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STATISTICAL INVESTIGATION OF miRNA ALTERATION IN HYPERTHYROIDISM PATIENTS IN AL-ANBAR PROVINCE

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By Omer Thamer Marzoog ALBORISHA

June 2023

We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science

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ABSTRACT

STATISTICAL INVESTIGATION OF miRNA ALTERATION IN HYPERTHYROIDISM PATIENTS IN AL-ANBAR PROVINCE

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Master of Science in Biology

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June 2023

Women of reproductive age often suffer from thyroid conditions and autoimmune diseases of the thyroid gland, which are linked to adverse pregnancy outcomes such as miscarriage and premature birth. Autoimmune thyroid disease (TD) is the leading cause of thyroid problems. Thyroid autoimmunity is characterized by the presence of thyroid antibodies (ATA), particularly thyroid peroxidase antibodies (TPO-Ab) and thyroglobulin antibodies (TG-Ab) (TAI). Women who test positive for thyroid antibodies tend to experience more frequent miscarriages in the first trimester of pregnancy. The aim of the study was to determine how thyroid disorders affect pregnant women who experienced recurrent miscarriage and to evaluate how thyroid antibodies affect abortion in the first trimester of pregnancy. Pregnant women who applied to the Women's and Children's Hospital in Ramadi were included in this study. 120 blood samples were taken from women with a history of miscarriage in the first trimester of pregnancy and were determined as the study group. The forty-five blood samples were taken from healthy women who did not have a miscarriage in the first trimester of their pregnancy and were used as a control group. Both groups completed the questionnaire, but all women with metabolic morbidity, hypertension, and diabetes were excluded. After statistical evaluation and comparison, there was a significant increase (P 0.05) in the thyroid stimulating hormone concentration of the study group as well as non-significant increases in Serbs t3 and free T4 concentrations (P=0.391 and P=0.628, respectively). It has been discovered that pregnant women who are overweight (n=35) according to body mass index have higher mean miscarriage rates than normal weight

pregnant women (85). This change was not statistically significant (P=0.524). Four age groups were examined in the study to determine the maximum abortion rate. Statistical analysis revealed a highly significant difference ($p<0.001$) in low levels between age groups (26-32 years) and other age groups (19-25, 33-40, 41-46). RT-PCR results were analyzed to detect expression of the *micRNA146A* gene and compared between control and patient groups. Comparative threshold cycle (CT) approach (2-Ct) was used to calculate relative changes in expression levels.

2023, 58 pages

Keywords: Hyperthyroidism, Real-time PCR, Hormones, ELISA, TSH, miRNA

ÖZET

AL-ANBAR İLİNDEKİ HİPERTİROİDİZM HASTALARINDA miRNA DEĞİŞİKLİĞİNİN İSTATİSTİKSEL İNCELENMESİ

Omer Thamer Marzoog ALBORISHA
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Üreme çağındaki kadınlar genellikle, düşük ve erken doğum gibi olumsuz gebelik sonuçlarıyla bağlantılı tiroid rahatsızlıklarından ve tiroid bezinin otoimmün hastalıklarından muzdariptir. Otoimmün tiroid hastalığı (TD), tiroid problemlerinin önde gelen nedenidir. Tiroid otoimmünitesi, tiroid antikorlarının (ATA), özellikle tiroid peroksidaz antikorlarının (TPO-Ab) ve tiroglobulin antikorlarının (TG-Ab) (TAI) varlığı ile karakterize edilir. Tiroid antikorları için pozitif test yapan kadınlar, hamileliğin ilk üç ayında daha sık düşük yapma eğilimindedir. Bu çalışmanın amacı, tiroid bozukluklarının tekrarlayan düşükler yaşayan gebeleri nasıl etkilediğini belirlemek ve tiroid antikorlarının gebeliğin ilk trimesterindeki düşükleri nasıl etkilediğini değerlendirmektir. Bu çalışmaya Ramadi Kadın ve Çocuk Hastanesine başvuran gebeler dahil edildi. Gebeliğin ilk trimesterinde düşük geçmişi olan kadınlardan 120 kan örneği alındı ve çalışma grubu olarak belirlendi. Gebeliğinin ilk trimesterinde düşük yapmamış sağlıklı kadınlardan alınan 45 kan örneği kontrol grubu olarak kullanıldı. Her iki grup da anketi doldurdu, ancak metabolik morbiditesi, hipertansiyonu ve diyabeti olan tüm kadınlar hariç tutuldu. İstatistiksel değerlendirme ve karşılaştırmadan sonra, çalışma grubunun tiroid uyarıcı hormon konsantrasyonunda önemli bir artış (P=0.05) ve Serbest t3 ve serbest T4 konsantrasyonlarında anlamlı olmayan artışlar (sırasıyla P=0.391 ve P=0.628) vardı. Vücut kitle indeksine göre fazla kilolu (n=35) gebelerin normal kilolu gebelere (85) göre düşük ortalama oranlarının daha yüksek olduğu saptanmıştır. Bu değişiklik istatistiksel olarak anlamlı değildi (P=0.524). Maksimum düşük oranını belirlemek için yapılan çalışmada dört yaş grubu

incelendi. İstatistiksel analiz, yaş grupları (26-32 yaş) ve diğer yaş grupları (19-25, 33-40, 41-46) arasında düşük seviyelerde oldukça anlamlı bir fark (P=0.001) ortaya çıkardı. *MIR146A* geninin ekspresyonunu saptamak için RT-PCR sonuçları analiz edildi ve kontrol ve hasta grupları arasında karşılaştırıldı. İfade seviyelerindeki göreli değişiklikleri hesaplamak için karşılaştırmalı eşik döngüsü (CT) yaklaşımı (2-Ct) kullanıldı.

2023, 58 sayfa

Anahtar Kelimeler: Hipertiroidi, Real-time PCR, Hormonlar, ELISA, TSH, miRNA

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Omer Thamer Marzoog ALBORISHA
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LIST OF SYMBOLS

Bmi	Body mass index
dL	Deciliter
g	Gram
gm	Gram
kg	Kilogram
L	Liter
µL	Micro liter
m^2	Square meter
mcg	Microgram
mg	Microgram
mIU	Milli-international units
mmol	Milli mole
mol	Mole
ng	Nanogram
nm	Nanometer
NS	Non-significant
rpm	Revolutions per minute
Sd	Standart Deviation
Tbg	Thyroxine binding globulin
-	Minus
%	Percent
**	Significant

LIST OF ABBREVIATIONS

AGO	Argonaute
ANTI-TG	Anti thyroglobulin antibody
ANTI-TPO	Anti-thyroid peroxidase
APL	Anti-phospholipid autoantibodies
BMI	Body mass index
ELISA	Enzyme-linked ımmune sorbent assay
FT3	Free triiodothyronine
FT4	Free thyroxin
hCG	Human chorionic gonadotropin
Mg	Magnesium
PIWI	P-element induced wimpy testis
PTU	Propylthiouracil
RAI	Radioactive iodine
RAIU	Radioactive iodine uptake
Se	Selenium
T3	Triiodothyronine
T4	Thyroxin
TBO	Thyroxine-binding globulin
TD	Thyroid disease
TFT	Thyroid function tests
TSH	Hormone that stimulates the thyroid
UTR	Untranslated region

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1. INTRODUCTION

In recent times, there has been a lot of interest in microRNAs, which are often referred to as miRNAs. MicroRNAs are non-protein-coding RNA molecules that are located in certain organs and are responsible for controlling gene expression in eukaryotic animals (Bavelloni *et al*. 2017).

