cytokines during inflammatory responses [133]. The TNF-α also has a direct impact on the intestinal epithelial barrier, since it directly disrupts the intestinal tight junctions [134].

The TNF-α belongs to the TNF superfamily. Proteins of this family can be released from the cell membrane by extracellular proteolytic cleavage and then function as cytokines. The TNF-α trimer exerts function by binding to the trimeric receptor TNFR-1 (CD120a) or TNFR-2 (CD120b). Interestingly, TNFR1 signalling is pro-inflammatory and apoptotic. It activates NF-κB, mitogen-activated protein kinases, and death signalling. The production of other pro-inflammatory cytokines (IL-1, IL-6, GM-CSF, etc.) may be induced subsequently. In contrast, TNFR2 signalling tends to be anti-inflammatory and promotes cell proliferation. It activates the canonical NF-κB signalling and PI3K/Akt pathways. Sino Biological has developed a panel of high-quality recombinant TNF-α and receptor proteins to support the study of this

cytokine. This protein may use the morpheein model of allosteric enzyme regulation [135].

1.2.2.3 Clinical Significance and Functions of Tumor Necrosis Factor Alpha

The TNF-α is an essential pro-inflammatory cytokine that plays an important role in the immune and inflammatory responses. It is involved in the conversion of T lymphocytes to T helper 1 (Th1) [136].

This factor is produced primarily by macrophages [137], but it is produced also by a broad variety of cell types including lymphoid cells, mast cells, endothelial cells, cardiac myocytes, adipose tissue, fibroblasts, and neurons [138]. Large amounts of TNF-α are released in response to lipopolysaccharide, other bacterial products, and interleukin-1 (IL-1). In the skin, mast cells appear to be the predominant source of pre-formed TNF, which can be released upon inflammatory stimulus [139].

Numerous reports suggest that factors such as stress, hematinic deficiency, trauma, genetics, and cytokines can be effective in the formation of RAS [140]. High levels of TNF-α have been reported in wound mucosa and peripheral blood of patients with aphthous ulcer [141-144]. High cytotoxic destruction of epithelial cells with TNF-α produced from peripheral blood mononuclear cells was shown in patients with RAS [116].

In addition, RAS can be prevented by inhibitors of endogenous TNF-α synthesis such as thalidomide and pentoxifylline [145]. Authors concluded that abnormal apoptosis of epithelial cells that progressed to necrosis, released the danger signals. Exposure of pathogen-specific

receptors such as TLR to these danger signals increases the production of IL-17C and TNF-α and leads to inflammation and RAS [146-148].

The effects of TNF-α were established to be associated with activation of a cascade of inflammatory events, enhancing expression of adhesion molecules and activation of neutrophils in addition to acting as a co-stimulator for T cell activation and antibody production [149].

A remarkable point is that, in most studies, salivary TNF-α levels are higher in the RAS group than in healthy individuals, so it suggests that TNF-α may be a potential salivary marker for this disease. Different results were obtained in studies that examined TNF-α level and this difference may be partly due to patient differences, RAS subtypes, research methods, or sample size [150].

It has a number of actions on various organ systems, generally together with IL-1 and IL-6. A local increase in concentration of TNF-α will cause the cardinal signs of Inflammation to occur: heat, swelling, redness, pain and loss of function. Whereas high concentrations of TNF-α induce shock-like symptoms, the prolonged exposure to low concentrations of TNF-α can result in cachexia, a wasting syndrome. This can be found, for example, in cancer patients [151].

1.2.2.4 Tumor Necrosis Factor Alpha Gene and Its Polymorphism

The gene encoding TNF-α is located in the short arm of chromosome 6 in the major histocompatibility complex class III region between the HLA-B and HLA-DR genes [152,153] figure (1-10).

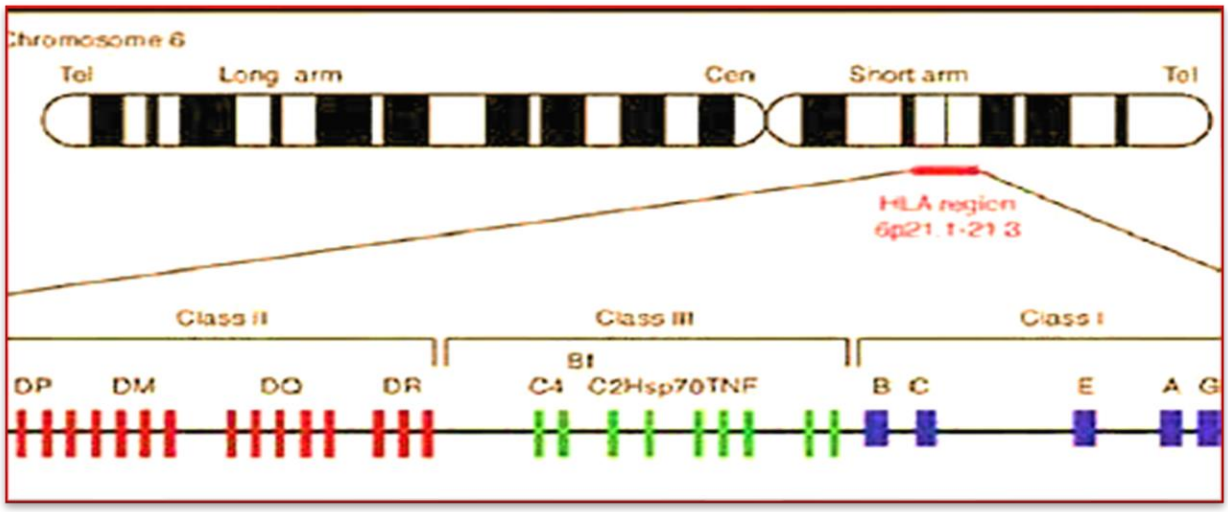


Figure (1-11): Localization of TNF- α Gene on Chromosome 6 (6p21.3) [152]

The gene for TNF-α include about 3 Kb and contains four exons (almost 80% of the protein is codified by the exon four) and three introns [151]. 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 [152].

The TNF binds to two types of outer membrane bound receptors on the target cells, TNFR1 and TNFR2, and triggers the cell survival and proinflammatory NF-κB and MAP kinases activations [154].

The roles that TNF-α play seem to be contradictory and this was related to the genetic polymorphisms in the genes regulating its production and effect [155], and the polymorphisms in TNF locus itself. It has been reported that the genetic alterations in the TNF-α locus are involved in high TNF-α production [156].

An important factor to consider in the development of RAS is the interaction of genes and cytokines as well as the effect of gene polymorphism on cytokine production. The TNF-α gene is located on the

chromosome 6 and several SNPs have been detected in its promoter region. Studies have indicated that a G-to-A mutation in the -308 promoter section is accompanied by an increase in TNF-α production [157,158].

Studies have also been conducted on the association between TNF-α gene polymorphism and susceptibility to aphthous ulcers. In some studies, a positive association was found between TNF-α polymorphism and susceptibility to aphthous ulcers [159,160]; and in other study, no association was found [161].

It was investigated that no association was found between TNF-α -308 G/A single nucleotide polymorphism and overall RAS risk except in recessive model. Recessive model is likely to be protective against RAS when compared to other models. This discrepancy in studies in some comparative models and inconsistent conclusions may be attributed to several factors. First, these studies included people from different populations in different countries (different races). Second, it may be the result of different aetiologies of RAS. Third, some studies did not use Hardy-Weinberg equilibrium (HWE) [150].

Several TNF-α polymorphisms have been identified inside the TNF-α promoter at the positions, 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) [162].

The TNF-α interacts with the TNF receptors TNF-RI and TNF-RII [163]. TNF-α receptors (TNF-Rs) are active both in membrane-bound and soluble forms, and the soluble receptors act as physiological attenuators of TNF activity [164]. With respect to their chromosomal

location, TNF-RI gene is located at 12p13 and the TNF-RII gene is located at 1p36.2 [163].

With respect to the relation between TNF-Rs and autoimmune diseases, no association was reported between TNF-RI +36 and rheumatoid arthritis (RA) in Dutch and UK Caucasian population as approved by Bayley et al. [163] and Barton et al. [167], respectively. No association was found between the TNF-RII +1690 polymorphism and susceptibility to or severity of RA in the Dutch population (Bayley et al. [163].

There is association between TNF-α genetic polymorphisms and autoimmune diseases. In systemic lupus erythematosus (SLE) patients, an increased level of TNF-α was reported and strongly correlated with the parameters of disease activity [166]. A significant genetic association between TNF-α promoter −308A/G polymorphism and SLE susceptibility in Asian populations, and in European-derived populations was detected in Zou et al. [167] and Lee et al. [168], meta-analysis respectively.

In RA, TNF-α is thought to play a central role in inflammation and it has been directly implicated in the pathogenesis of this condition [169]. High concentrations of TNF-α were detected in serum and synovial fluid of RA patients and TNF-α blood concentration correlated with RA disease activity [170].

Also, studies on the relation between TNF-α polymorphisms and RA showed conflicting results. For example, susceptibility to RA was associated with the −308A allele in some studies [171,172] with the G allele in others [173], but neither with A allele nor G allele in others [174-177].

In Ankylosing spondylitis (AS), TNF-α level in blood and its expression by peripheral T cells correlated well with AS activity [178, 179]. Studies on the relation between TNF-α SNPs and AS have shown controversial results. For example, TNF-α polymorphisms had no independent effect on AS susceptibility [180, 181] but their modulating effect on TNF-α expression were well relevant to the phenotypic diversity in AS [182, 183]. In contrast, Vargas-Alarcón et al. and Shiau et al. [184], showed an association of −308G/A polymorphism with susceptibility to AS. Moreover, the A allele was thought to have a protective role against AS [185], and was associated with a lower risk of developing AS, and with the age at disease onset, disease severity and response to anti-TNF treatment [186].

So, several studies show that TNF-α gene promoter polymorphisms affect the susceptibility and/or severity of autoimmune diseases. Polymorphisms in the TNF receptors does not seem to be associated with the development of autoimmune diseases. The −308 G/A TNF-α promotor polymorphism seems to be highly associated with the development of these diseases, however some discrepant results have been recorded. Other TNF-α gene polymorphisms had little or no association with autoimmune diseases. This discrepancy might be explained by the differences in the ethnic origin or number of the studied individuals. TNF-α gene expression is controlled by the presence of some polymorphisms in its promoter region and by several types of signalling molecules or nuclear factors that interact with the TNF-α promotor region or other elements [154].

❖ *Hypothesis*

1. The *SLC6A4* gene polymorphism is implicated development of recurrent aphthous stomatitis.

2. Tumor necrosis factor alpha plays a very important mediatory role in the pathogenesis of recurrent aphthous stomatitis. So, it is a useful diagnostic marker.

3. Polymorphisms of TNF-α gene were associated with increased risk of recurrent aphthous stomatitis.

❖ *Aims of the study*

1. To evaluate the risk of *SLC6A4* gene polymorphism G>T (rs 6354) and TNF-α gene polymorphism G>A (rs 1800629) with the development of recurrent aphthous stomatitis.
2. To estimate the relationship of *SLC6A4* and TNF-α genes with the related serum levels of their proteins.

Chapter Two

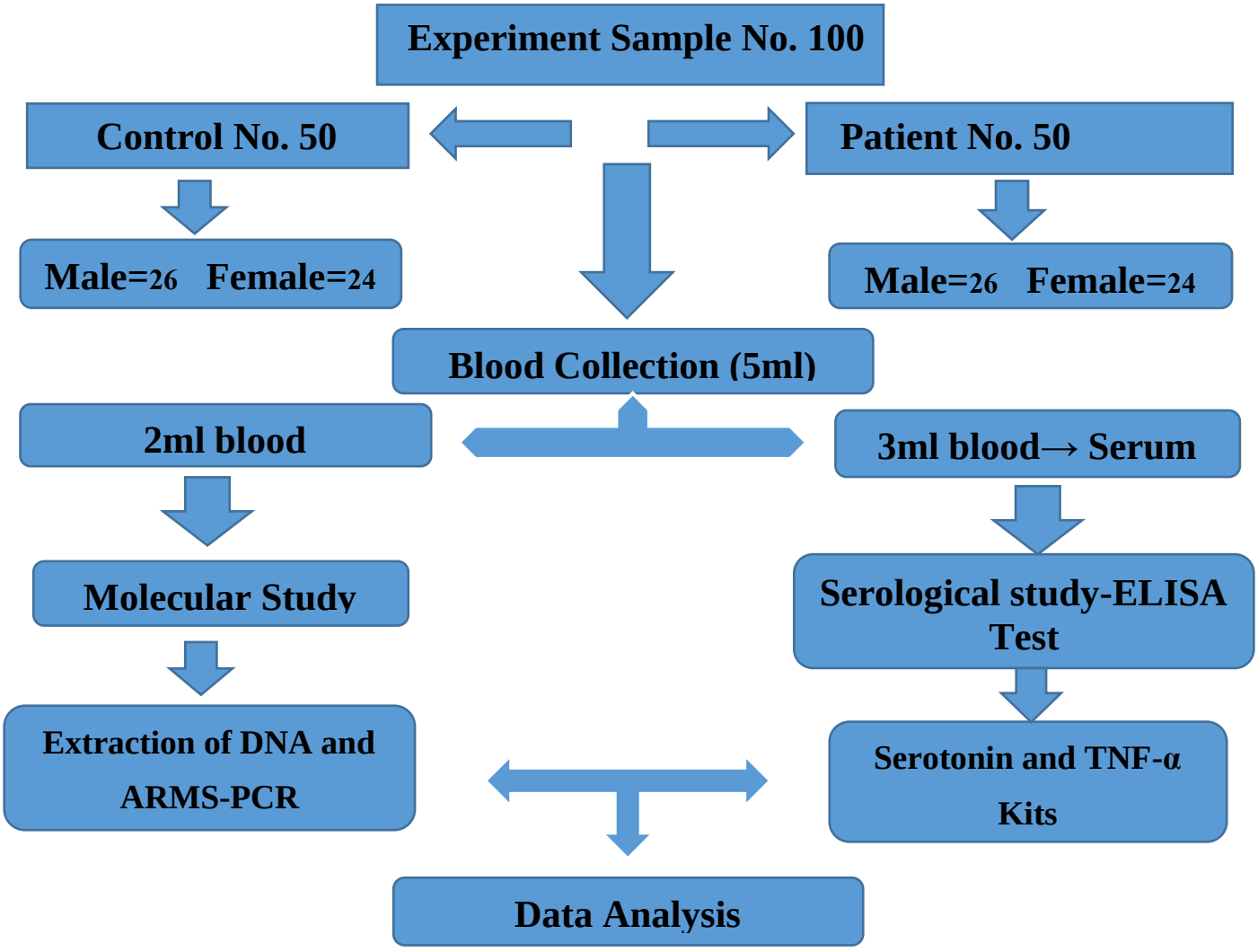
Materials & Methods

2.1 Materials

2.1.1 Study Settings

This study was carried out on patients attended to Al-Hussain Medical City and Al Hur Riahi Specialized Dental Center in Karbala Province/ Karbala city. All of patients had been diagnosed by specialist dentist during the period from 1/5/2018 to 20/5/2019. The practical side of the study was performed at the laboratory of Biochemistry Department in College of Medicine/ University of Babylon.