These microRNA molecules are tiny and their changing concentrations are diagnostic or causative of a number of pathological disorders, such as cancer or cardiovascular ailments. miRNAs are an example of a prospective new class of biomarkers that have the potential to be used in the diagnosis of life-threatening conditions as well as the tracking of the progression of illness in patients (Ganju *et al.* 2017). In addition, a significant number of the therapeutically targetable microRNAs contribute significantly to the process of immune response control. Because their reduction has an influence on the overactive response to infection, this suggests that the control of the immune response, which is also important in regulation of genes, is crucial. This highlights the importance of gene expression regulation (Brosnan and Mitter 2021, Correia *et al*. 2019).

This is due to the important roles that miRNAs play in the body's biological processes. A significant number of the therapeutically targetable microRNAs contribute significantly to the process of immune response control. Because their reduction has an influence on the overactive response to infection, this suggests that the control of the immune response, which also important in regulation of genes, is crucial. This highlights the importance of gene expression regulation (Brosnan and Mitter 2021).

The thyroid is a butterfly-shaped gland that sits in the centre of the neck, above the clavicles and below the larynx (voice box). Triiodothyronine (T3) and thyroxine (T4), two hormones made by the thyroid, control how the body consumes and stores energy (Beynon and Pinneri 2016). The immune system creates an antibody similar to TSH in hyperthyroidism patients, which causes the thyroid gland to overproduce thyroid

hormone. This can happen to men or women of any age, although it most frequently affects women between the ages of 20 and 40. Hyperthyroidism symptoms are brought on by the thyroid gland enlarging and producing excessive amounts of thyroid hormone (Stathatos 2012, Biondi and Kahaly 2010).

A highly frequent refractory endocrine and metabolic disorder known as hyperthyroidism results from an overproduction of thyroid hormone and has a variety of internal and external causes. It encourages an accelerated metabolic rate and enhanced bodily system excitability, especially in the neurological, circulatory, and digestive systems. For therapeutic therapy of hyperthyroidism, early and accurate biomarkers to predict the return of hyperthyroidism are therefore extremely important. The role of microRNAs (miRNAs), a group of endogenous non-coding short RNAs, has garnered increased attention in recent times. In humans, miRNAs are located in the intron region of coding genes. These miRNAs can influence gene expression by acting on the target gene's promoter region, preventing the binding of suppressor genes and inhibitory factors, thereby increasing gene expression. Several miRNAs have been identified to play a role in the development of onychomycosis (Kartamihardja *et al.* 2016, Ai *et al.* 2020).

1.1 Aim of Study

Given this information, the objective of the study was to explore the link between thyroid antibodies (antithyroid peroxidase and antithyroid) and thyroid hormones in a specific group of women who had hyperthyroidism and gene expiration of microRNA (miRNA) with hyperthyroidism. In the city of Anbar, we are planning to collect a total of 45 control samples, 120 from women patients (women with a history of miscarriage in the first trimester of pregnancy). Each sick and a healthy individual will each have 5 milliliters of whole blood drawn from them for the collection that will take place.

2. LITERATURE REVIEW

The thyroid gland is made up of two lobes that are related to each other and is one of the biggest endocrine glands in the body. The condition known as hyperthyroidism is characterized by an overactive thyroid gland that leads to a persistent rise in the production and discharge of thyroid hormones. The hypermetabolic condition of thyrotoxicosis is the end result of a group of illnesses known collectively as hyperthyroidism (Qadir and Faheem 2017). An unusually high level of thyroid hormones (T3 and T4) generated and released by the thyroid gland characterizes these illnesses (Wilczynska and Bushell 2015).

Both underactive and overactive thyroids may have a negative impact on fertility. However, there is minimal evidence to support the hypothesis that hyperthyroidism is linked to infertility, and this data is sometimes inconsistent. Thyroid hormone has a range of effects on human reproduction, both at the central and the peripheral levels, and these effects are caused by different processes. It is possible for men and women who have hyperthyroidism to have infertility; however, this condition is often reversible once euthyroidism has been achieved (Paraskevopoulou and Hatzigeorgiou 2016). Certain microRNAs that are therapeutically targetable play a crucial role in modulating the immune response. Their depletion has an effect on the hyperactive response to infection, which demonstrates that the regulation of the immune response, which also plays an important role in the regulation of gene expression, is essential (Ludwig *et al*. 2016).

2.1 MicroRNAs

A recently uncovered phenomenon of gene expression that is associated with RNA has recently been defined thanks to the development of more sophisticated molecular tools. MicroRNAs, also known as miRNAs, are short sequences of endogenous RNA that act as regulatory molecules but do not code for proteins. It has been discovered that they serve important roles in the regulatory activities of gene expression for the majority of eukaryotic organisms. These naturally occurring RNA sequences are the focus of a

substantial amount of investigation in a wide variety of model species, ranging from plants to mammals (Turchinovich *et al*. 2016). MicroRNAs are involved in a wide variety of key biological activities in plants, including the control of growth and development, as well as the response to abiotic stress. There is mounting evidence that microRNAs, which are found in a wide variety of animals, influence gene expression at post-transcriptional levels. More recent studies have showed that the sequences of miRNAs found in plants are highly conserved and have almost perfect complementarities with the messenger RNA (mRNA) targets that they are designed to regulate (Vishnoi and Rani 2017).

In the nucleus, stem-loop structures of around 70 nucleotides in length called pre-miRNAs are processed into mature microRNAs with a length of 21 to 24 nucleotides. MicroRNAs have a length of 21–24 nucleotides. Base pairings, which are determined by sequence homologies, are responsible for the formation of the stem and loop structures (Katar *et al*. 2017). Since it is believed that Pri-miRNA is longer than pre-conserved miRNA's stem-loop structures, the subsequent step in the processing involves some modification. In addition, it has been shown that microRNAs may, under some circumstances, stimulate the expression of genes. Recent research has prompted scientists to hypothesize that microRNAs are transported from one subcellular compartment to another in order to modulate the pace of translation and maybe even transcription (Riolo *et al*. 2020).

MicroRNAs are crucial for the regular development of animals and play significant roles in a variety of biological processes. In humans, abnormal expression of miRNAs has been linked to a wide variety of illnesses. In addition, microRNAs may be found in extracellular fluids since they are released there. Extracellular miRNAs function as signaling molecules that mediate interactions between cells (McGuire *et al*. 2015).

2.1.1 Biogenesis

The initial step in miRNA synthesis involves the processing of RNA polymerase II/III transcripts, either following or simultaneously with transcription. MicroRNAs can

occasionally be produced in clusters, which are large single transcripts. Since their seed regions are probably relatively similar, the miRNAs are classified as belonging to the same family in this instance. There are two types of miRNA biogenesis pathways: canonical pathways and non-canonical pathways. Among these, the canonical biogenesis route is by far the most predominant and significant miRNA processing pathway (Lukasik *et al*. 2016). The RNase III endonuclease Dicer is responsible for the processing of the pre-miRNAs after they have been produced and subsequently exported to the cytoplasm by a complex consisting of exportin 5 (XPO5) and RanGTP. As a result of this processing step, a mature miRNA duplex is generated, involving the removal of the terminal loop. The mature version of the miRNA is named based on the fact that the miRNA strand can be read in either orientation. These variations may range from almost equal amounts to predominately one or the other (Mishra *et al*. 2016).

The level of thermodynamic stability at the 5' ends of the miRNA duplex and the presence of a 5' U at nucleotide position 1 play significant roles in determining whether the 5p or 3p strand will be utilized. The passenger strands of miRNA (Figure 2.1), which do not contain any mismatches, are selectively cleaved by AGO_2 and subsequently degraded by cellular machinery, leading to a potential bias towards a particular strand. This happens when the miRNA duplexes have a central mismatch or lack AGO_2-loaded miRNA (Lekchnov et al., 2016). After processing, the miRNA is incorporated into the RISC (RNA-induced silencing complex) complex with the aim of preventing target mRNA translation or cleaving the target mRNA. The AGO_1 protein, containing a PAZ domain, an RNA-binding domain, and an RNaseH-like PIWI domain, is a crucial component of the RISC complex responsible for degrading miRNA* strands. The AGO protein family is vital and indispensable for the functionality of the miRNA-RISC complex, as its absence would render the complex ineffective (Militello *et al.* 2017).

In human cells, we were able to establish both their capacity to inhibit protein production and their connection with miRNAs. The particular targeting of the mRNA sequence is made possible thanks to the presence of the Piwi domain, as well as the fact that the target mRNA sequence and the single-stranded miRNA contained in the RISC

complement one another. Afterward, the AGO component has the ability to cleave the miRNA-mRNA duplex (or siRNA-mRNA duplex), resulting in a decrease in the rate of gene expression for the targeted mRNA (Segal and Slack 2020).

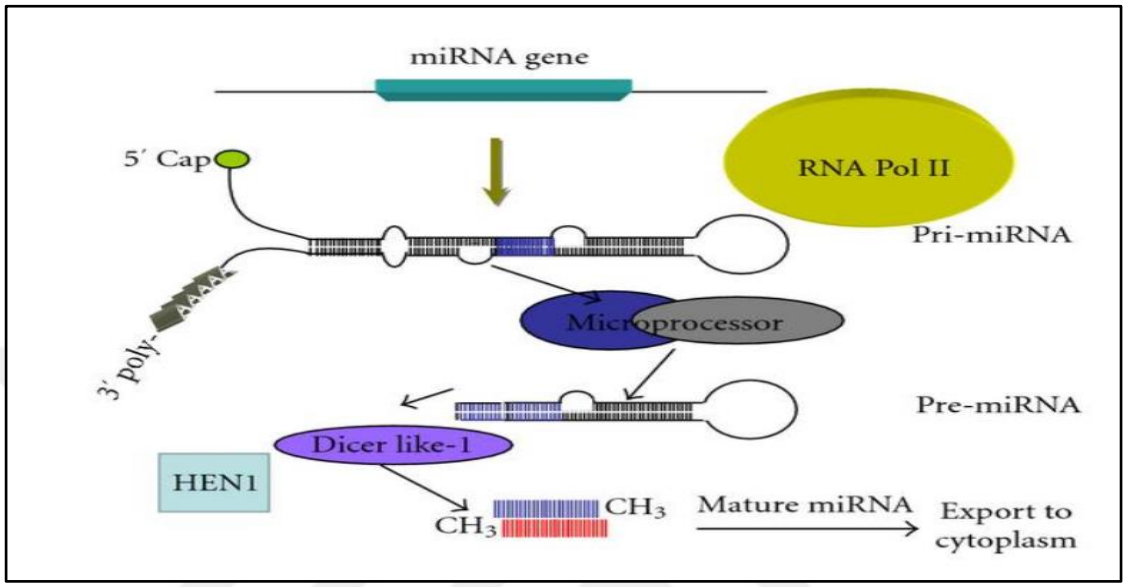

Figure 2.1 MicroRNA biogenesis (Achkar *et al*. 2016)

2.1.2 Mechanisms

MicroRNAs, upon binding to specific sequences in the 3'untranslated region (UTR) of their target mRNAs, initiate translational repression, mRNA deadenylation, and decapping (Dori and Bicciato 2019) as shown in Figure 2.2. Numerous research studies have consistently demonstrated the validity of this observation. Apart from the 5' ends of miRNA duplexes, miRNA binding sites have also been identified in various regions of mRNA, including the 5' untranslated region, the coding sequence, and even within promoters. However, further research is necessary in order to get a complete comprehension of the practical importance of this kind of interaction (Michlewski and Cáceres 2019).

AGO and the guide strand make up the miRISC, which is an abbreviation for the minimum miRNA-induced silencing complex. The degree of complementarity between MREs and target mRNAs is what determines whether or not there is AGO_2-dependent slicing of target mRNA or miRISC-mediated translational inhibition and degradation of target mRNA. The induction of AGO_2 endonuclease activity and mRNA cleavage may be attributed to an interaction between completely complementary miRNA and MRE (Rupaimoole *et al*. 2016).

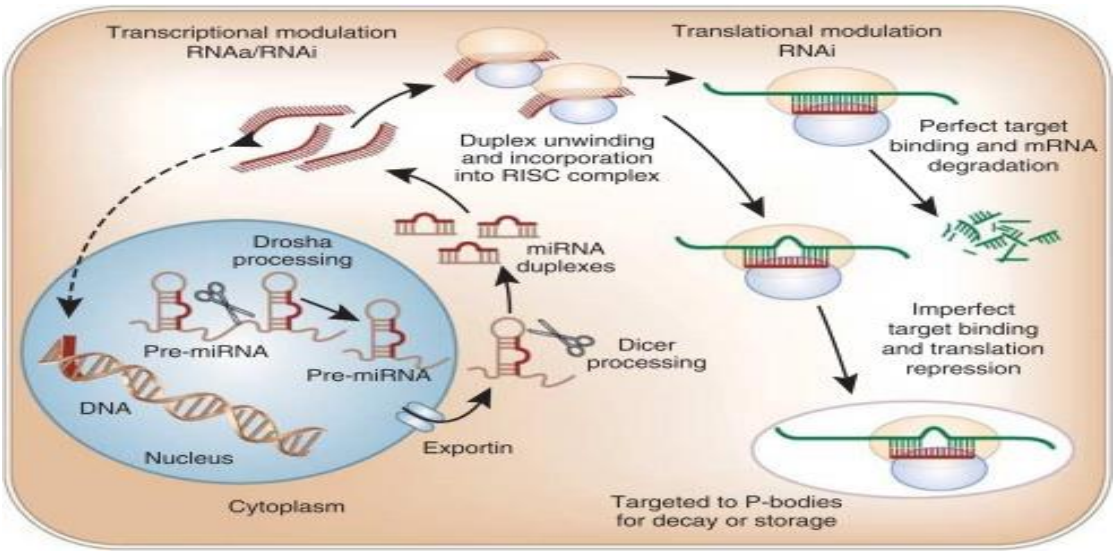

Figure 2.2 The role of miRNA (Cambiagno *et al*. 2021)

The majority of interactions between miRNA and MRE in animal cells are only partially complimentary. The vast majority of MREs have at least one central mismatch to their respective guide miRNA, which inhibits the action of the AGO2 endonuclease. As a consequence of this, AGO2 plays a role analogous to that of a mediator in RNA interference, much like the other non-endonucleolytic members of the AGO family (AGO1, 3, and 4 in humans) (Swarbrick *et al*. 2019).

The 5' seed region (nucleotides 2–8) is the location in which, in many instances, a functional miRNA: MRE interaction takes place. However, further trimming at the 3' end improves the interaction between the miRNA and its target in terms of both its stability and its specificity. Some studies have indicated that miRNAs may also

stimulate the expression of genes, despite the fact that the majority of research focuses on how miRNAs suppress gene expression (Chakraborty *et al*. 2017).

During cell cycle arrest, miRNAs like let-7 have been shown to cooperate with AGO_2 and FXR1 to increase translation, while in proliferating cells, they act as translational inhibitors. There was also evidence that miRNAs may upregulate gene expression in cells that were in a quiescent state, such as oocytes. Instead of GW182, AGO_2 and FXR1 are responsible for the activation of translation that is mediated by miRNA. MiRNAs may also activate genes by binding to the 5' untranslated region (UTR) of mRNAs producing ribosomal proteins when amino acids are in short supply (Zhang *et al*. 2021).

2.2 Hyperthyroidism

The condition known as hyperthyroidism, or an overactive thyroid, occurs when the gland responsible for thyroid function produces an abundance of thyroid hormones. Situated at the front of the neck, the thyroid, resembling a small butterfly in shape, plays a vital role in controlling energy utilization in nearly all organs, including the heartbeat. Excessive levels of thyroid hormones in the body lead to an acceleration of various physiological processes (Kravets 2016).