2.1.2. Study Design: It is a case control study.



Scheme (2-1): Major steps for the research project

2.1.3. Study Individuals

The present study included one hundred (100) participants; male and female. Their ages ranged between (18-48) years. For serological and genetic studies, these subjects were divided into two groups: the first group included 50 patients with RAS, the diagnosis was carried out according to the criteria based on the suggested signs and symptoms of RAS which were confirmed by the specialist dentist in addition to the medical history.

While the second group included apparently healthy individuals (control group) were selected from relatives and staff of Al-Hussain Medical City. The level of controls was evaluated according to the absence of clinical manifestation of RAS approved by the specialist dentist and the negative history of this condition known from their health questionnaire. Number of participants of each group, for biochemical part was (44) while for genetic part was (50).

2.1.4 Research and Sampling Ethics

The project proposal and sampling method were approved by the committee of publication ethics at College of Medicine, University of Babylon/ Iraq.

Also, this project achieves the permission of research ethics from Head of Karbala Health Department / Training and Development Center. Written informed consent was obtained from all participants prior to entering the study.

2.1.5. Data Collection

The inclusion and exclusion criteria for this study were as follows:

A-Inclusion Criteria

The participators in the present study were patients with RAS, in addition to apparently healthy controls. Ages of patients were between (18-48) years old.

B-Exclusion Criteria

Any subject with any of the followings was excluded from the study:

- Subjects with age < 18 years.
- Non-agreement.
- Family history of RAS regarding to control individuals.
- Subjects with chronic systemic diseases; like documented hypertension, proved ischemic heart disease, proved heart failure, dyslipidaemia and documented cancer patients or on chemotherapy.
- Females with pregnancy, in menopause and who take oral contraceptives

❖ ***Questionnaire***

The socio- demographic characteristics composed of age, height, weight, gender, family history, and medical history (Appendix 1).

❖ ***Anthropometric Measurements***

The participant's weight and height were measured by electronic balance and measuring tape respectively. Body Mass Index (BMI) was calculated by weight (kg) divided by the square of height (m); as shown

in equation below; weight and height were measured by the same scale for the all subjects.

$$BMI = (\text{weight in kg}) / (\text{height in meters})^2$$

2.1.6. Samples Collection

The five millilitres of venous blood were obtained by 5ml disposable syringe; 2ml of the blood were drained into EDTA tube and mixed gently and then stored at -20 ˚C and used for DNA extraction. The rest 3ml of blood were drained into gel plain tube for serum preparation, which would be used for 5-HT and TNF-α serological tests.

2.1.7. Chemicals

The chemical substances used throughout the study were listed in the table below:

Table (2-1): Chemical Substances Used in the Study

No.	Chemical substances	Origin
1	Acrylamide C_3H_5NO	Himedia (India)
2	Agarose	Conda Pronadisa (spian)
3	Ammonium persulfate $(NH_4)_2S_2O_8$	(BDH) UK
4	Bis acrylamide $C_7H_{10}N_2O_2$	SCR (China)
5	DNA extraction kit (blood)	Favorgen (Taiwan)
6	100 bp DNA ladder	Biolab (England)
7	Loading dye (bromophenol blue)	SCR (china)
8	N,N,N-,N— Tetrmethylenediamine	Bio Pure (Korea)
9	PCR Master Premix kit	Promega (USA)
10	Primer F, R	Bioneer (Korea)
11	Proteinase K	Promega
12	TNF-α and serotonin ELISA Kit	PARS BIOCHEM (China)
13	Red safe	INTRON (Korea)
14	TBE buffer	Promega (USA)
15	Water free nuclease	Bioneer (Korea)

2.1.8. Instruments and Tools

The instruments and tools used in this study with their origins were shown in the table (2-2).

Table (2-2): Instruments and tools used in this study

No.	Instruments and Tools	Origin
1	Autoclave	Haramaya/ Japan
2	Deep Freeze	GFL / Germany
3	Distiller	GFL / Germany
4	EDTA tube (5ml)	AFCO, Jordan
5	ELISA reader and washer	Biotech /USA
6	Hood	labtech / Korea
7	Horizontal gel electrophoresis (agarose)	Autto/ Japan
8	Magnetic Stirrer with Hot plate	Grant/ England
9	Incubator	Fisher Scient./ Germany
10	Micropipettes	Slamed/ Germany
11	PCR Thermo cycler	Bioneer/ Korea
12	Photo documentation	E-Graph/ Japan
13	Scandrop	Analytikjena/ Germany

14	Sensitive balance	Sartorius/ Germany
15	Vertical gel electrophoresis (SSCP)	Cleaver/ UK
16	Vortex (Electronic)	Kunkel /Germany
17	Water bath	GFL / Germany

2.2 Methods

2.2.1 Biochemical Part

The levels of 5-HT and TNF-α were assessed in the sera of RAS patients and controls by means of enzyme linked immunosorbent assay (ELISA) technique. The assessment was carried out at the Biochemistry laboratory by using an ELISA kits and the manufacturer's instructions were followed.

2.2.1.1 The Quantitative Measurement of Serotonin in Serum by Enzyme Linked Immunosorbent Assay (ELISA) Technique.

A- Assay Principle

The blood was allowed to clot for 10-20 minutes at room temperature and then centrifuged at 3000 Xg for 20 minutes. The supernatant was collected.

The kit was an ELISA. The plate had been pre-coated with human 5-HT antibody. The 5-HT present in the sample was added and bonded to antibodies coated on the wells. And then biotinylated h uman 5-HT Antibody was added and bonded to 5-HT in the sample. Then Streptavidin-HRP was added and bonded to the Biotinylated antibody. After incubation, unbound Streptavidin-HRP was washed away during a washing step. Substrate solution was then added and colour developed. The reaction was terminated by addition of acidic stop solution and absorbance was measured at 450 nm [187].

❖ Reagent Preparation

➢ Standard: the 120µl of the standard (80ng/ml) was reconstituted with 120µl of standard diluent to generate a 40ng/ml standard stock solution.

The standard was allowed to sit for 15 mins with gentle agitation prior to making dilutions. Duplicate standard points were prepared by serially diluting the standard stock solution (40ng/ml) 1:2 with standard diluent to produce 20ng/ml, 10ng/ml, 5ng/ml and 2.5ng/ml solutions. Standard diluent served as the zero standard (0 ng/ml). Dilution of standard solutions were as follows (figure 2-1 and table 2-3):

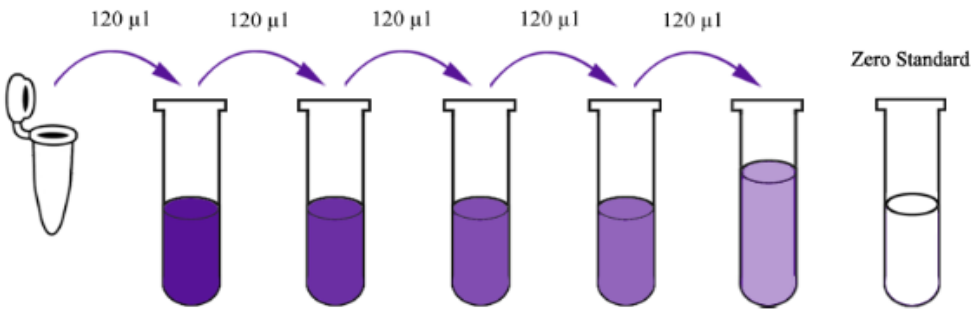


Figure (2-1): Serial dilution of standard sample of serotonin

Table (2-3): Serial dilution of standard sample of serotonin

Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
80ng/ml	40ng/ml	20ng/ml	10ng/ml	5ng/ml	2.5ng/ml

Wash Buffer: 20ml of Wash Buffer Concentrate 25x was diluted into distilled water to yield 500 ml of 1x Wash Buffer.

B- Assay Procedure

1. All reagents were brought to room temperature before use. The assay was performed at room temperature.
2. The strips were inserted in the frames for use.
3. The 50µl standard was added to standard well.
4. The 40µl sample was added to sample wells and then 10µl antibody was added to sample wells, then 50µl streptavidin-HRP was added to sample wells and standard wells. These were mixed well. The plate was covered with a sealer. Incubated 60 minutes at 37°C.
5. The sealer was removed and the plate was washed 5 times with wash buffer. Wells were soaked with 0.35 ml wash buffer for 1 minute for each wash. For automated washing, all wells were aspirated and washed 5 times with wash buffer, overfilling wells with wash buffer. Plate was blotted onto paper towels.
6. The 50µl substrate solution A was added to each well and then 50µl substrate solution B was added to each well. Plate covered with a new sealer was incubated for 10 minutes at 37°C in the dark.
7. The 50µl Stop Solution was added to each well, the blue colour changed into yellow immediately.
8. The optical density value of each well was determined immediately by using a microplate reader that set to 450 nm within 10 minutes after adding the stop solution.

❖ **Calculation of Sample Results**

The sample results were calculated by interpolation from a standard curve that was performed in the same assay as that for samples by using standard curve fitting equations for human 5-HT (figure 2-2). The equation and drawing of the standard curve were carried out using Microsoft Excel 2019.

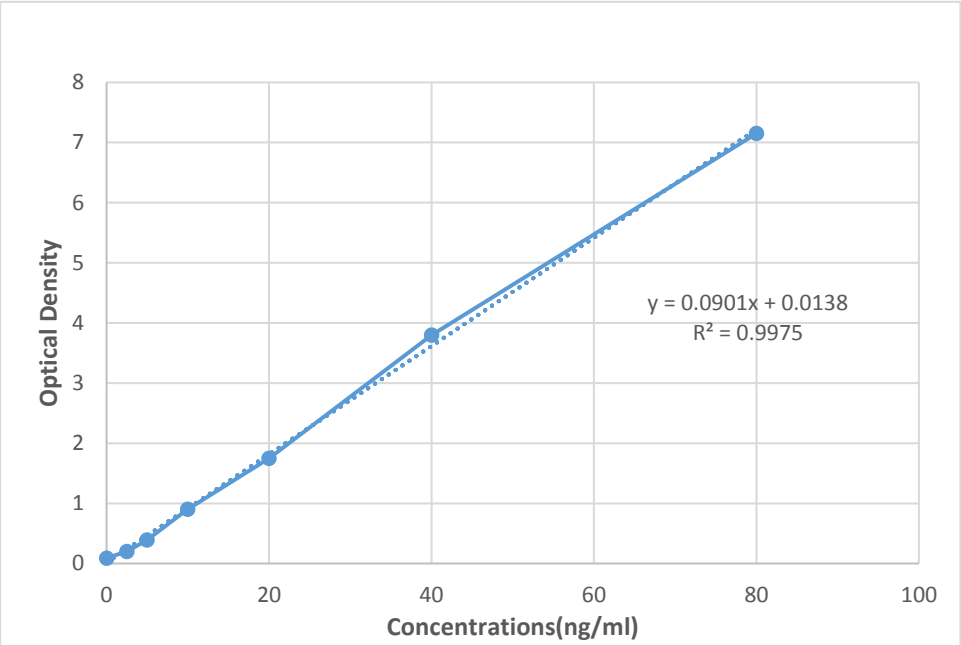


Figure (2-2): Standard curve of serotonin

2.2.1.2 The Quantitative Measurement of Tumor Necrosis Factor Alpha in Serum by Enzyme Linked Immunosorbent Assay (ELISA) Technique.

A- Assay Principle

The blood was allowed to clot for 10-20 minutes at room temperature and then centrifuged at 3000 Xg for 20 minutes. The supernatant was collected.

The TNF-α was measured by TNF-α kit which is an ELISA. The plate has been pre-coated with human TNF-α antibody. TNF-α present in the sample is added and binds to antibodies coated on the wells. And then biotinylated human TNF-α Antibody is added and binds to TNF-α in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated TNF-α antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution was then added and colour developed in proportion to the amount of human TNF-α. The reaction is terminated by addition of acidic stop solution and absorbance was measured at 450 nm [188].

❖ **Reagent Preparation**

- Standard: the 120µl of the standard (960ng/L) was reconstituted with 120µl of standard diluent to generate a 480ng/L standard stock solution.

The standard was allowed to sit for 15 mins with gentle agitation prior to making dilutions. Duplicate standard points were prepared by serially diluting the standard stock solution (480ng/L) 1:2 with standard diluent to produce 240ng/l, 120ng/L, 60ng/L and 30ng/L solutions. Standard diluent served as the zero standard (0 ng/L). Dilution of standard solutions were as follows (figure 2-3 and table 2-4):

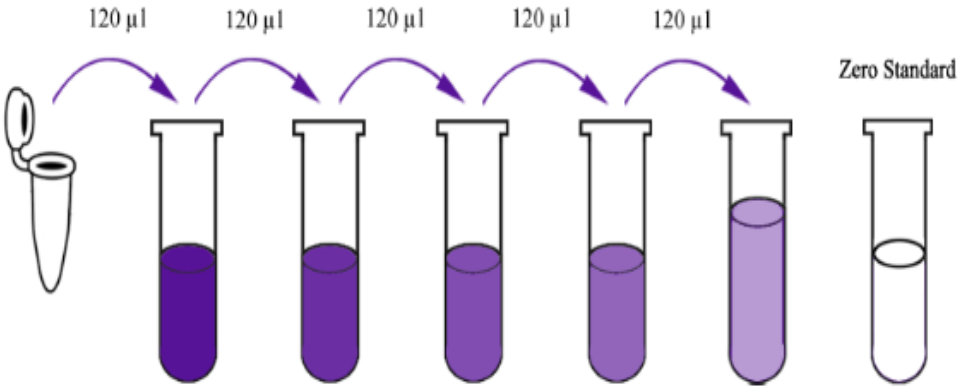


Figure (2-3): Serial dilution of standard sample of TNF-α

Table (2-4): Serial dilution of standard sample of TNF-α

Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
960ng/L	480ng/L	240ng/L	120ng/L	60ng/L	30ng/L

- **Wash Buffer:** 20ml of Wash Buffer Concentrate 25x was diluted into distilled water to yield 500 ml of 1x Wash Buffer.

B-Assay Procedure

1. All reagents, standard solutions and samples were prepared as instructed. All reagents were brought to room temperature before use. The assay was performed at room temperature.

2. The number of strips required for the assay was determined. The strips in the frames were inserted for use.

3. The 50µl standard was added to standard well.

4. The 40µl sample was added to sample wells and then 10µl anti-TNF-α antibody was added to sample wells, then 50µl streptavidin-HRP was added to sample wells and standard wells and then mixed well. The plate was covered with a sealer, incubated 60 minutes at 37°C.

5. All wells were aspirated and washed 5 times with wash buffer, overfilling wells with wash buffer. The plate was blotted onto paper towels or other absorbent material.

6. The 50µl substrate solution A was added to each well and then 50µl substrate solution B was added to each well. Plate was incubated and covered with a new sealer for 10 minutes at 37°C in the dark.

7. The 50µl Stop Solution was added to each well, the blue color changed into yellow immediately.

8. The optical density value of each well was determined immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

❖ Calculation of Sample Results

The standard curve was constructed by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and a best fit curve was drawn through the points on the graph. These calculations were performed with computer-based curve-fitting software and the best fit line had been determined by regression analysis. Figure (2-4) showed the standard curve of TNF-α.