It refers to a disorder in which your thyroid gland produces and secretes excessive amounts of the hormone thyroid. Because of this condition, your metabolism may become more active. The condition known as hyperthyroidism (Figure 2.3) is characterized by a fast heartbeat, decreased weight, increased hunger, and feelings of worry. Surgery, antithyroid medications, radioactive iodine, and beta blockers are some of the treatment options available for hyperthyroidism (Osuna *et al*. 2017).

In general, the thyroid is responsible for the production of two primary hormones that have an effect on how your body functions. Thyroxine (T4) and triiodothyronine are the names given to these two hormones (T3). Things like the rate at which your heart beats

and the rate at which you burn calories are both under the control of your thyroid. It achieves this by releasing hormones that govern your metabolism, which is the process by which the body converts food into energy and maintains its vitality (Srinivasan and Misra 2015).

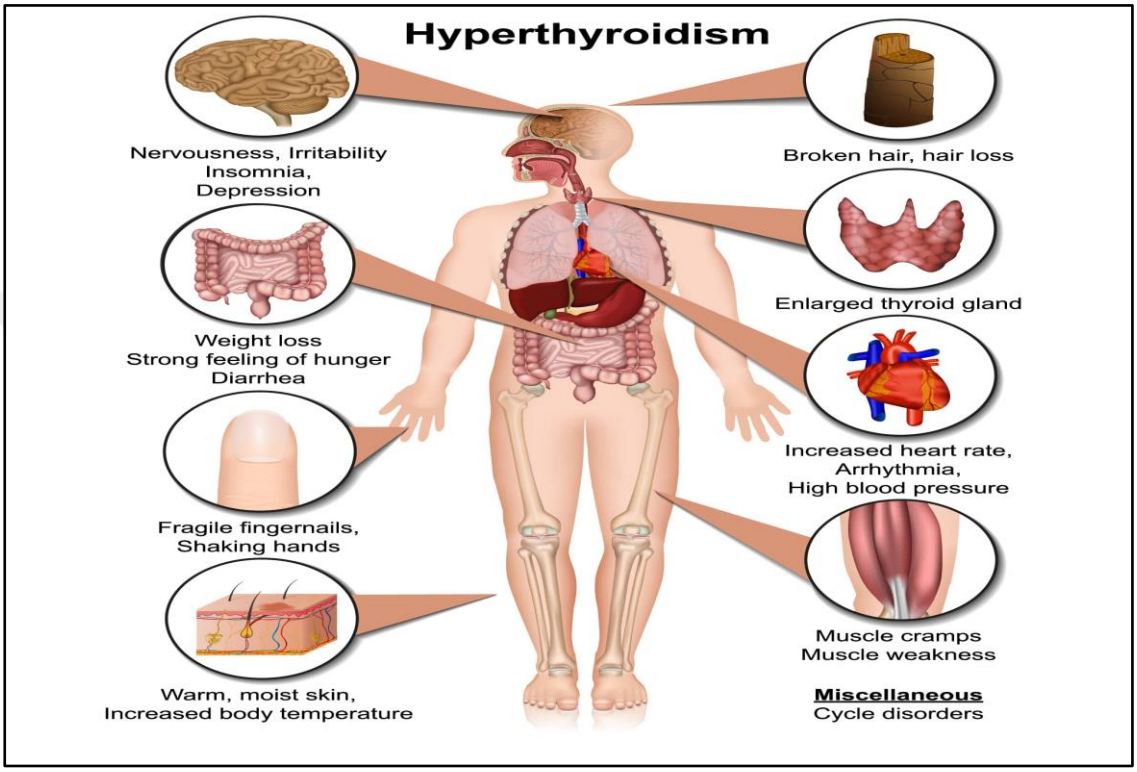

Figure 2.3 Overview of hyperthyroidism (Sjölin *et al*. 2019)

The parathyroid hormone that your thyroid produces is yet another vital hormone in your body. This contributes to the maintenance of a normal level of calcium in your blood. The condition known as hyperthyroidism, commonly referred to as an overactive thyroid, may cause the metabolism to speed up and manifest in a variety of uncomfortable symptoms (Reddy *et al*. 2017).

2.2.1 Causes

Hyperthyroidism can result from various disorders, including Graves' disease, Plummer's disease, and thyroiditis. The thyroid gland, resembling a butterfly, is a small gland situated near the base of your neck, just below the Adam's apple. It exerts a

significant impact on your overall health. Thyroid hormones play a vital role in regulating every aspect of the metabolic process (LiVolsi and Baloch 2018).

The thyroid gland produces two primary hormones: thyroxine (T4) and triiodothyronine (T3). Your body's cells are all affected by these hormones. They assist maintain the pace at which your body burns fats and carbs, contribute to the regulation of your body temperature, have an effect on the rate of your heartbeat, and contribute to the management of the manufacture of protein. Moreover, your thyroid gland plays a vital role in producing a hormone called calcitonin, which contributes to maintaining a healthy balance of calcium in your blood (Moleti *et al*. 2019).

The thyroid usually secretes the appropriate quantity of hormones, but there are instances when it produces an excessive amount of T4. There are a variety of possible explanations for this, some of which are included below. Graves' disease is an autoimmune disorder where antibodies produced by your immune system cause the thyroid to overproduce T4, leading to the symptoms of the disease. Hyperthyroidism may be traced back to this factor more often than any other (Kitahara *et al*. 2019).

You can discover that your thyroid gland gets irritable after giving birth for unknown reasons, such as an autoimmune condition. The inflammation may cause an excessive amount of thyroid hormone, which would ordinarily be kept in the gland, to leak into the circulation. Thyroiditis comes in a variety of forms, some of which can be painful while others are completely non-lethal (Biondi and Cooper 2018).

Inflammation of the thyroid gland is referred to as thyroiditis. Some forms of thyroiditis may cause thyroid hormone to escape from the thyroid gland and into the circulation. This can be a dangerous condition. As a direct consequence of this, you run the risk of developing signs of hyperthyroidism. Some forms of thyroiditis have been linked to the development of hyperthyroidism. Subacute thyroiditis is characterized by a painfully swollen thyroid that is enlarged with inflammation. Postpartum thyroiditis is an inflammation of the thyroid that may occur after a woman has given birth (Nguyen *et al*. 2018).

Painless thyroiditis is a kind of thyroiditis that may develop at any time, and unlike postpartum thyroiditis, it is not caused by pregnancy. It's possible that your thyroid is too big. The experts agree that autoimmune disease is most likely the cause of painless thyroiditis. The symptoms of hypothyroidism, often known as an underactive thyroid, may also be caused by thyroiditis. When your thyroid is hyperactive over an extended length of time, there is a possibility that it could eventually become underactive (Banigé *et al*. 2018).

2.2.2 Complications

If left untreated, hyperthyroidism might raise a person's chance of experiencing a thyroid storm. A thyroid storm is an extremely uncommon health condition that may develop if hyperthyroidism is left untreated for an extended period of time. Because there are an excessive amount of thyroid hormones in your system, your body goes into overdrive. Having a fast heart rate, elevated blood pressure, and fever are all symptoms that may be seen during a thyroid storm, which is a potentially life-threatening combo of symptoms (Ferrari *et al*. 2019).

Women who already have thyroid issues or who acquire hyperthyroidism during pregnancy are both susceptible to developing pregnancy-related difficulties. Both the pregnant woman and the baby are at risk when there is an excess of the hormone thyroid. There is a possibility of having an ectopic pregnancy or giving delivery too soon. During pregnancy, having your thyroid hormone levels checked on a regular basis might help identify any anomalies, at which point your physician may recommend treatment with medication (Andersen and Laurberg 2016).

If you have hyperthyroidism, your bones may become fragile and thin, which might put you at risk for developing osteoporosis. Bone health may be improved by taking vitamin D and calcium supplements both during and after treatment for an illness. Getting the recommended amount of exercise or engaging in any kind of physical activity on a regular basis may also help avoid osteoporosis (Quérat *et al*. 2015).

People who have hyperthyroidism have an increased risk of developing thyroid cancer, often known as thyroid carcinoma. According to a study summary published in 2018, hyperthyroid people with thyroid cancer had a more "aggressive" form of the disease, which was associated with a direr prognosis than euthyroid patients (those with a healthy thyroid). Atrial fibrillation is a potentially fatal arrhythmia (irregular heartbeat) that may be caused by hyperthyroidism. This condition can also induce congestive heart failure and increase the risk of having a stroke (Delitala 2017).

Thyroid conditions that aren't properly managed may put a significant strain on your body and, if left untreated, might lead to life-threatening complications. There are a number of uncomplicated blood tests that are used in the diagnosis of hyperthyroidism and other thyroid problems (Bartalena *et al*. 2016).

2.2.3 Diagnosis

Due to the fact that many of its symptoms are shared by those of other disorders, hyperthyroidism cannot be diagnosed solely on the basis of its symptoms. Besides ordering imaging scans, your physician might also request a series of thyroid blood tests to help confirm the diagnosis and determine the underlying cause. For women facing difficulties in getting pregnant, it is advisable to undergo thyroid level examination, as hyperthyroidism has been associated with various fertility-related challenges. (Kaplowitz and Vaidyanathan 2020).

In the condition of hyperthyroidism, the individual experiences elevated levels of the thyroid hormones T3 and T4, while the levels of the thyroid-stimulating hormone (TSH) are reduced. The following imaging tests may be used to diagnose hyperthyroidism (Figure 2.4) and investigate the thyroid: a test known as the radioactive iodine uptake (RAIU). In this test, you will take a low amount of radioactive iodine, which is also known as a radiotracer, by mouth in order to determine the percentage of this substance that is absorbed by your thyroid (Vaske *et al*. 2016).

Healthcare professional will do a scan of your neck using a machine known as a gamma probe after a certain length of time has passed, often six and twenty-four hours later, to determine how much of the radioactive iodine your thyroid has absorbed. If a significant amount of radioactive iodine has been taken up by your thyroid, this indicates that your thyroid gland is creating an excessive amount of thyroxine (T4). If this describes your condition, you almost certainly suffer from Graves' disease or thyroid nodules (King *et al*. 2016).

The gamma camera will take these images while you are in the same position as during the RAIU. On the screen, the radioactive substance may make the whole of your thyroid, or only select areas of it, seem "bright." An ultrasound is a diagnostic procedure that creates pictures of your thyroid by using sound waves that have a high frequency. It is a treatment that is not intrusive and provides your clinician with the ability to see your thyroid on a screen. This test might be used by your healthcare professional to search for nodules on thyroid (Goichot *et al*. 2016).

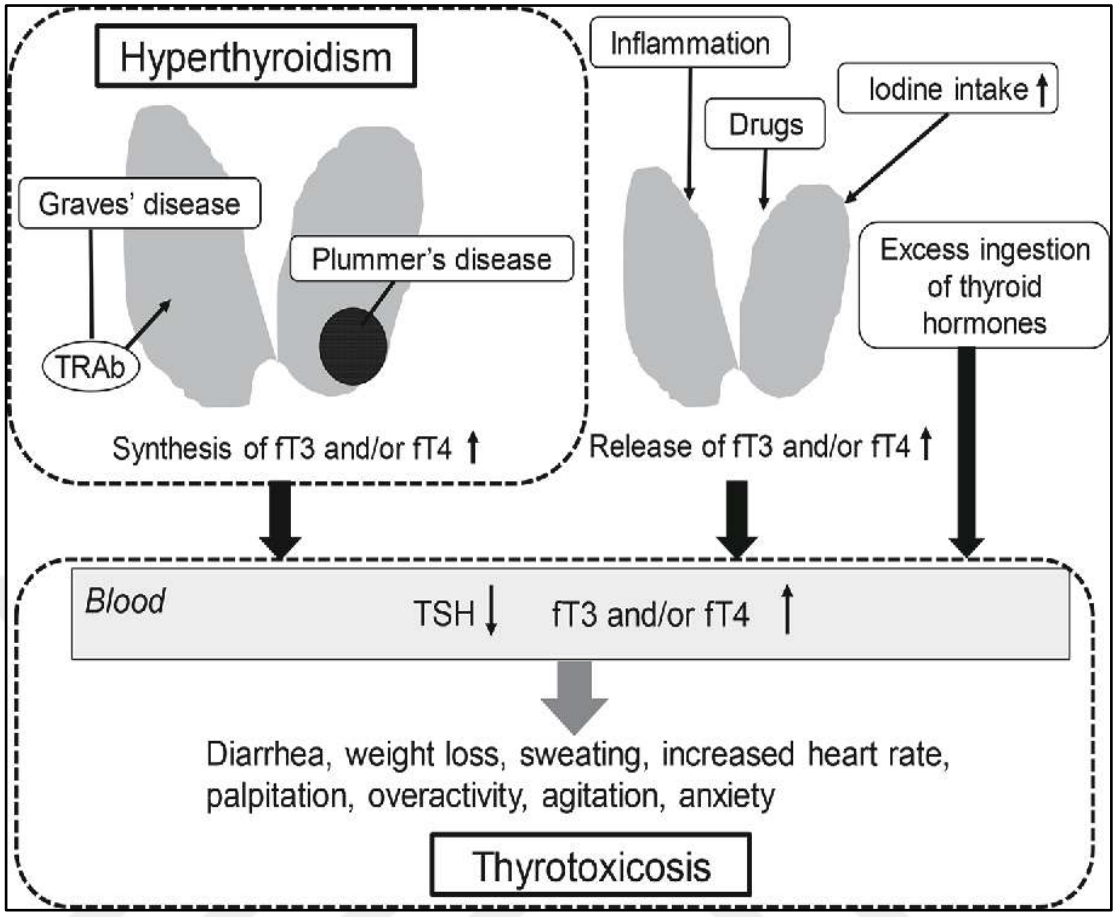

Figure 2.4 Diagnosis of hyperthyroidism (Watad *et al*. 2016)

2.2.4 Treatment

Hyperthyroidism may be treated with a wide variety of different approaches. It's possible that some treatments will work better for you than others, depending on what brought on your hyperthyroidism in the first place. Your healthcare professional will go through all of your options with you and help you choose the approach that will bring the most benefit. The following are some of the treatment options available for hyperthyroidism: Methimazole (Tapazole) or propylthiouracil (PTU), both of which are antithyroid medications: These medications suppress the thyroid's hormone production in the body, offering rapid control over your thyroid condition (Donangelo and Suh 2017).

Overactive thyroid cells may be treated with an oral radioactive iodine therapy. Over a span of several weeks, the radioactive iodine effectively eliminates these cells, leading to a reduction in the size of your thyroid and a decline in thyroid hormone levels. This treatment typically results in the permanent destruction of the thyroid, effectively treating hyperthyroidism. In contrast to the radioactive iodine uptake (RAIU) test and the diagnostic scan, the amount of radiation absorbed by the body as a result of taking this drug is different (Dekkers *et al*. 2017).

The majority of individuals receiving this medication must continue taking thyroid hormone prescriptions for the remainder of their lives to keep their hormone levels at normal levels. These medications, also referred to as beta blockers, prevent thyroid hormone effects from taking place within the body. Although they do not modify the existing hormone levels in your bloodstream, these treatments can aid in alleviating symptoms caused by hyperthyroidism, such as rapid heartbeat, anxiety, and tremors. When treating hyperthyroidism throughout the course of a patient's lifetime, this therapy is almost always combined with one or more other approaches (Turunen *et al*. 2020).