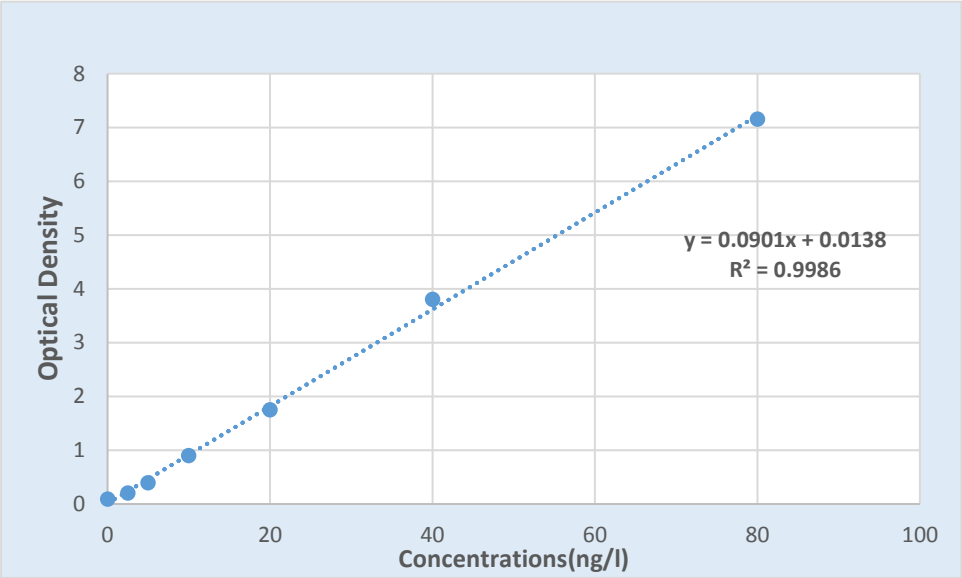


Figure (2-4): Standard curve of TNF-α

2.3 Genetic Part

2.3.1 DNA Extraction

The DNA was extracted from frozen whole blood of both control and patient groups by Faverogen Genomic DNA Purification Kit by following the protocol [189], briefly as follow:

1- A volume of 200 µl of whole blood in 1.5ml tube was added.

2- The blood tube was mixed thoroughly by pulse-vortexing with 20µl Proteinase K and 200 µl FABG Buffer.

3- The mixture was incubated at 60 ºC for 15 minutes to lyse the leukocytes, the tube was inverted 2-3 times during the incubation period.

4- The tube was centrifuged in 15000 xg for 20 seconds at room temperature.

5- Ethanol (99 %) 200 µl was added, and then mixed thoroughly by pulse-vortexing for 10 sec.

6. The tube was centrifuged in 15000 xg for 20 seconds at room temperature.

7. A Mini Column FABG was Placed to a collection tube, the mixture was transferred (including all precipitate) carefully to the FABG Mini Column.

8. Centrifugation at 6,000 x g for 1 min, then FABG Mini Column was placed to a new collection tube.

9. After that 400 µl of W1 buffer was added to the FABG mini column.

10. The tubes were centrifuged at full speed (15,000 x g) for 40 sec.

11. The supernatant was discarded.

12. Wash buffer 750 µl was added to the FABG mini column and the latter was centrifuged at full speed for 30 sec then the supernatant was discard.

13. To dry the column, it was centrifuged at full speed for an additional 3 minutes.

14. DNA was eluted by placing the FABG Mini Column in sterile 1.5 ml micro centrifuge tube and has been added of preheat elution buffer to 65°C, 150 µl of Elution Buffer was added to the membrane centre of FABG mini column, incubated at room temperature for 3 minutes and centrifuged at full speed for 2 minutes to elute total DNA.

15. The tube contain genomic DNA was stored at -20°C for long storage.

2.2.3 Estimation of Integrity and Molecular Weight of Extracted DNA

The molecular weight and the integrity of extracted DNA were determined by agarose gel electrophoresis, the electrophoresis was carried out according to [190] as described below in brief:

1. The gel (2%) was prepared by dissolving 2 gm. of agarose in100 ml of 1X TBE buffer and heated for 5 minutes.

2. A volume of 4.5µl of red safe was added to the gel and mixed by swirling

3. The homogenized agarose then cooled to 55 ºC by water bath.

4. The gel then poured to the gel try and let to polymerize for 20 minutes

5. The polymerized gel then transferred to the electrophoresis devise and submerged with 1X TBE running buffer.

6. Seven microliters of extracted DNA were mixed with 3 µl of loading dye and loaded carefully by mechanical pipet to the gel wells.

7. The electrophoresis was carried out by setting the device on 100 volts and 50 milliamp for 30 minutes.

8. The gel then imaged and the image analysed by CS analyser® software to determine the extracted DNA molecular weight. Figure (2-5) shows the electrophoresis equipment.

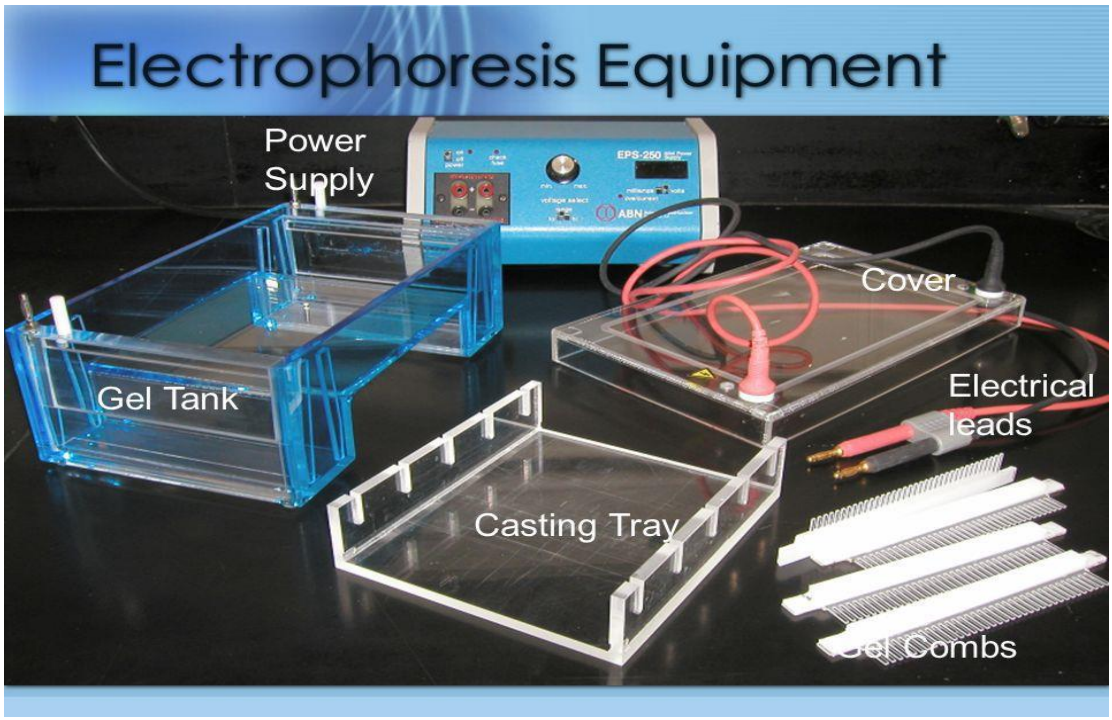


Figure 2-5: The Electrophoresis Equipment

2.2.4. Primers Designing

The Tri Allele-specific primers had been designed for the detection of G/T polymorphism of *SLC6A4* gene (rs6354) and G/A polymorphism of TNF-α gene (rs1800629). This was achieved according to the following:

1- The sequence of the rs6354 G>T of the *SLC6A4* gene, and rs1800629 G>A of the TNF-α gene was obtained from dbSNP with a maximum sequence of 200 flanking nucleotides.

2. The flanking sequence was applied in the blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure the efficacy of sequence of the rs6354 G>T of the *SLC6A4* gene, and rs1800629 G>A of the TNF-α gene, as in figures (1) and (2) in Appendix (2), recepectively.

3. To obtain suitable primers, flanking nucleotides were taken from a complete gene sequence.

4. The flanking sequence was transferred to ApE software and applied the designing primers based on the allele-specific primer technology (tri-allele specific primers), taking into consideration the bases of allele-specific primer design related to the last three nucleotides in the allele primers.

5. The ApE output primers results were transferred to the primer-blast for checking the specificity.

6. Oligo nucleotides of interest were analyzed by sequence manipulation suit (SMS) bioinformatics.

7. Optimase Protocol Writer™ software was applied to get the amplicon size for the studied SNPs with their conditions. This was illustrated in figures (1) and (2) in Appendix (3) for *SLC6A4* (rs6354, G/T) and TNF-α (rs1800629, G/A) genes and in tables (2-5) and (2-6), respectively.

Also, it turns out that Functional Consequence for *SLC6A4* gene (rs6354) *[Homo sapiens]* is: 5' untranslated region (5' UTR) variant. While Functional Consequence for TNF-α gene rs1800629 *[Homo sapiens]* is: 2KB_upstream_variant,upstream_transcript_variant, as had been shown in figures (1) and (2) in Appendix (4), recepectively.

Table (2-5): Primer sequence for alleles of serotonin transporter gene *SLC6A4* (rs6354, G/T)

Alleles of *SLC6A4* gene	Sequences of Primers (5 ′ -3 ′)	bp No.	Tm (°C)	Product length (bp)
G allele	TAAGCCCCTTGTTATTCTGCGAG	23	57.15	325
T allele	TAAGCCCCTTGTTATTCTGCGAT	23	55.37	
Common reverse (R)	GAAACAGAGTGAGCAGTTGAACAG	25	57.24	

Tm=melting temperature; bp=base pair

Table (2-6): Primer sequence for alleles of TNF-α gene (rs1800629, G/A)

Alleles of TNF-α gene	Sequences of Primers (5 ′ -3 ′)	bp No.	TM (°C)	Product length (bp)
G allele	ATAGGTTTTGAGGGGCATCG	20	52.73	275
A allele	ATAGGTTTTGAGGGGCATCA	20	54.78	
Common reverse (R)	AAGAATCATTCAACCAGCGG	20	52.73	

Tm=melting temperature; bp=base pair

2.2.5 Reconstituting and Diluting Primers:

A primer working solution was prepared from the lyophilized primers that illustrated above in tables (2-5) and (2-6).

The nuclease-free H_2O added to each primer to obtain a master stock that used again to obtain a working stock.

The following steps followed for reconstituting and diluting the primers:

1. The tubes were spin down before opening the caps.
2. The desired amount of free nuclease water added according to the guidelines of manufacturer to obtain a 100 Pico moles/µl (master stock).
3. The tubes had vortexed properly for re-suspend the primers evenly.
4. A volume of 10 µl of each primers stock transferred to a 1.5 ml Eppendorf tube that contains 90µl of sterile, nuclease-free water (working stock).
5. The master stock was stored at -20 ˚C.
6. The working stock was stored at -20 ˚C.
7. The working stock was thawed on ice and vortexed before using in PCR and then stored at -20 ˚C.

2.2.6 Polymerase Chain Reaction (PCR) Amplification

The SLC6A4 gene and TNF-α gene in the current study were amplified by PCR-amplification performed in a programmable thermal cycler gradient PCR system.

The amplification of each target region was first optimized by gradient PCR and the best efficient and specific annealing temperature

that produce the most efficient, specific product was chosen for further PCR amplification procedure. The latter was performed according to addition the components for amplification of each SNP and the master mix is of Biolap was used, that containing dNTP (dATP, dCTP, dGTP, dTTP), KCl, $MgCl_2$, Stabilizer and tracking dye, Tag DNA polymerase and Tris-HCl (pH 9.0). Protocols for PCR reaction mixture volume and components of PCR working were given in the tables (2-7) and (2-8) respectively.

Table (2-7): Protocols for PCR reaction mixture volume

SNPs	Nuclease free-water	Master mix 2X	Primer mix	DNA µl	Total volume µl
rs6354	7	10	1	2	20
rs1800629	6	10	2	2	20

Table 2-8: Working solution components of PCR

Reagent Master Mix	20 µl
AccuPower® PCR PreMix	5
DNA template	3
Forward primer	0.5
Reverse primer	0.5
Nuclease free water	Up to 20

2.2.7 Amplification Refractory Mutation System–ARMS-PCR

The allele-specific PCR, also known as an amplification refractory mutation system (ARMS-PCR) or PCR amplification of specific alleles (PASA or AS-PCR), was used to detect SNPs of *SLC6A4* gene (rs 6354 G>T) and TNF-α gene (rs 1800629 G>A). Tri-ARMS PCR was designed to find laboratory methods for detecting SNPs or mutations that are fast, reliable, cost-effective and is mainly used in developing countries [191].

2.2.8 PCR Optimization Programs

The optimization of PCR reaction for each marker with the thermo-cycling condition was listed in table (2-9).

Table (2-9): The starting thermo-cycling conditions for PCR products in SNPs studied

No.	Stages	Temperature (°C)	Time	Number of cycles
1	**Initial denaturation**	91	6 min	1
2	**Denaturation**	91	36 sec	37
3	**Annealing**	Gradient±5^{0}C of the lowest primer Tm	36 sec	
4	**Elongation**	73	>1min/kb product	
5	**Final elongation**	73	6 min	1

2.2.9 Principles of ARMS-PCR

The ARMS-PCR technique is a molecular technique for producing thousands to millions of copies of a demanding DNA fragment [192].

The amplification is a multiplex relation of two sets of primers, and certain reagents and components are required for ARMS-Pecan. Theme components include a Duma target (Dell template) that includes the region of Daft to be amplified, and the amplification is a multiplex reaction of two sets of primers [193].

Theory's aquatics DNA polymerase (the polymerase) enzyme, deoxynucleotide triphosphates (dNTPs), and buffer solution provide a chemical environment that allows the DNA polymerase to maintain its optimal stability and activity [194].

The ARMS PCR is typically used to detect a mutation or polymorphism (a polymorphism is simply a variation in DNA sequence between two similar species, such as two individuals). Polymorphisms can be divided into two types: those involving base changes and those involving a difference in the length of the corresponding DNA region [195].

It is also important that it be able to determine whether the DNA shift is heterozygous or homozygous. ARMS primers for the mutant/polymorphic and natural (wild type) alleles are used to distinguish between heterozygote and homozygote. In most cases, the mutant and normal allele reactions are carried out in different tubes [196].

The following are the basic ARMS-PCR steps: Initiation, Denaturation, Annealing, Extension / Elongation, and Final Extension. The reaction was heated to around 94-96 °C during the Initialization Phase. It is needed for the activation of DNA polymerases. The

denaturation step involves raising the temperature of the reaction to around 94-98°C in order to melt the DNA template but disrupting hydrogen bonds. The single stranded DNA molecules are yielded in this step. The temperature of the reaction is lowered to 5-65 °C in the Annealing step, allowing the primers tab anneal with single-stranded DNA template. The Taq polymerase binds the primer- template hybrid band starts the DNA synthesis process. Taq polymerase is most active at 72-80 °C during the Extension/Elongation step, and this enzyme is typically used at 72 °C.DNA polymerase produces a new DNA strand that is complementary to the DNA template strand by aiding dNTPs in the 5' to 3' direction [197].

2.3 Statistical Analysis

Data of the study participants were transferred into computerized database, revised for errors or inconsistency and then managed, processed and analysed using the statistical package for social sciences (IBM-SPSS) version 23. All continuous (scale) variables were expressed as the mean ± standard error (mean ± SE).