The length of time needed to cure hyperthyroidism might vary widely depending on the underlying etiology of the condition. It is possible that your healthcare professional would opt to treat you with large dosages of non-radioactive iodine drops. This treatment would restore your thyroid levels in seven to ten days. However, this is just a temporary cure, and in the long run, you will most likely need a treatment that is more permanent, such as surgery. This is a highly efficient and long-lasting method of treating hyperthyroidism; nevertheless, it is possible that you may have to wait to be scheduled for thyroid surgery (thyroidectomy). It is widely regarded as a long-term treatment option for hyperthyroidism (Léger and Carel 2018). The majority of therapies come with a warning label stating that they may have adverse effects. Before settling on a course of therapy, it is essential to discuss all of your options with your primary care physician and carefully consider the benefits and drawbacks of each. Among these potential dangers are the following: Methimazole and propylthiouracil are the two drugs that are able to cure hyperthyroidism (Gronich *et al*. 2020).

Both of these treatments have negative effects (PTU). It's common knowledge that these medicines can cause a wide range of unwanted consequences. However, PTU may cause liver injury, albeit this only occurs in a very small number of patients (less than 1% of the population). Agranulocytosis, a severe decrease in white blood cell count, is a further harmful side effect that happens less than 1% of the time. Any age group is susceptible to experiencing these adverse consequences (Sundaresh *et al*. 2017).

This medication, if taken by a pregnant woman, has the potential to pass through the placenta and into the developing fetus. This has the potential to give the unborn child hypothyroidism, as well as the formation of a goiter. Because of this potential adverse effect, pregnant women are subject to strict observation. Additionally, there is the chance of experiencing an adverse response to these treatments, which happens in around 5% of patients (Kobaly and Mandel 2019).

When radiation is present, there is always the risk of developing cancer as an adverse outcome. At this time, there is no evidence to suggest that hyperthyroidism treatment with radioactive iodine increases the risk of getting cancer. This has a low risk and is not very likely to occur. There is a recognized danger that may be passed on from a mother who is pregnant or nursing to their child. While you are pregnant or nursing, you should avoid using radioactive iodine since it may have an effect on the thyroid gland of your kid. After radioactive iodine (RAI) treatment, it is possible that you may have a loss of feeling in your mouth on occasion. This happens rather often. But there's no need to fear; the feeling will eventually return to your lips, even if it may stay with you for as long as a few months at a time (Scappaticcio *et al*. 2021).

There are always going to be some dangers associated with surgery, such as the chance of getting an infection or bleeding. The condition known as hyperthyroidism may often be successfully treated via surgical procedures. In very unusual cases, patients may have consequences such as paralysis of the vocal cords (which makes it impossible for them to speak) and damage to their parathyroid glands, which leads to a calcium deficiency in their blood (Graves 2017).

Following therapy, it is quite probable that you will have to continue to take a synthetic thyroid hormone for the rest of your life. This is due to the fact that some of these therapies, particularly surgery, either lower the amounts of thyroid hormone in your body to very low levels or remove this hormone entirely by removing your thyroid. It is then required to start taking the medicine on a regular basis in order to reintroduce the thyroid hormones into body (Asban *et al*. 2018).

2.3 Housekeeping Genes

Housekeeping genes are frequently described as necessary, conserved, stably expressed in all cells and circumstances, and members of cellular maintenance pathways. The idea of housekeeping genes has benefited the study of evolution as well as theoretical and applied biology. Housekeeping genes, on a broad scale, are the set of genes necessary to maintain life (Marie and Carlotti 2009). In practical terms, housekeeping genes can be described as genes that exhibit consistent expression in every cell of an organism, independent of tissue type, developmental stage, cell cycle state, or external signals. They serve as indicators of an organism's biological well-being. These genes offer valuable insights into species-specific and higher taxonomic level-specific genomic characteristics and gene functions that may influence conservation or evolutionary changes. Consequently, a thorough understanding of housekeeping genes can significantly enhance exploratory, fundamental, and translational research. However, despite their essential and translational significance, their definition has remained unchanged for over 40 years (Bartha *et al.* 2018, Kiat *et al.* 2019).

Housekeeping genes have frequently been linked to the following four biological characteristics: evolutionarily conserved, essentiality, engagement in cellular maintenance, and stability in expression across samples. The connection between these characteristics, however, has not received much attention. The study of housekeeping genes is popular in both fundamental and translational science. Therefore, it is crucial to demonstrate the experimental foundations for these standards (Joshi *et al.* 2022).

Housekeeping genes serve the crucial role of maintaining consistent expression levels in all cells and under various conditions, as they are essential for fundamental cellular maintenance. Their discovery allows for a clearer understanding of the underlying cellular architecture and enhances comprehension of various structural genomic aspects. Additionally, these genes play a vital role in calibrating numerous biotechnological processes and genomic investigations. The number of housekeeping genes has steadily increased as a result of improvements in our ability to quantify RNA expression. Here, we discuss the discovery of housekeeping genes in the RNA-seq and massively parallel sequencing era (Eisenberg and Levanon 2013).

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipments

The general laboratory equipments, instruments, and chemicals that used during the current study to achieve the objective of the current study are arranged in Table 3.1.

Table 3.1 Equipment and tools that were used

Equipment and instruments	Company	Country
Automatic micro pipette (100-1000µL)	Slamed	German
Automatic micropipette (2-20 µL)	Eppendrof	German
Centrifuge	Hattich	Germany
Refrigerator	Kelon	Turkey
Micro plate Elisa Reader	Rayto	China
Micro plate Elisa washer	Allsheng	China
VIDAS	Bio Merieux	France
Vortex shaker	INNOCOM	China
96-wall ELISA Plate	Bioneer	Korea
Vortex Mixer	TOPSCIEN	China

3.1.2 Kits

Below is Table 3.2, which provides the kits used in the procedures of this study along with their respective sources:

Table 3.2 The kits and their sources

Kit	Company	Origin
Thyroid stimulating hormone ELISA (TSH) kit	Bio merieux	France
VIDAS® FT4 kit	Bio merieux	France
VIDAS® FT3 kit	Bio merieux	France
VIDAS Anti-Tg ki	Bio merieux	France
VIDAS Anti-Tpo kit	Bio merieux	France
Anti-phospholipid ELISA kit	Aeskulisa	Germany

3.1.3 Studied groups

The study was carried out between February 1, 2022, and July 1, 2022. The ethical committees of the Department of Biology granted approval for the study.The total number of participants women in was 165 samples from healthy and patiens women, and divieded into 45 control samples 120 from women patients (women with a history of miscarriage in the first trimester of pregnancy). Their ages ranged between 19- 46 years. Clinical information for the patient was presented directly from the patients. The purpose of this research was to evaluate thyroid hormone and antithyroid hormone levels in a study group compared to a control group. Additionally, the study aimed to determine the percentage of thyroid disorders in women with a history of miscarriage during the first trimester of pregnancy.

Secondly, we will study weight changes, as pregnant women who have miscarriages in the first three months of pregnancy will be divided according to average results into women of normal weight and women of overweight to compare between the two groups and find out the percentage of which is more likely to miscarry under the influence of weight changes.

3.1.4 Collection of Blood Samples

A plastic disposable syringe collected 5 mL of venous blood from pregnant individuals at three trimesters. The collected blood was then transferred into a gel tube and placed in a water bath, where it was allowed to clot for ten minutes at 37°C. After clotting, the samples were centrifuged at 3000 g for five minutes. The resulting clear serum was stored frozen in a refrigerator at -10°C. This serum was dispensed into separate portions for further analysis.

- Hormone analytical.
- Immunological parameters assay.
- Molecular analytical.

3.2 Methods

3.2.1 Determining thyroid function tests (TFT) for TSH, FT4, FT3, ANTI-TPO, and ANT-TG

Hormonal test was performed by using (VIDAS) BioMerieux Company, France, through an enzyme linked fluorescent assay" (ELFA).