To compare the studied parameters between studied groups, student ***t*** test for two groups was used, while for more than two groups analysis of variables test (ANOVA) was applied [198].

Correlation coefficient (r) was used to find the relationship between two continuous variables. A *p*-value of ≤ 0.05 was considered as a significant.

Test for Hardy-Weinberg equilibrium of allelic or genotypic association in cases versus control were evaluated by Chi – square (x^2) test, this analysis was performed for all genotypes in this study using Hardy-Weinberg equilibrium online calculator.

To assess the predictability of RAS, logistic analysis of two SNPs was applied, this yielded odds ratio (OR). Also, the 95% confidence interval was calculated which is good estimator for the significance of the OR; when the value of "one" included within interval, this is an indicator that the OR is not significant, web sites were used for statistical analysis [199], as shown in below.

1- odds ratio was measured by an online software program (https://www.medcalc.org/calc/odds ratio.php)
2- Hardy Weinberg equilibrium was measured by (https://scienceprimer.com/hardy-weinberg-equilibrium-calculator)

Receiver operating characteristic (ROC) curve was used to evaluate the diagnostic value of TNF-α RAS, the sensitivity and specificity of biochemical parameter and calculate the optimal cutoff according to "Youden Index" by select the point that is closest to the top-left corner of the ROC curve giving equal weight to sensitivity and specificity when picking a cut-off point is a typical practice. This idea is often referred to as the Youden Index [200]. The area under the curve (AUC) provides a useful tool to compare different biomarkers as Table (2-10).

Table (2-10) List of AUC ranges and their classification levels

AUC Range	Classification Level
0.90 - 1.00	Excellent
0.80 - 0.90	Good
0.70 - 0.80	Fair
0.60 - 0.70	Poor
0.50 - 0.60	Failure

AUC=Area under curve

Chapter Three

Results
&
Discussion

3. Results and Discussion

3.1 The Demographic Characteristics of the Studied Subjects

The current study included 100 subjects (50 patients with RAS and 50 controls). Table (3-1) shows mean differences of the studied variables, expressed as mean ± standard error (SE). Variables were including (age and body mass index) while gender was shown in figure (3-1). There was non significant (p-value 0.78) difference in age between patients and control groups, mean ± SE were (32.64 ± 1.25 and 32.41 ± 1.26) respectively.

Also, the difference of BMI between patients and control groups was non significant (P-value=0.64), mean ± SE for patients and control were (31.48 ± 0.64, 31.06 ± 0.63) respectively.

Table (3-1): Demographic characteristics of the patients and control groups.

Variable	Study groups	No.	Means± SE	P-value
Age(years)	Patients	50	32.64± 1.25	0.78
	Control	50	32.41± 1.26	
BMI(Kg/m^2)	Patients	50	31.48± 0.64	0.64
	Control	50	31.06± 0.63	

P-value ≤0.05 was significant, P-value >0.05 was no significant, BMI (body mass index), SE (Std. error).

3.1.1 Age

Table (3-1) demonstrated the age matching by showing the means of ages for both 50 RAS patients and 50 control in this study. This age matching helps to eliminate differences in parameters results that may be originated due to the significant variation in age [201].

3.1.2 Body Mass Index (BMI)

BMI is the ratio of body weight expressed in kilograms, divided by height expressed in metres squared [202].

The difference of BMI between patients and control groups was non-significant showing a matching in this index between those groups. So, the differences in parameters results, that may be created from the significant variation in BMI, were excluded, as in table (3-1).

3.1.3 Gender

The patients group comprised of 52.0% males and 48% females, as well as control group comprised of 52% males and 48% females, as seen in figure (3-1). This indicates a complete matching in gender distribution between the study groups. This sex matching helps to eliminate differences in parameters results that may be originated due to the significant variation in sex [203].

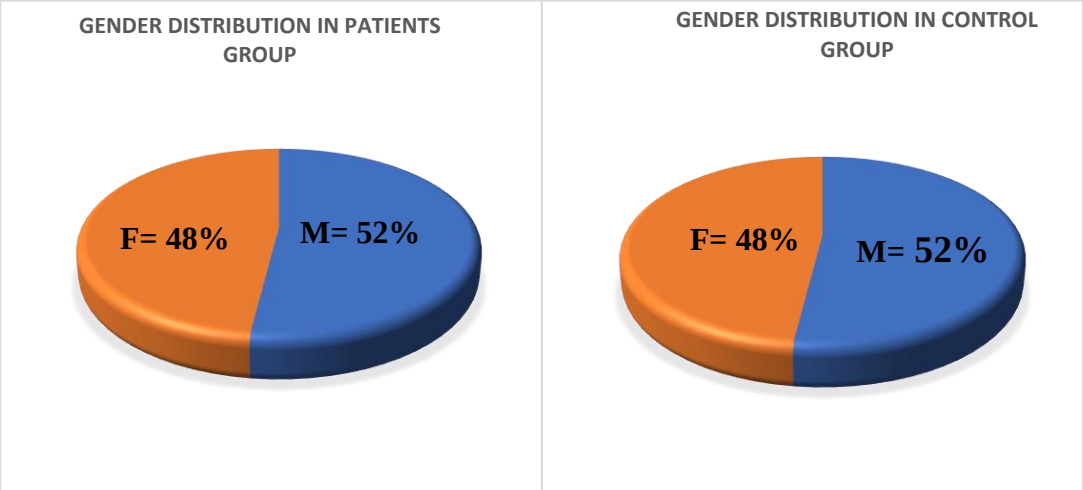


Figure (3-1): Gender Distribution in Patients and Control Group. M=Male, F=Female

One of certain studies reported more males (54.5%) presenting with RAS [31]. This is similar to another report done in the same Center which revealed a higher recurrent aphthous experience among males with the most commonly affected age group [204]. However, this contradicted the results of other investigators that reported female preponderance, with patients in the third and fourth decades of life most commonly affected [205, 206] that similar to some authors results who found that the annual prevalence of RAS in women was higher than in men (13.1% women, 8% men) (p=0.000) [201]. In relation to female predisposition to RAS, some authors have suggested that this association is related to hormonal rates. The incidence of RAS is related to the luteal phase of the menstrual cycle and also a decrease in its incidence during pregnancy, thus relating the episodes of RAS to progesterone levels [207, 208]. Actually, this study was designated to eliminate the differences between genders that may interfere with discussion of present results.

3.2. Biochemical Characteristics of the Studied Subjects

In the current study, certain biochemical characteristics of the recruited individuals were investigated.

3.2.1 Serotonin Concentration in Patients and Control Groups

It was demonstrated that non significant difference between patients and control groups regarding 5-HT concentrations. Mean ± SE for patients and control was (41.43±2.45, 35.35±2.74) respectively with P-value > 0.05, as seen in table (3-2).

Table (3-2): Mean ± SE of Serotonin Concentrations in Patients and Control

Dependent Variable	Study groups	No.	Mean± SE	P- value
Serotonin concentration (ng/ml)	Patients	44	41.43±2.45	> 0.05
	Control	44	35.35±2.74	

SE=Standard Error

Aphthous stomatitis and angular cheilitis are considered as common nonspecific extraintestinal manifestations (EIMs) of many diseases such as irritable bowel diseases in addition to effects of drugs and vitamin deficiency. From review of previous studies, it was found

that 5-HT was not measured in anyone of these researches dealing with RAS [209].

From that, in the present study, it was attempted to evaluate the level of 5-HT in RAS patients. Present results did not show any significant changes that considered as accepted results when comparing with others that recorded the postprandial circulating 5-HT likely originates from the gut, interpretation of circulating levels is more complex, and recent data suggest that there may be differences in platelet SERT function [209].

Also, one of the important facts aphthous stomatitis occurs in general population. If aphthous ulcerations are present, the presence of inflammatory bowel disease must be suspected, although intestinal symptoms may not yet be present [110].

From that, it can be expected the indirect relationship between 5-HT and aphthous development. Indeed, 5-HT in present study altered insignificantly that may confirm these facts due to selection of present patients without any active disease. In general, this evidence will need further investigations with large number of patients with different clinical states to explore the actual role of 5-HT [111].

3.2.2 Tumor Necrosis Factor Alpha Concentrations in Patients and Control Groups

Numerous studies have been performed to show the role of TNF-α in RAS [212].

3.2.2.1 Tumor Necrosis Factor Alpha Concentrations in Studied Groups

As shown in table (3-3), there is a significant increase (P-value < 0.05) of TNF-α concentrations in the patient group (as a mean). Mean ± SE for patients (515.05±26.49) while for control (421.97±33.29).

Table (3-3): Mean± SE of TNF-α Concentrations in Patients and Control

Dependent Variable	Study groups	No.	Mean± SE	P- value
TNF-α concentration (ng/l)	Patients	44	515.05±26.49	<0.05
	Control	44	421.97±33.29	

SE=Standard Error

The present results indicated increase of TNF-α, that agreed with some studies which showed same results [213-215]. While others did not show any significant results [216,217].

One certain study indicated that TNF-α has important effects on the RAS development Numerous effective factors on the production of TNF-α have been reported in RAS. In addition, TNF-α can cause RAS lesions through its effect on keratinocyte cells [212].

The TNF-α is a main pro-inflammatory cytokine that plays an important role in immune and inflammatory responses [218]. TNF-α actually shows important immune-modulatory activities and studies have shown its relationship with RAS. Thus, high levels of TNF-α have been reported in wound mucosa and peripheral blood of patients with aphthous ulcer [214,215,219,220]. High cytotoxic destruction of epithelial cells with TNF-α produced from peripheral blood mononuclear cells was shown in patients with aphthous ulcer [214]. In addition, RAS can be prevented by inhibitors of endogenous TNF-α synthesis such as thalidomide and pentoxifylline [221].

The effects of TNF-α were established to be associated with activation of a cascade of inflammatory events, enhancing expression of adhesion molecules and activation of neutrophils in addition to acting as a co-stimulator for T cell activation and antibody production [222].

It was found that different results were obtained in studies that examined TNF-α level. This difference may be partly due to patient differences, RAS subtypes, research methods, or sample size. Another important factor to consider is the interaction of genes and cytokines as well as the effect of gene polymorphism on cytokine production.

A remarkable point is that, in most studies, salivary TNF-α levels are higher in the RAS group than in healthy individuals, so it suggests that TNF-α may be a potential salivary marker for this disease [201].

Al-Samadi *et al.* concluded that abnormal apoptosis of epithelial cells that progressed to necrosis, released the danger signals. Exposure of pathogen-specific receptors such as TLR to these danger signals increases the production of IL-17C and TNF-α and leads to inflammation and RAS [146-148].

3.2.2.2 Receiver Operating Characteristic (ROC) Analysis of Tumor Necrosis Factor Alpha Concentrations in Patients Group.

The area under the curve (AUC) was 66.7, 95%CI= (0.549-0.784), P-value=0.007 (< 0.01). The sensitivity and specificity of the test at the cut-off value of TNF-α (equal to 421 ng/l)) were 75 and 66.9 respectively, as seen in figure (3-2) and table (3-4).

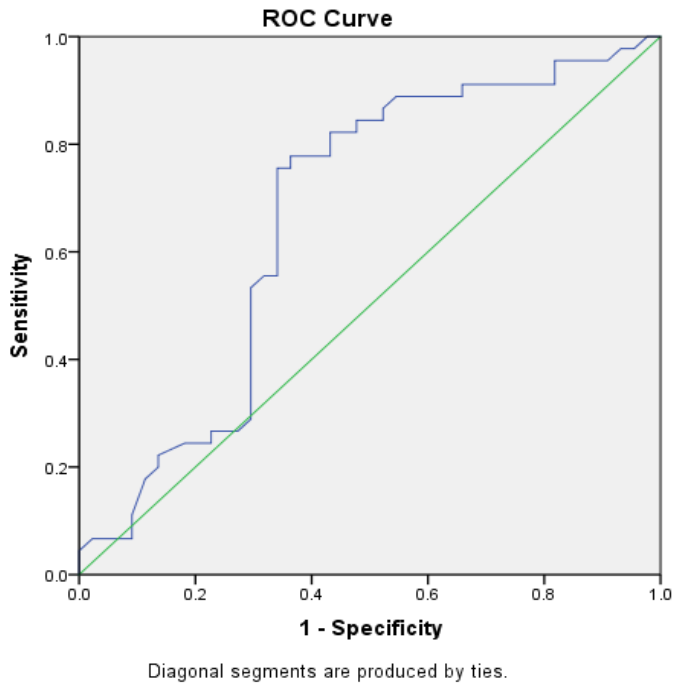


Figure (3-2): Roc Curve of TNF-α; AUC=66.7

Table (3-4): Area Under the Curve for TNF-α

Area under the curve (AUC)	Std. Error	P-value	95% Confidence Interval
0.667	0.060	0.007	0.549 - 0.784

For TNF-α, results of the present study stated that it had poor diagnostic value in the diagnosis of RAS.

3.2.3 Correlations between Studied Variables.

3.2.3.1 Correlation of Serotonin with Tumor Necrosis Factor Alpha Concentrations in Patients Group

As seen in table (3-5) and figure (3-3), there is a significant (p-value=0) positive correlation between 5-HT and TNF-α concentrations with $r^2 = 0.9344$.

Table (3-5): Correlation of Serotonin with TNF-α Concentrations in Patients Group

Dependent Variable		TNF-α
Serotonin (ng/l)	Pearson Correlation	0.967
	P-value	0.000
	No.	44

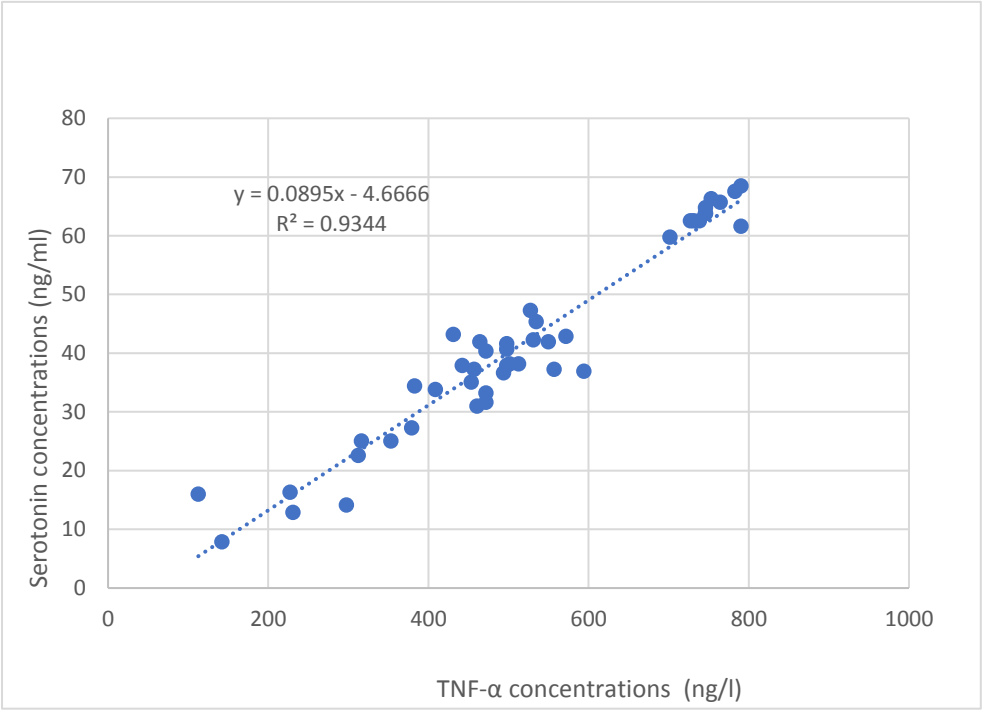


Figure (3-3): Correlation between Serotonin and TNF-α Concentrations in Patients Group

Accurate aetiology of RAS still remains unknown [223]. Controversy exists among different authors about the pathogenesis of RAS [224]. Various factors predispose for its occurrence. Genetic background, stress, anxiety, food allergens, local trauma, smoking cessation, menstrual cycle, chemicals and microbes were identified as predisposing factors [225-227]. Also, the process is initiated by antigenic stimulation of the mucosal keratinocytes which leads to stimulation of T-lymphocytes, cytokine release as well as migration of other lymphocytes, neutrophils and Langerhans cells. Cytotoxic trigger causes ulceration of the mucosa. TNF α is considered as the most significant cytokine implied in the development of new RAS lesions [213,228].