Principle: The enzyme-linked fluorescence immunoassay (ELFA) used in the VIDAS assay is performed using automated equipment. The apparatus control temperature and individual assay steps. In this particular investigation, the Solid Phase Receptacle (SPR®) acts not only as a pipetting tool but also as the solid phase since it is a disposable alternative to a pipette. During production, mouse anti-TSH antibodies, mouse anti-T4 antibodies, and sheep monoclonal anti-T3 antibodies are coated onto the Solid Phase Receptacle (SPR) that will be used for TSH analysis. In the event that the sample contains Tg antibodies, those antibodies will form a particular bond with the protein that covers the inside of the SPR. This binding occurs as the Tg antibodies specifically interact with the protein coating on the SPR's interior surface.

The reagents required for the assay are contained within sealed reagent strips. A fluorescent substrate called 4-methylumbelliferyl phosphate is passed through the Solid Phase Receptacle (SPR). The process that converts the substrate into the fluorescent product 4-methylumbelliferone will be catalyzed by enzymes that have not been released from the SPR's walls. The TSH concentration in the sample may be deduced from the result of the optical scanner's measurement of the fluorescence's intensity, which is directly proportional to that concentration. The equipment automatically analyzes and prints a report for each sample when the VIDAS, TSH, FT4, FT3, ANTI-TPO, and ANTI-TG tests are completed. The automated analysis ensures efficient and precise data processing, delivering comprehensive reports that facilitate further evaluation and interpretation.

3.2.2 Determination of thyroid stimulating hormone (TSH)

- Only the necessary items were removed from the package, and those that weren't utilized were put back into storage at 2-8 degrees Celsius as soon as possible.
- Waiting about thirty minutes to allow the ingredients to reach room temperature.
- Each test sample, control, and calibrator required one "TSH" strip and one "TSH" SPR. The bag was then resealed after being used for storage.
- The instrument's "TSH" code serves as a test identification. The calibrator needs to be tested twice and marked with "S1." The control should be identified by C1 if it has to be tested.
- A vortex-style mixer was used to mix the calibrator, the control, and the samples (for serum or plasma that had been separated from the pellet).
- The total volume of the experiment's calibration, control, and test samples is 200 liters.
- SPRs and "TSH" Reagent Strips were positioned in their usual spots on the instrument. SPRs and reagent strips with the assay code on them were compared for label color consistency.
- All of the assay steps were made to be carried out automatically by the instrument, and the assay procedure was started as instructed in the operator's manual.
- The test took around 40 minutes, and when it was finished, the vials were re-sealed and brought back to the proper temperature. The SPRs and strips are taken out of the device following the scan.
- The used SPRs and strips were appropriately discarded in the designated container. The normal range for micro-international units per milliliter is 0.38 – 4.31 LU/mL.

3.2.3 Determination FT3 and FT4

- After removing the necessary components from the kit, any other components that were not needed were placed back into storage at a temperature between 2 and 8 degrees Celsius.
- Waiting about thirty minutes to allow the ingredients to reach room temperature.

- After using one "FT3" strip (or FT4 strip) and one "FT3" SPR (or FT4 SPR) for each sample, control, or calibrator that was examined, the storage bag was resealed. This was done in order to prevent any contamination.
- The test on the instrument was labeled with the "FT3" code (or FT4 code). "S1" was determined to be the calibrator, and it was evaluated three times. In cases when the control was examined, it was given the designation "C1".
- A mixer of the vortex type designed specifically for use with serum or plasma after it has been separated from the pellet was used to combine the calibrator, the control, and the samples.
- During the test, the calibrator, control, and sample test portion were measured at 100 µL for FT3 and 200 µL for FT4.
- The "FT3" SPRs (FT4 SPR) and "FT3" strips (FT4 strip) were inserted into the instrument. The color labels on the SPRs and Reagent Strips were checked to ensure they matched with the corresponding assay code.
- The procedure for the test was started by following the directions that were given in the User's Manual. The gadget carried out the entirety of the procedure on its own without human intervention.
- After pipetting, the vials were securely re-closed and placed back in storage at 2-8°C.
- The test was finished in a little less than half an hour. After the results of the test were analyzed, the SPRs and strip cassettes were taken out of the device.
- Normal ranges for FT3 = 2.17 – 3.34 pg/mL (picogram/milliliter).
- Normal ranges for FT4 = 0.82 – 1.63 ng/dL (nanogram/deciliter).

3.2.4 Determination of anti thyroglobulin antibody and anti-thyroid peroxidase

- Required reagents were removed from a refrigerator, that can be used immediately.
- Each sample, control, and calibrator was examined with one "ATG" strip (ATPO strip), and one "ATG" SPR® (ATPO SPR). Carefully reseal the storage pouch after removing the necessary SPRs.

- The "ATG" code (ATPO code) on the device was used to find out what test was being done. The calibrator is marked with "S1" and checked three times. If the control is tried, it should be marked with "C1".
- A mixer of the vortex type was utilized in order to combine the calibrator, the control, and the samples.
- For this test, the calibrator, control, and sample test portion is 100 µL for both ATPO and ATG.
- We loaded the instrument with an ATG, some SPRs (ATPO SPRs), and some ATG strips (ATPO strips). The SPRs and Reagent Strips' color labels were examined to guarantee they corresponded to the assay code.
- The test was started after reading the manual's recommendations. The equipment carried out the entire assay procedure mechanically.
- After pipetting, the vials were re-closed and placed back in storage at 2-8°C.
- The test was finished in a little over a quarter of an hour. After the conclusion of the test, the SPRs and strips were removed from the instrument, and then the used SPRs and strips were disposed of in the container that was designated for that purpose.
- Normal ranges of Anti Thyroglobulin Antibody (ANTI-TG) = 2.68 - 33.2 ng/mL.
- Normal ranges of Anti-thyroid peroxidase (ANTI-TPO) < 8.0 IU/mL.

3.2.5 Determination of immunological parameters

The estimation of anti-phospholipid autoantibodies (APL) screen IgM was performed using ELISA kits following the manual procedure provided by Aeskulisa Company (Germany). The analysis was carried out in human serum using a microplate enzyme immunoassay for quantitative determination.

Principle: Serum samples are diluted at a ratio of 1:101 and incubated in microplates coated with a specific antigen. If the patient's antibodies are present in the specimen, they will bind to the antigen. Any unbound fraction is removed through washing in the subsequent step. Following this, anti-human immunoglobulins conjugated to horseradish peroxidase (conjugate) are added and incubated with the antigen-antibody

complex in the microplates. The next step involves washing away any unbound conjugate that may have been present. After this, the inclusion of TMB-substrate triggers an enzymatic colorimetric reaction that is characterized by a blue coloration. The addition of acid that has been diluted puts an end to this process and causes the hue to change to yellow. The initial concentration of each antibody in the patient sample is directly proportional to the quantity of conjugate that is attached to the antigen-antibody complex that is formed as a result of the reaction. This indicates that the quantity of conjugation that is connected with the antigen-antibody combination is directly linked to the degree to which the chromogen contributes to the creation of color intensity.

3.2.6 Molecular studies

Extraction of Genomic RNA; The RNA was obtained by utilizing a commercial TRIzol extraction kit, following the manufacturer's instructions through the subsequent steps

A. Separation of the RNA

1. 750 µL of Trizol reagent was added to the blood sample, and the mixture was homogenized by pipetting several times.
2. In order to fully separate the nucleoprotein complex, the homogenized material was incubated for 5 minutes at room temperature.
3. The homogenized sample tube had 150 µL of chloroform added to it, and after that, the tube was securely capped.
4. The tube was mixed vigorously by hand for 15 sec., and then incubated for 3 minutes at room temperature, and the sample was spine at 12,000 xg for 15 min.
5. The sample's liquid component was separated out and placed in a fresh test tube.