Inflammation and metabolism of free radicals in RAS patients and healthy controls were evaluated by Avci *et al.* [228]. They investigated TNF-α, interleukin-2 (IL-2), IL-10, and IL-12 using ELISA and emphasized their importance in the occurrence of RAS. Similar pattern was observed in present study in regard to TNF-α only [229].

The 5-HT plays a significant role in regulation of mood and cognition and helps to relay messages from one area of the brain to another one of important roles in the body including:

• Functioning as a natural mood stabiliser by helping to reduce levels of anxiety and depression, in addition to:

• Helping to heal wounds by the formation of clots. The 5-HT is released by platelets when there is a wound, it helps to narrow blood vessels, which reduces blood flow and helps blood clots to form [230].

Various causative factors such as genetic tendency, immunologic basis, nutritional deficiency, emotional stress, hematologic and hormonal disturbances, local injury, microbial agents and other influences have been suggested in previous studies [226, 231].

However, eating a carbohydrate-rich meal triggers your body into releasing insulin. This causes any amino acids in the blood to be absorbed into the body rather than the brain, with the exception of tryptophan. It remains in the bloodstream at high levels following a carbohydrate meal, which means it can freely enter the brain and cause 5-HT levels to rise [232]. All of these observations may explain the positive correlation result in present study between TNF-α and 5-HT that play an important role, synergically, in RAS occurrence due to their effects in inflammatory immune cascades.

3.2.3.2 Correlations of Serotonin and Tumor Necrosis Factor Alpha Concentrations in Patient Group with Age and BMI.

Table (3-6) shows that there is no correlation between 5-HT concentrations and both age and BMI, with (p-value=0.938 and 0.607) respectively. As well as there is no correlation between TNF-α concentrations and both age (p-value=0.965) and BMI (p-value=0.867).

Table (3-6): Correlations of Serotonin and TNF-α Concentrations in Patients Group with Age and BMI

Dependent variables		Age	BMI
Serotonin (ng/ml)	Pearson Correlation	0.012	0.079
	P-value	0.938	0.607
	No.	44	44
TNF-α (ng/l)	Pearson Correlation	0.007	0.026
	P-value	0.965	0.867
	No.	44	44

3.2.3.3 Correlations between Age and BMI in Patients Group.

In the present study, there is a significant correlation between age and BMI with p-value < 0.01 (0.009). This correlation was demonstrated in figure (3-4).

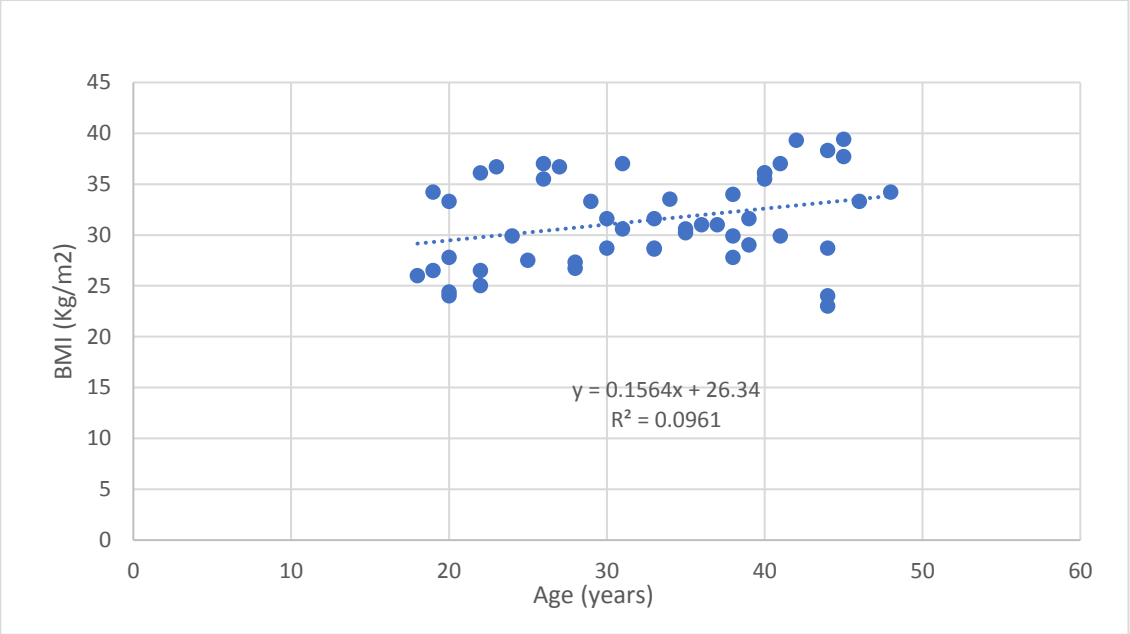


Figure (3-4): Correlations Between Age and BMI in Patients Group

3.3 Genetic Analysis

3.3.1 Optimization of Polymerase Chain Reaction Conditions

3.3.1.1 Optimization of Serotonin Transporter Gene *SLC6A4* (rs 6354)-PCR

Programms used in PCR preperation depended on changes of annealing temperature. As shown in figure (3-5), the results were indicated at (325 bp).

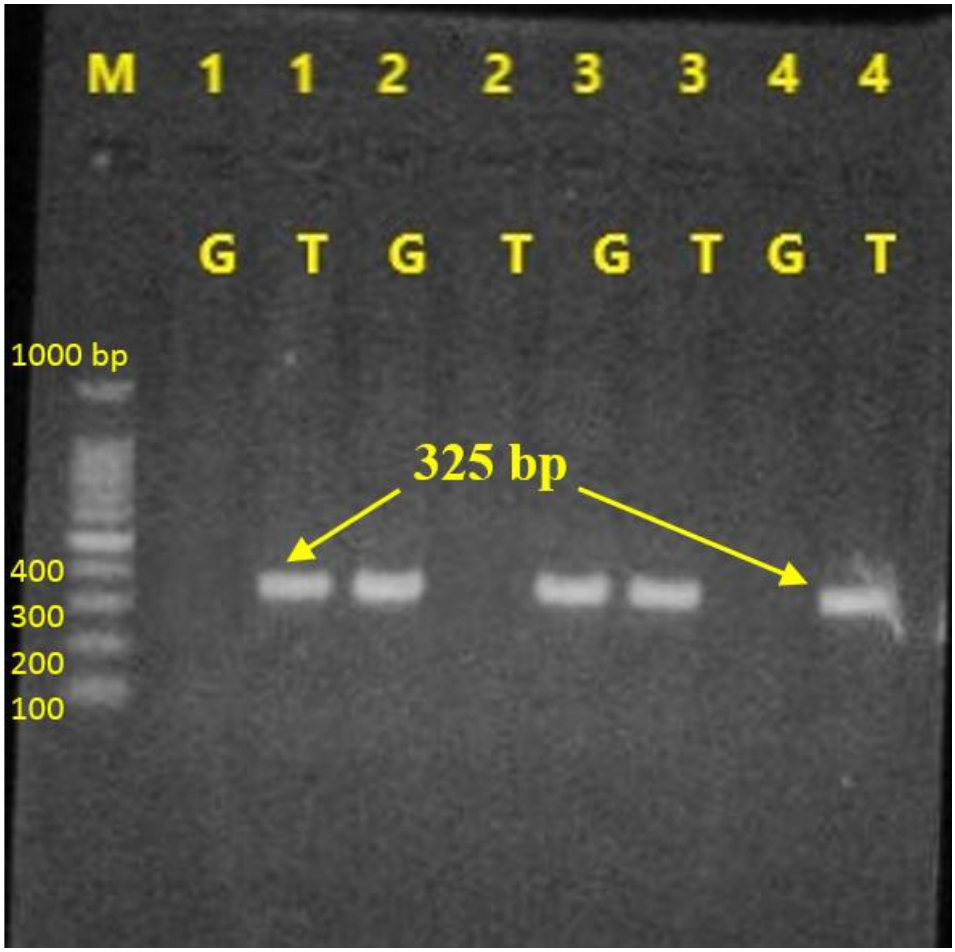


Figure 3-5: Tri-primer ARMS-PCR optimization of Serotonin Transporter Gene (*SLC6A4*) rs6354; M = DNA marker (100-1000 bp), 70 Volt and genotypes are G and T. Time: 50 minutes.

3.3.1.2 Optimization of DNA of Tumor Necrosis Factor Alpha (rs1800629)

Programms used in PCR preperation depended on changes of annealing temperature. As shown in figure (3-6), the results were indicated at (275 bp).

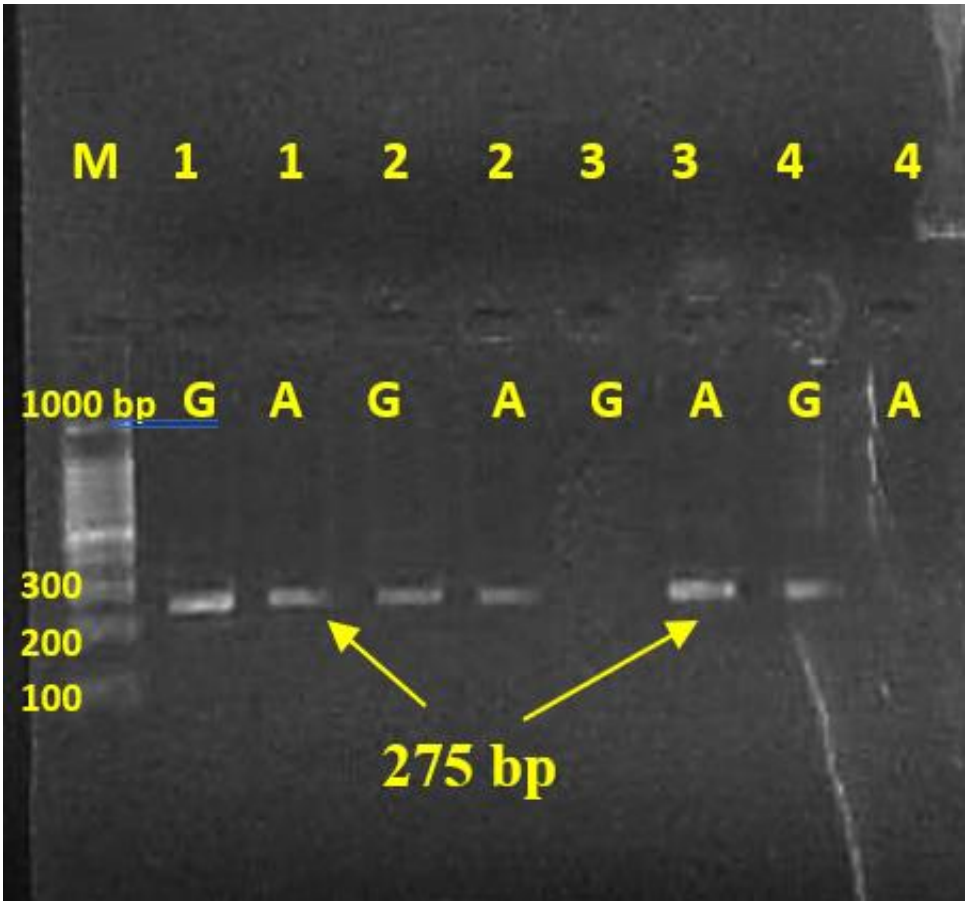


Figure 3-6: Tri-primer ARMS-PCR Optimization of TNF-α gene (rs1800629); M = DNA marker (100-1000 bp), 70 Volt and genotypes are G and A. Time is 50 minutes

3.3.2 Serotonin Transporter Gene (*SLC6A4)* Variants (rs 6354) G>T

A- Hardy–Weinberg Equilibrium and Inheritance Model for rs6354 G>T

Genotype frequencies of rs6354 for *SLC6A4* gene polymorphism do not consistent with Hardy Weinberg Equilibrium ($P<0.05$) in RAS-patients and control groups, Chi-square values were (13.15) and (17.57) for patients and controls respectively as shown in Table (3-7).

Table 3-7: Hardy-weinberg equilibrium law of *SLC6A4* gene polymorphism rs6354 observed and expected genotype frequency for control and patients

rs6354	GG	GT	TT	Chi-square value	P -Value
Control	58% (n=29)	18% (n=9)	24% (n=12)	17.57915	0.0001
Patients	32% (n=16)	24% (n=12)	44% (n=22)	13.15778	0.0013

G- major allele, T-minor allele, P value ≤0.05 was significant

B- Genotyping and Alleles Frequency of *SLC6A4* Gene (rs6354)

Table (3-8) shows the comparative genotypes and alleles frequency of *SLC6A4* gene between the patients and control groups.

The genotype (TT) showed a significant correlation (P<0.05) with odd ratio=3.32 and C.I.= (1.31 - 8.43). While the genotype (GT) showed no significant correlation (P>0.05) with odd ratio= 2.42 and C.I.= (0.84 - 6.96).

Also, in this table, it was showed that G-allele frequency is with a significant value as P-value (<0.05), odd ratio (3.32) and C.I (1.45 - 4.58).

Table (3- 8): Comparative Genotypes and Alleles Frequency of *SLC6A4* Gene Between the Study Groups

Genotypes			Groups		Total (100)	Statistics	OR (C.I. 95%)
			Patients (50)	Control (50)			
Serotonin Transporter Gene rs (6354)	GG	No.	16	29	45	References value	
		%	32%	58%	45%		
	GT	No.	12	9	21	z = 1.64 (P>0.05)	2.42 (0.84 - 6.96)
		%	24%	18%	21%		
	TT	No.	22	12	34	z =2.53 (P<0.05)	3.32 (1.31 - 8.43)
		%	44%	24%	34%		
	Allele frequencies						
	T	No.	56	33	89	z = 3.24 (P<0.01)	2.58 (1.45 - 4.58)
		%	56%	33%	44.5%		
	G	No.	44	67	111		
		%	44%	67%	55.5%		

X^2 = Chi-square, P= probability, OR= odd ratio, C.I.= confidence intervals

The subjects enrolled in present study were reported into three genotypes, these are homozygous for the G allele (GG) wild type, heterozygous (GT) and the last one was homozygous for the allele (TT), as in Figure (3-7).

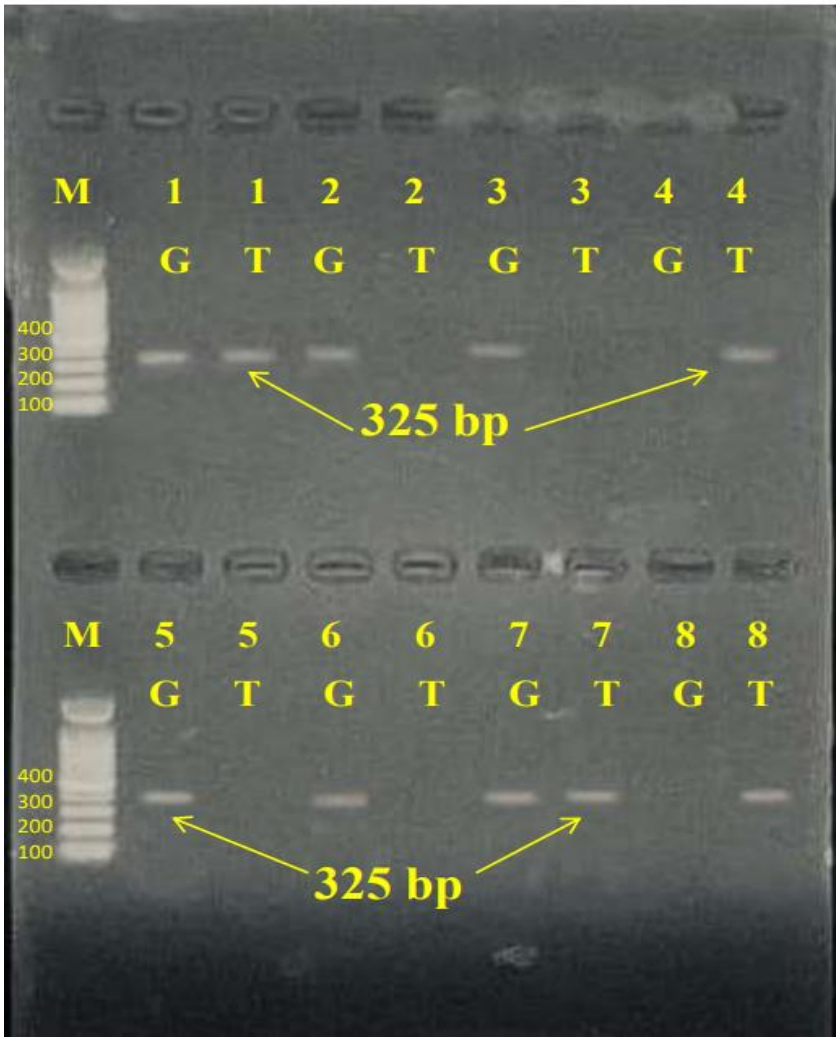


Figure 3-7: Tri-ARMS-PCR bands of Serotonin Transporter Gene *SLC6A4* rs6354 G/T SNP; M = DNA marker (100-1000 bp). Time: 50 minutes

Lane (1 and 5): Homozygote wild type which seen as one band and represent (GG Genotype) at (325 bp).

Lane (2 and 8): Heterozygosity type which seen as two bands and represent (GT Genotype) at (325 bp).

Lane (3,4,6 and 7): Homozygote mutant type seen as one band and represent (TT Genotype) at (325 bp).

Table (3-9) shows the association of *SLC6A4* genotypes with RAS under different models of inheritance. As seen in the current study, the Co. dominant model explore that the genotype TT shows a significant result with p-value < 0.05 (p-value=0.011; Odd ratio (95% CI) was 3.32(1.30 to 8.43)).

In the dominant model, the GT>TT genotype shows a significant effect p-value < 0.01(p-value=0.009; Odd ratio (95% CI) was 2.93 (1.29 to 6.64)). Also, as seen in recessive model, there is a significant result given by the genotype TT with p-value < 0.05 (p-value=0.036; Odd ratio (95% CI) was 2.48 (1.05 to 5.85)).

Table 3-9: Genotypes of *SLC6A4* (rs 6354) under different models of inheritance

Model	Genotype	Control (No. 50)	Patients (No. 50)	Odd ratio (95% CI)	P-Value
Co. dominant	GG	58% (no=29)	32% (n=16)	References (OR=1)	
	GT	18% (no=9)	24% (n=12)	2.41(0.83 to 6.96)	0.102
	TT	24% (no=12)	44% (n=22)	3.32(1.30 to 8.43)	0.011
Dominant	GG	58% (no=29)	32% (n=16)	References (OR=1)	
	GT-TT	42% (no=21)	68% (n=34)	2.93(1.29 to 6.64)	0.009
Recessive	GG-GT	76% (no=38)	56% (n=28)	References (OR=1)	
	TT	24% (no=12)	44% (n=22)	2.48(1.05 to 5.85)	0.036
Over dominant	TT-GG	82% (no=41)	76% (n=38)	References (OR=1)	0.462
	GT	18% (no=9)	24% (n=12)	1.43(0.54 to 3.79)	

Chi-Square test was applied to detect allelic frequency, G (Guanine)- major allele, T (Thymine) -minor allele.

The RAS is not recognized as a single disease, but a clinical manifestation of several pathologic conditions such as hematologic and immunologic diseases, stress, and psychological disorders [111-113].

In previous studies, the levels of depression, anger, and stress in the patients suffering from RAS were determined using Hamilton Anxiety Depression (HAD) scale [114], and the psychological factors were demonstrated to be highly associated with RAS pathogenesis [233]. Approximately, 46% of the patients with RAS have a positive family history. Local trauma, iron deficiency anemia, folic acid deficiency, vitamin B12 resorption defect, neutropenia and psychological factors such as stress and anger can play a role in RAS aetiology [234].

The *5-HTT* gene modulates the intensity and duration of serotonergic neurotransmission, thus, this gene polymorphism can influence the anxiety related behaviours. It has been recently reported that the L and S variants of the promoter polymorphism modulate SLC6A4 differently. The S allele is associated with reduced transcription of SLC6A4 and consequently a reduction in 5-HT reuptake [119].

Several studies have evaluated the correlation between a variety of polymorphisms and aphthous lesions; however, none of them has assessed *5-HTT* gene polymorphism related to this disease among Iranian population. In one of studies, RAS patients and healthy subjects were evaluated. The genotypes of *5-HTTLPR* were divided into three groups by PCR using specific primers: homozygote for SS, homozygote for LL, and heterozygote for LS. L and S allele frequencies showed no statistically significant relation with RAS patients as compared to the healthy subjects. Totally, the results of this study demonstrated that the frequency of *5-HTTLPR* genotype and L/S alleles could be considered as

a risk factor for RAS development and progression. According to the results of this study, *5-HTTLPR* genotypes and S/L allele frequencies seem to be associated with some of stress-related or auto-immune diseases among Iranian population [235].

Victoria *et al* evaluated the polymorphism of *SLC6A4* gene in patients suffering from RAS and in healthy subjects in Brazil. The results of their study showed a significant increase in incidence of SS genotype, S allele, and *5-HTTLPR* polymorphism in the patients with RAS compared to the healthy subjects [236].

Researchers in Michigan University performed a systematic review on the relation between the polymorphism in the promoter region of the *SLC6A4* gene (*5-HTTLPR*) and anxiety/ anger related behaviours. They evaluated 23 studies applying meta-analysis. The results revealed a significant relation between the polymorphism of *5-HTTLPR* and neuroticism [237].

Many noticeable observations were obtained from the comparison of present results with other researches. The TT genotype of (*5-HTTLPR*) in current study recorded a highly risk association with disease development that agreed with previous investigations [236, 237]. also, insignificantly high odd ratio was recorded of GT genotype: that indicated the effective role of *SLC6A4* gene in development of RAS.

Several studies recorded the effective of *5-HTT* gene in many predisposing factors for the development of RAS, (Zeyuan Cao *et al*) observed a statistically significant interaction between 5-HTT rs6354 and work stress on burnout [238].

The *SLC6A4* gene and genes involved in the serotonergic system are candidate genes for susceptibility to depression given that many antidepressant medications act on these systems. Several studies have implicated the *SLC6A4* gene (*SLC6A4*) in major depressive disorder (MDD) [239-241].

C- Multiple Comparisons of *SLC6A4* Genotypes (rs 6354) with Serotonin Concentrations

There were non-significant changes in 5-HT according to different characters of *SLC6A4* genotypes that involved in this study as shown in table (3-10).

Table (3-10): Multiple comparisons of *SLC6A4* genotypes (rs 6354) with serotonin concentrations

Genotype (1)	Genotype (2)	Mean Difference (1-2) ±SE	P-value
GG	GT	0.58±6.64	0.930
	TT	9.56±5.88	0.112
GT	GG	-0.58±6.64	0.930
	TT	8.98±5.88	0.135
TT	GG	-9.56±5.88	0.112
	GT	-8.98±5.88	0.135

As mentioned in previous section (3.3.2-C), there were no effects of 5-HT levels directly on RAS development, in addition to minimal effect of *SLC6A4* on the levels of 5-HT in serum.

3.3.3 Tumor Necrosis Factor Alpha Gene Variants (rs 1800629) G>A

A- Hardy–Weinberg Equilibrium and Inheritance Model for (rs1800629) G>A

Genotype frequencies of rs1800629 for TNF-α gene polymorphism do not consistent with Hardy Weinberg Equilibrium ($P<0.05$) in RAS-patients and control groups, Chi-square values were (12.03) and (21.80) for controls and patients respectively as shown in Table (3-11).

Table 3-11: Hardy-weinberg equilibrium law of TNF-α gene polymorphism rs1800629 observed and expected genotype frequency for control and patients

rs1800629	GG	GA	AA	Chi-square value	P -Value
Control	50% (no=25)	24% (no=12)	26% (no=13)	12.03	0.0024
Patients	54% (no=27)	16% (no=8)	30% (no=15)	21.80	0.00002

G- major allele, A-minor allele, P value ≤0.05 was significant

B- Genotypes and Alleles Frequency for TNF-α Gene

Table (3-12) shows comparative genotypes and alleles frequency of TNF-α gene between patients and control groups. There was no

significant correlation of the genotypes GG, GA, and AA with serum TNF-α in the study groups.

Table (3-12): Comparative Genotypes and Alleles Frequency of TNF-α Gene (rs 1800629) between the Study Groups

Genotypes			Groups		Total (100)	Statistics	OR (C.I. 95%)
			Patients (50)	Control (50)			
TNF-α Gene rs (1800629)	GG	No.	27	25	52	References value	
		%	54%	50%	52%		
	GA	No.	8	12	20	z = 1.64 (P>0.05)	0.62 (0.22-1.76)
		%	16%	24%	20%		
	AA	No.	15	13	28	z =0.14 (P>0.05)	1.07 (0.42-2.68)
		%	30%	26%	28%		
	Allele frequencies						
	A	No.	38	38	76	z= 0.00 (P>0.05)	1.00 (0.56-1.77)
		%	38%	38%	38%		
	G	No.	62	62	124		
		%	62%	62%	62%		

X^2 = Chi-square, P= probability, OR= odd ratio, C.I.= confidence intervals

The subjects enrolled in present study were reported into three genotypes, these are homozygous for the G allele (GG) wild type, heterozygous (GA) and the last one was homozygous for the allele (AA).

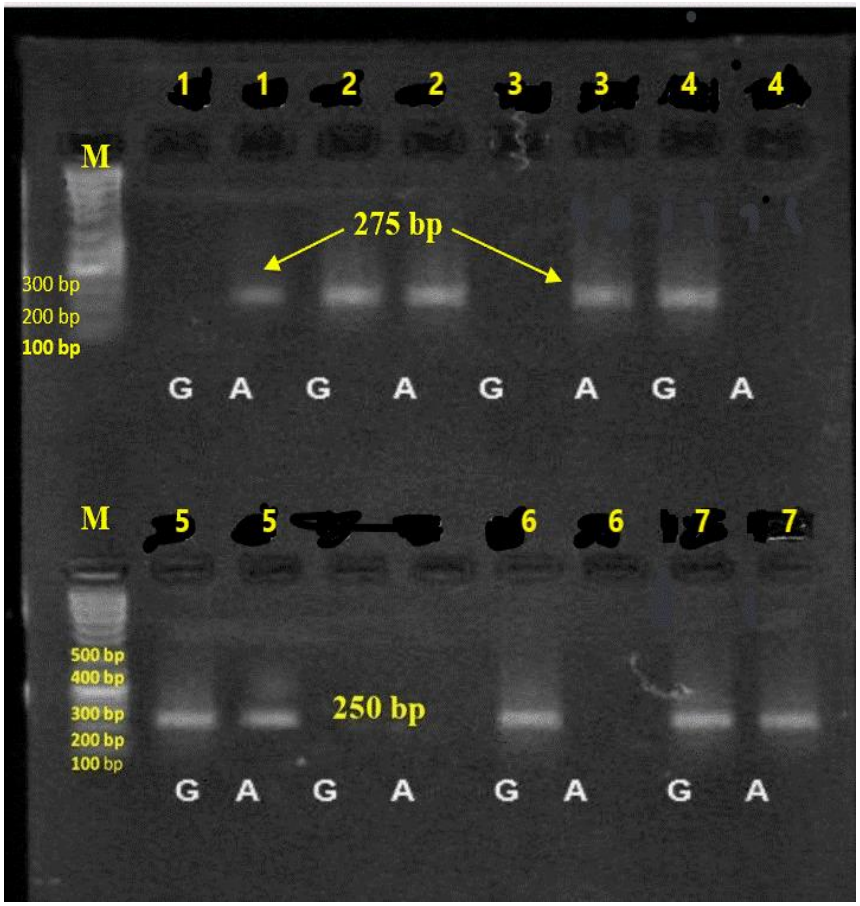


Figure 3-8: Tri-ARMS-PCR bands of Tumor Necrosis Factor Alpha gene rs 1800629 G/A SNP; M = DNA marker (100-1000 bp). Time: 50 minutes

Lane (4 and 6): Homozygote wild type which seen as one band and represent (GG Genotype) at (275 bp).

Lane (2, 5 and 7): Heterozygosity type which seen as two bands and represent (GA Genotype) at (275 bp).

Lane (1 and 3): Homozygote mutant type seen as one band and represent (AA Genotype) at (275 bp).

Table (3-13) shows the association of TNF-α genotypes with RAS under different models of inheritance. None of these inherited models gave a significant relationship to disease progression compared to the other genotypes.

For Co. dominant, Dominant, Recessive and finally Over dominant models, p-value > 0.05.

Table (3-13): Genotypes of TNF-α (rs1800629) under Different Models of Inheritance

Model	Genotype	Control (No.50)	Patients (No.50)	Odd ratio (95% CI)	P-Value
Co. dominant	GG	50% (no=25)	54% (no=27)	References (OR=1)	
	GA	24% (no=12)	16% (no=8)	1.61 (0.21 to 1.75)	0.366
	AA	26% (no=13)	30% (no=15)	1.06 (0.42 to 2.68)	0.888
Dominant	GG	50% (no=25)	54% (no=27)	References (OR=1)	0.689
	GA-AA	50% (no=25)	46% (no=23)	0.85 (0.38 to 1.86)	
Recessive	GG-GA	74% (no=37)	70% (no=35)	References (OR=1)	0.656
	AA	26% (no=13)	30% (no=15)	1.21 (0.50 to 2.92)	
Over dominant	AA-GG	76% (no=38)	84% (no=42)	References (OR=1)	0.320
	GA	24% (no=12)	16% (no=8)	0.60 (0.22 to 1.63)	

Chi-Square test was applied to detect allelic frequency, G- major allele (Guanine), A-minor allele (Adenine).

The TNF-α gene is located on the chromosome 6 and several single nucleotide polymorphisms have been detected in its promoter region. Studies have indicated that a G-to-A mutation in the -308-promoter section is accompanied by an increase in TNF-α production [157,158].

Studies have also been conducted on the association between TNF-α gene polymorphism and susceptibility to aphthous ulcers. In some studies, a positive association was found between TNF polymorphism and susceptibility to aphthous ulcers [159,160]; and in other study, no association was found [161].

This discrepancy in studies in some comparative models and inconsistent conclusions may be attributed to several factors. First, these studies included people from different populations in different countries and could be the result of differences in the race of the individuals studied. Second, it may be the result of different aetiologies of RAS. Third, some studies did not use Hardy-Weinberg equilibrium (HWE). Another reason may be related to the low statistical population of some studies. In certain meta-analysis, no association was found between TNF-α -308 G/A single nucleotide polymorphism and overall RAS risk except in recessive model. Recessive model is likely to be protective against RAS when compared to other models [150].

C- Multiple Comparisons of Tumor Necrosis Factor Alpha Genotypes with serum TNF- α concentrations

As shown in table (3-14), the multiple comparisons of TNF-α genotypes exhibited that there is a highly significant association of genotypes GG and GA with TNF-α concentration with mean

difference=184.14±58.94 and P=0.003. Also, there is a significant association of genotypes GA and AA with the mean difference=191.24±70.73 and P=0.01. On the other hand, there is no association between genotypes GG and AA.

Table (3-14): Multiple Comparisons of TNF-α Genotypes

Genotype (1)	Genotype (2)	Mean Difference± SE (1-2)	P-value
GG	**AA**	7.09±60.93	0.91
	GA	184.14±58.94	0.003
GA	**AA**	191.24±70.73	0.01
	GG	184.14±58.94	0.003

The TNF-α polymorphism is widely studied by previous researchers and many of them recorded the clinical effects of G-308A SNP of TNF-α gene in RAS development. As a result of certain study, the prevalence of polymorphisms in the gene for the anti-inflammatory cytokine of the polymorphic variant rs1800629 of the TNF-α gene of the locus G-308A was established. The OR calculation of this study showed that the highly productive A allele and its heterozygous variant (G/A) are associated with RAS and its severity, while the wild G allele and its homozygous G/G genotype are protective against the development of the disease. The present study showed significant difference in mean of TNF-α serum levels among the genotypes of GA with GG and AA [242].

One of the agreement previous results of the 308 G/A TNF-α gene polymorphism, encoding the expression of the proinflammatory cytokine TNF-α, showed that it is pathogenetically significant in the development of RAS. Polymorphism of 308 G/A rs1800629 of the TNF-α gene, carriage of allele A and homozygous genotype G/A can be considered as a hereditary predisposition to RAS and a criterion for the severity of its clinical course.

❖ Conclusions

1. Serum serotonin has no direct role in RAS development.

2. Serotonin transporter gene *SLC6A4* (rs6354) polymorphism at mutant homozygous TT may be one of the underlying causes of RAS development.

3. Serum TNF-α has an effective role in RAS occurrence but it is a poor diagnostic marker for RAS.

4. At the level of genotypes mutant GA and AA of rs1800629 play an important role on the elevated levels of TNF-α, that consider as a predictive factor for developing RAS.

5. Presence of positive correlation between serotonin and TNF-α synergistically strengthen its relation to occurrence of RAS.

❖ Recommendations

1. Further studies involving large samples are suggested along with extensive predisposing factors for RAS, such as stress, bowel diseases and vitamin B12 deficiency.

2. Estimation of 5-HTT/ 5-HT ratio to determine the exact role in RAS development.

3. Study of the gene expression of 5-HTT with different categories to explain the effects on protein synthesis and functions.

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Appendices

Appendices

Appendix (1): Questionnaire

اسم المراجع/ العمر:

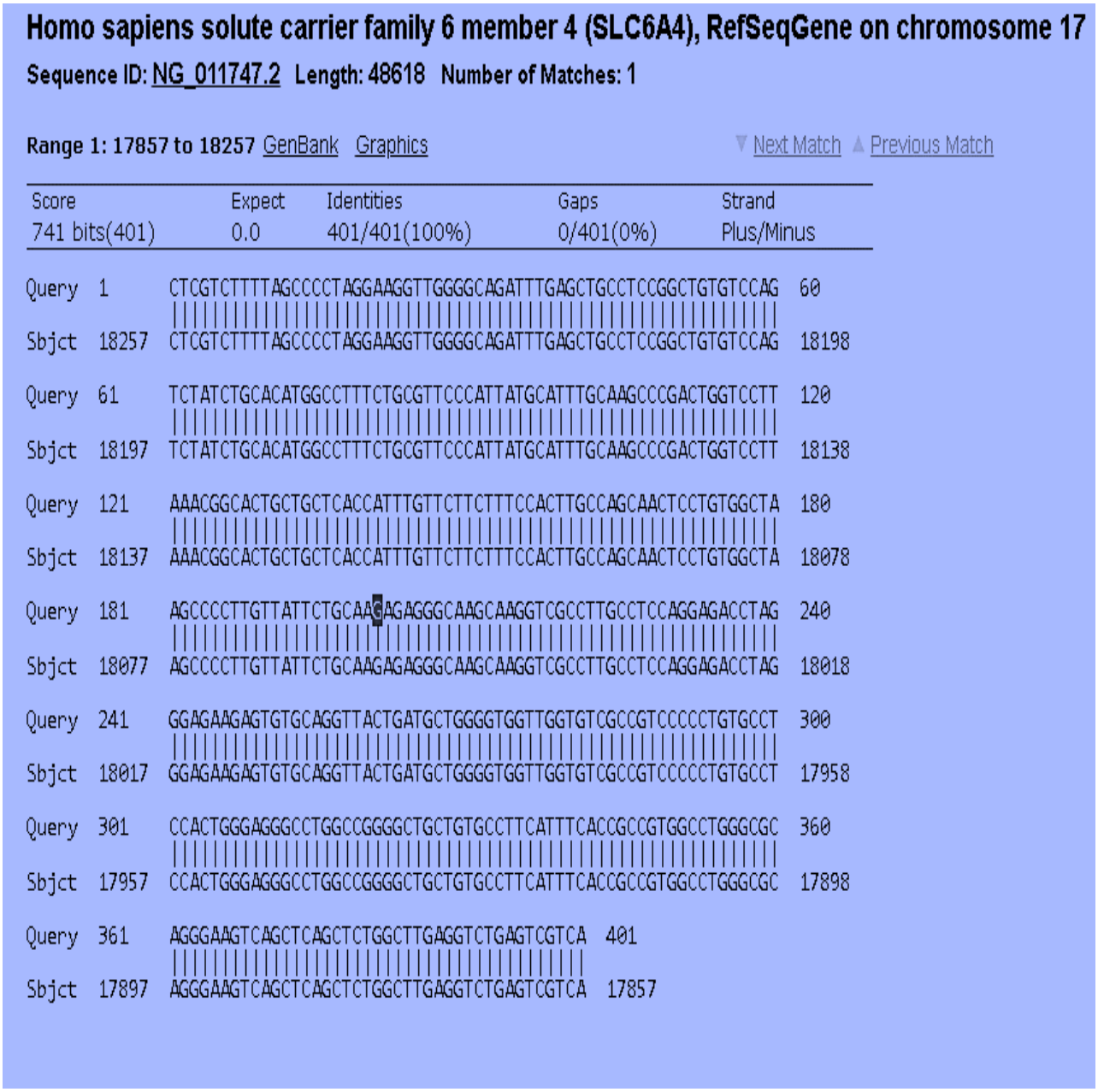
التاريخ واليوم: الجنس:

السكن الحالي رقم العينة:

رقم الهاتف:

1	المستوى التعليمي والمهني	
2	عدد مرات حدوث المرض في السنة وبداية ظهوره	
3	هل يوجد تاريخ عائلي بوجود التهاب الفم القلاعي المتكرر	
4	هل يتم اخذ أدوية مزمنة	
5	هل يعاني الشخص من أمراض مزمنة	
6	الطول	
7	الوزن	

<u>Appendix (2):</u> Figures of Sequences of *SLC6A4* (rs6354) and *TNF-α* (rs1800629) genes.



Homo sapiens solute carrier family 6 member 4 (SLC6A4), RefSeqGene on chromosome 17

Sequence ID: NG_011747.2 **Length:** 48618 **Number of Matches:** 1

Range 1: 17857 to 18257 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
741 bits(401)	0.0	401/401(100%)	0/401(0%)	Plus/Minus

```
Query  1      CTCGTCTTTTAGCCCCTAGGAAGGTTGGGGCAGATTTGAGCTGCCTCCGGCTGTGTCCAG  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  18257  CTCGTCTTTTAGCCCCTAGGAAGGTTGGGGCAGATTTGAGCTGCCTCCGGCTGTGTCCAG  18198

Query  61     TCTATCTGCACATGGCCTTTCTGCGTTCCCATTATGCATTTGCAAGCCCGACTGGTCCTT  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  18197  TCTATCTGCACATGGCCTTTCTGCGTTCCCATTATGCATTTGCAAGCCCGACTGGTCCTT  18138

Query  121    AAACGGCACTGCTGCTCACCATTTGTTCTTCTTTCCACTTGCCAGCAACTCCTGTGGCTA  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  18137  AAACGGCACTGCTGCTCACCATTTGTTCTTCTTTCCACTTGCCAGCAACTCCTGTGGCTA  18078

Query  181    AGCCCCTTGTTATTCTGCAAGAGAGGGCAAGCAAGGTCGCCTTGCCTCCAGGAGACCTAG  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  18077  AGCCCCTTGTTATTCTGCAAGAGAGGGCAAGCAAGGTCGCCTTGCCTCCAGGAGACCTAG  18018

Query  241    GGAGAAGAGTGTGCAGGTTACTGATGCTGGGGTGGTTGGTGTCGCCGTCCCCCTGTGCCT  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  18017  GGAGAAGAGTGTGCAGGTTACTGATGCTGGGGTGGTTGGTGTCGCCGTCCCCCTGTGCCT  17958

Query  301    CCACTGGGAGGGCCTGGCCGGGGCTGCTGTGCCTTCATTTCACCGCCGTGGCCTGGGCGC  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  17957  CCACTGGGAGGGCCTGGCCGGGGCTGCTGTGCCTTCATTTCACCGCCGTGGCCTGGGCGC  17898

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              |||||||||||||||||||||||||||||||||||||||||
Sbjct  17897  AGGGAAGTCAGCTCAGCTCTGGCTTGAGGTCTGAGTCGTCA  17857
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Figure (1): Sequences of *SLC6A4* gene (rs6354)

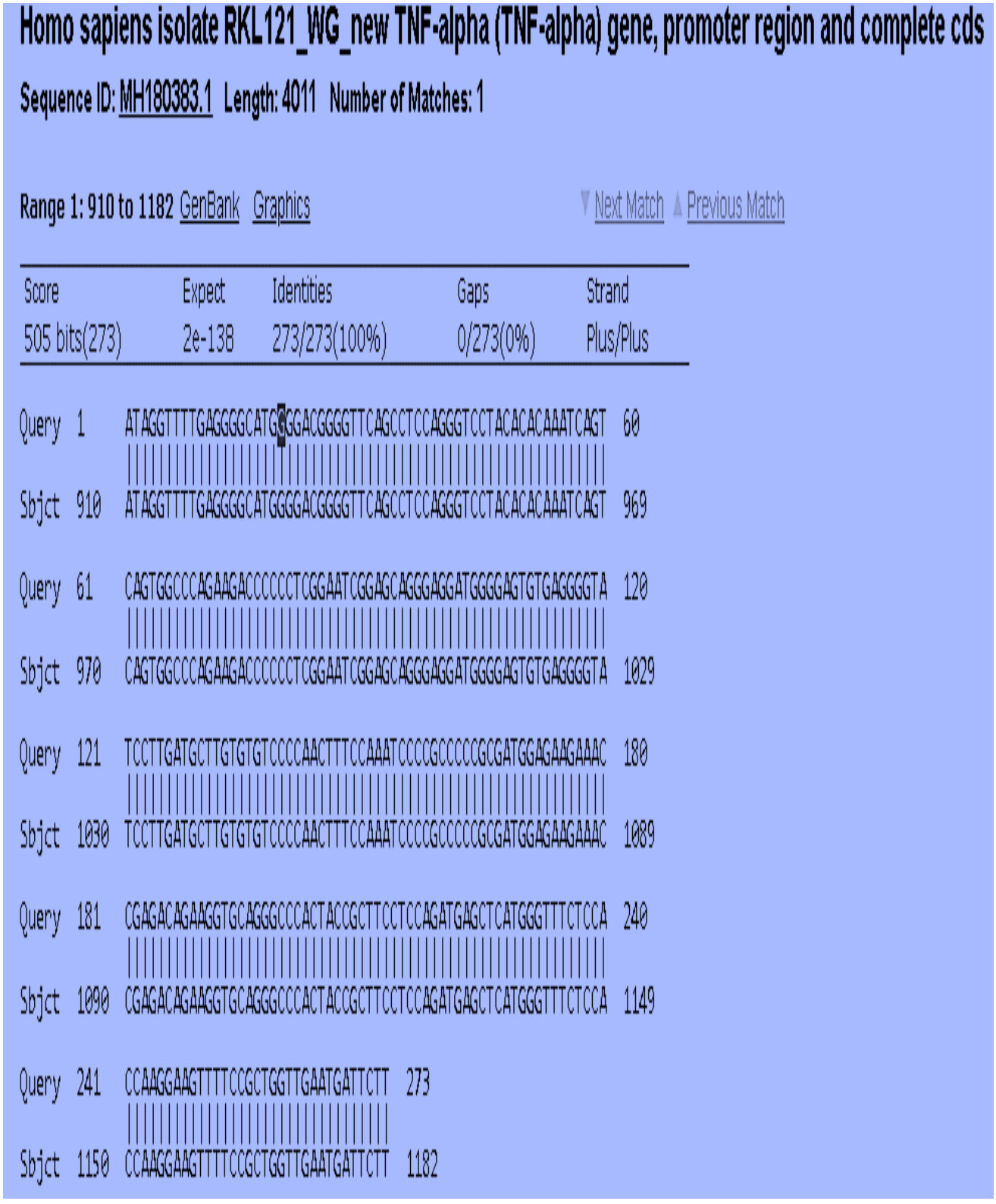

Figure (2): Sequences of *TNF-α* gene (rs1800629)

<u>Appendix (3):</u> Schemes of tri allele specific primer of the SLC6A4 gene; SNP: rs6354 G>T and the *TNF* gene; SNP:rs1800629 G>A

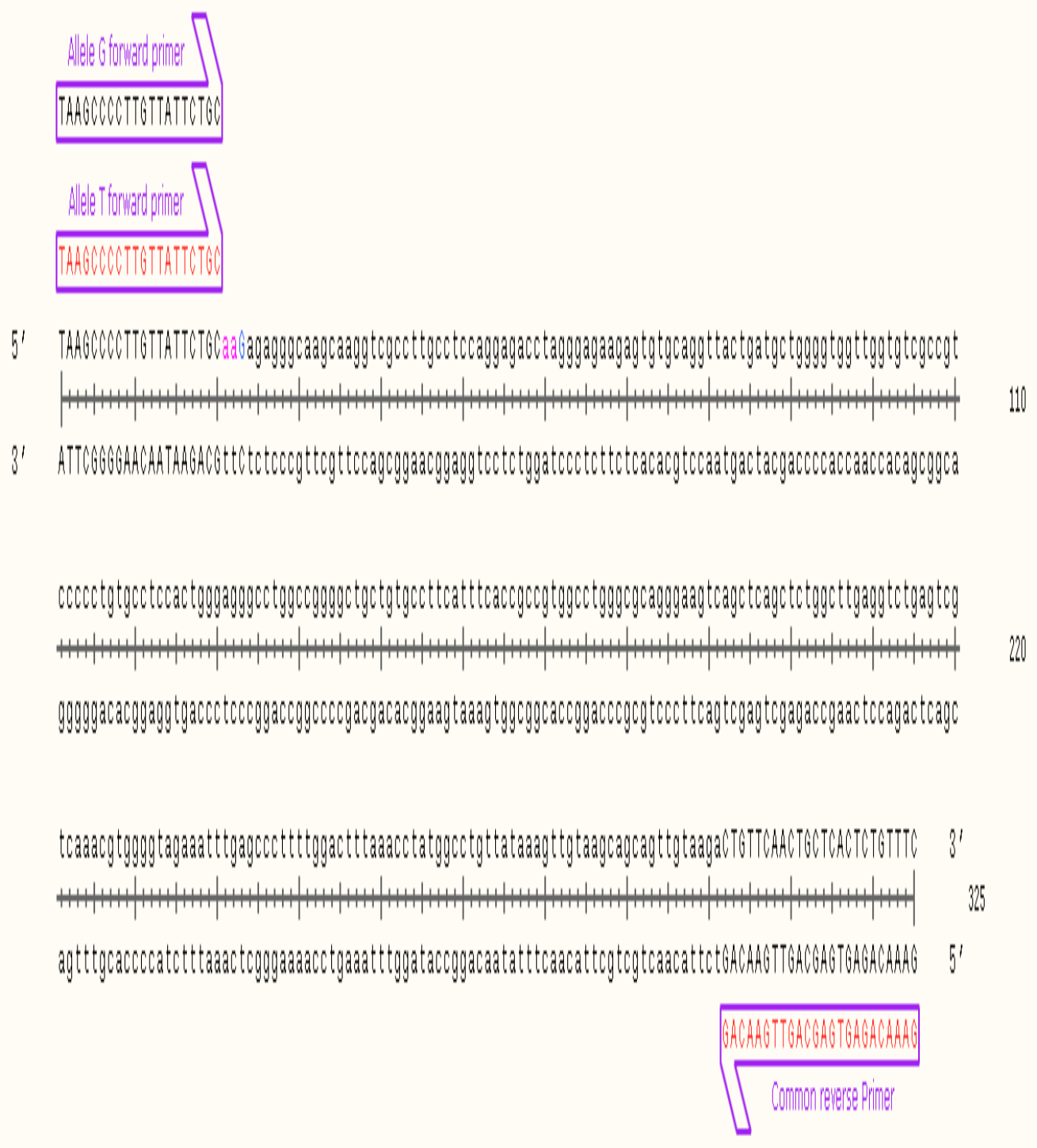

Figure (1): The scheme of tri allele specific primer of the SLC6A4 gene; SNP: rs6354 G>T

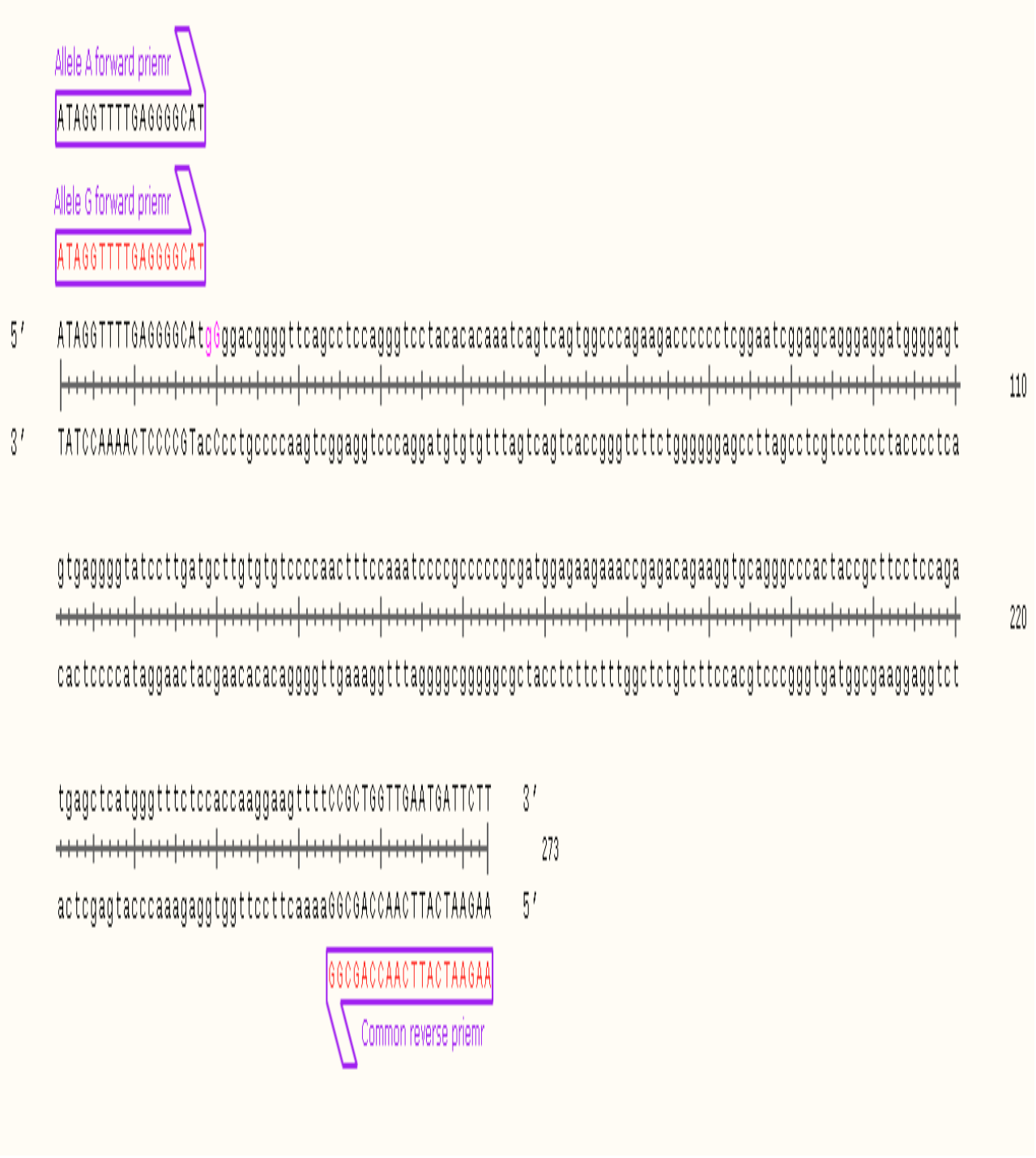

Figure (2): The scheme of tri allele specific primer of the *TNF* gene;

SNP:rs1800629 G>A

<u>Appendix (4):</u> Figures of Functional Consequences for *SLC6A4* (rs6354) and *TNF-α* (rs1800629) genes

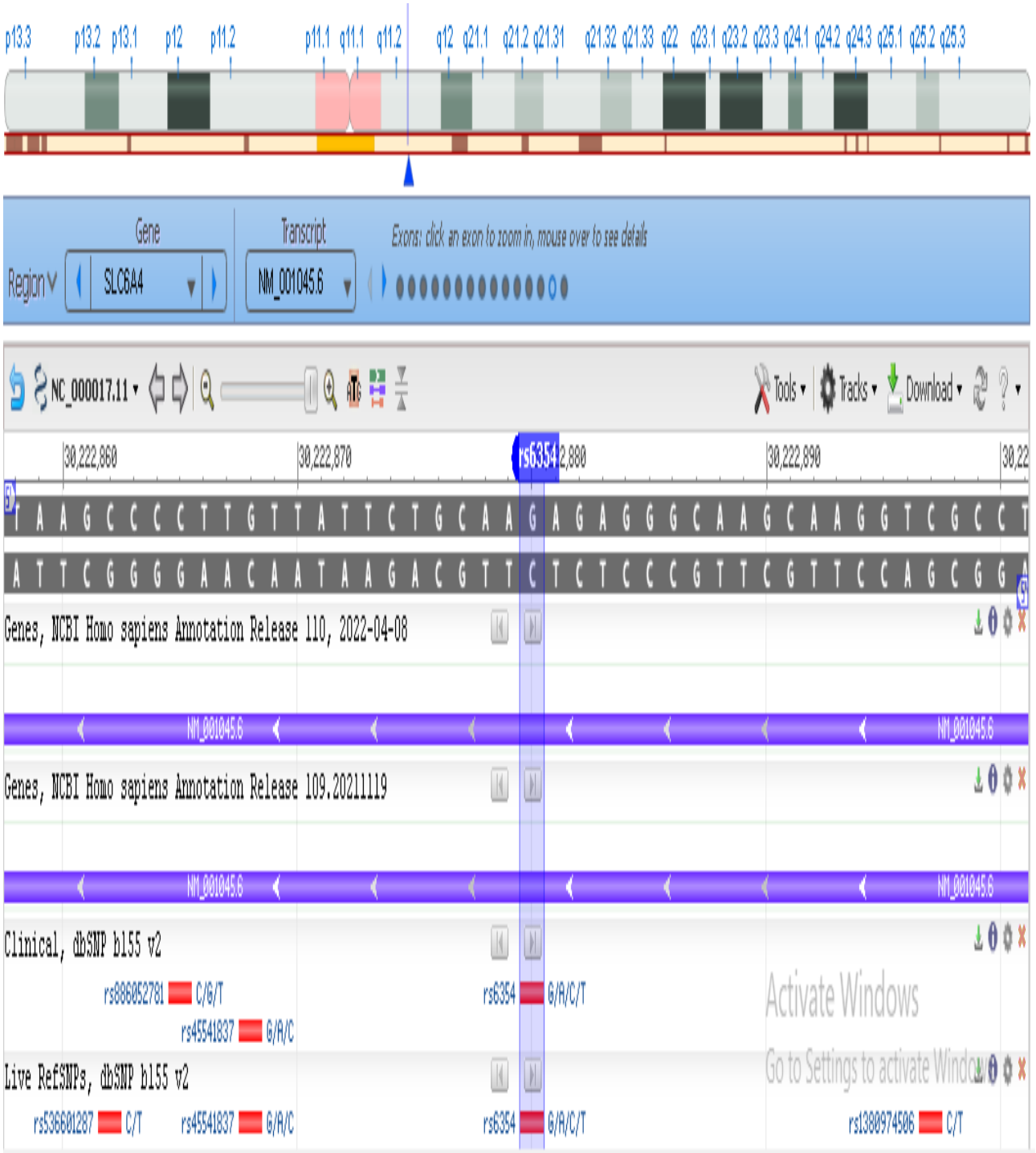

Figure (1): Functional Consequences for *SLC6A4* gene (rs6354)

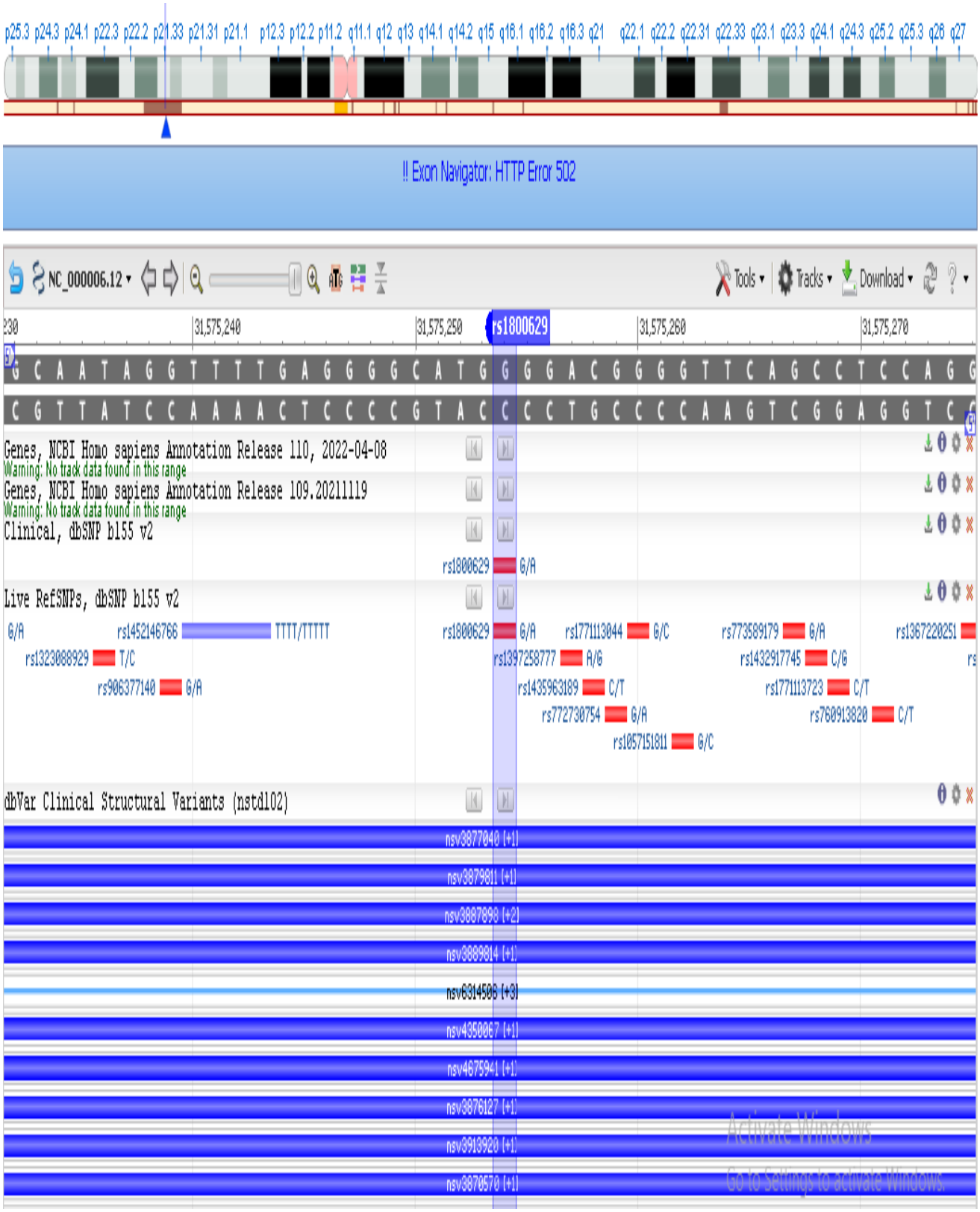

Figure (2): Functional Consequences for TNF-α gene (rs1800629)