B. Precipitate the RNA

The lysis solution contained 1 mL of TRIzolTM Reagent and 0.5 mL of isopropanol. The mixture was then incubated for 10 minutes before being centrifuged for 10 minutes at 12,000 xg and 4°C.

C. Wash the RNA

The sample was quickly vortexed, the pellet was resuspended in 1 mL of 75% ethanol for every 1 mL of the TRIzolTM reagent used for lysis, and the centrifugation cycle was completed for 5 minutes at 7500 x g at 4°C. Following that, the supernatant was removed, and the RNA pellet was dried for 5–10 minutes.

D. Solubilize the RNA

The RNA pellet was mixed with 50 L of RNase-free water several times with a pipette, and the tube was put in a heat block set to 55°C for 15 minutes.

3.2.7 Complementary DNA synthesis

The RNA was converted into complementary DNA (cDNA) through a reverse transcription (RT) reaction to be used as a template in the PCR reaction. AccuPower Rocket Script RT PreMix from Bioneer/Korea was utilized for the RT reaction. This product is a lyophilized master mix that is ready for immediate application. This kit has everything you need to synthesize first-strand cDNA from an RNA template, including all of the essential components.

1. The total RNA and primers were added into the AccuPower® RocketScript™ RT PreMix tubes.
2. DEPC-water was added to the AccuPower® RocketScript™ RT PreMix tubes, bringing the total volume to 20 μL.

3. Through the use of a vortex, the lyophilized pellet was able to completely disintegrate and spin down.
4. The reaction was then carried out under the subsequent conditions (Table 3.3).
5. The samples were stored at -20°C until use.

Table 3.3 RT-PCR program to complementary DNA synthesis

Step	**Temperature**				**Time**
	dN6	**dN12**	**dT20**	**Specific primer**	
Primer annealing	15°C	30°C	37°C	Tm of primer	10min
cDNA synthesis	42°C				30min
Heat inactivation	95°C				5min

3.2.8 RT-qPCR

As demonstrated in the following section, RT- PCR was used to detect the gene expression of the microRNA gene.

1. RT-qPCR Primers

A. micRNA146a Primers Selection

The species-specific primers micRNA146a were utilized to develop RT-PCR primers sensitive enough to detect gene expression of the blood micRNA gene.

B. Primer's preparation

The primers were supplied by Macrogen Company as lyophilized products of different picomoles concentrations. Macrogen company protocol was adopted for primers preparation, by adding the desired volume of Tris-EDTA buffer (TE buffer), pH 7.5 - 8.0, this buffer was prepared by dissolving 3.93 g of Tris- HCl and 3.72 g EDTA-Na2 using distilled water then the pH was adjusted to 8.0 and used to the lyophilized primers

to get stock solution of primers with 100 µM concentrations and stored -20ºC, some these detials are shown in Table 3.4.

Table 3.4 RT-PCR master mix used to detect the micRNA146a gene expression

Component	Concentration	Amount (µL)
Master Mix	-	10
micRNA146a -F primer	10 µM/µL	1
micRNA146a –R primer	10 µM/µL	1
Nuclease free water	-	6
cDNA sample	-	2
Total	-	20

3.2.9 RT-PCR program

The following RT-PCR protocol, developed to determine the expression of the micRNA146a gene, was implemented (Table 3.5).

Table 3.5 RT-PCR program used to detect the micRNA146a genes expression

Step	Temperature	Time	Cycles
RT inactivation/Hot-start activation	95 °C	2 Minutes	1
Step qPCR a. Denaturation b. Annealing and extension	95 °C 72 °C	15 Seconds 1 Minute	40
Dissociation	72 °C 95 °C	30 Seconds 30 Second	

3.2.10 Delta Ct method

The simplest way was the delta delta Ct (Ct) method, which compares the Ct values of the target gene with the reference gene directly (Schmittgen and Livak 2008). The selection of a calibrator sample is necessary for relative quantification. Initially, as depicted in Equation (3.1), the Ct value between the target gene and the reference gene was calculated for every sample, including both the unknown samples and the calibrator sample.

$$\Delta Ct = Ct \text{ target gene} - Ct \text{ reference gene} \quad (3.1)$$

Subsequently, the disparity between the ΔCt of the unknown sample and the ΔCt of the calibrator was determined, resulting in the ΔΔCt value, as illustrated in Equation (3.2).

$$\Delta\Delta Ct = (Ct \text{ target-}Ct \text{ reference}) \text{ sample-}(Ct \text{ target-}Ct \text{ reference}) \text{ calibrator} \quad (3.2)$$

3.3 Statistical Analysis

To detect the impact of various factors on study parameters, the Statistical Analysis System (SAS) program from 2012 was employed for data analysis. The mean and the standard deviation were calculated for all parameters. After conducting the Kruskal-Wallis test to determine whether the data are normally distributed or not, the student t-test was employed to assess the significance of the differences between normally distributed variables. In statistical comparisons, 0.05 significance level was considered.

4. RESULTS AND DISCUSSION

4.1 The Levels of TSH and Thyroid Hormones

Serum hormone concentrations, including FT3, FT4, TSH, ANTI-TPO, and ANTI-TG, were measured using the Vidas method. A comparison was made between the study groups and control groups, and the significance was evaluated using analysis of variance (ANOVA) and t-test. The results are presented in Table 4.1 and Table 4.2.

4.2 Thyroid Stimulating Hormone (TSH)

The results demonstrated that the mean ± SD of TSH during the first trimester was (4.417 ± 0.613) µIU/mL, which was higher than the mean ± SD of (1.889 ± 0.222) µIU/mL observed in the control group. The present study shows a significant difference ($P<0.05$) for the levels of TSH in control and patient (Table 4.1 and Figure).

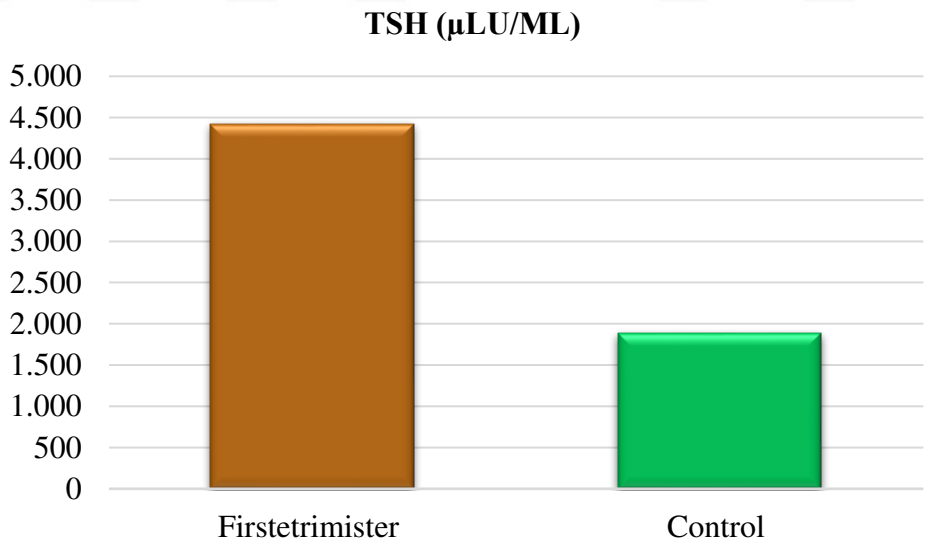

Figure 4.1 Comparison of TSH level in study group with control group

4.3 Free Triiodothyronine (FT3)

The results showed the mean ± SD of FT3 at first trimester was (3.092 ± 0.463), was lower significant when compared to control group mean ± SD (2.945 ± 0.603) pg/mL of control group (Table 4.1). Also, the present study shows not significant difference (P = 0.628) for level of FT3 in control and patients (Table 4.1 and Figure 4.2).

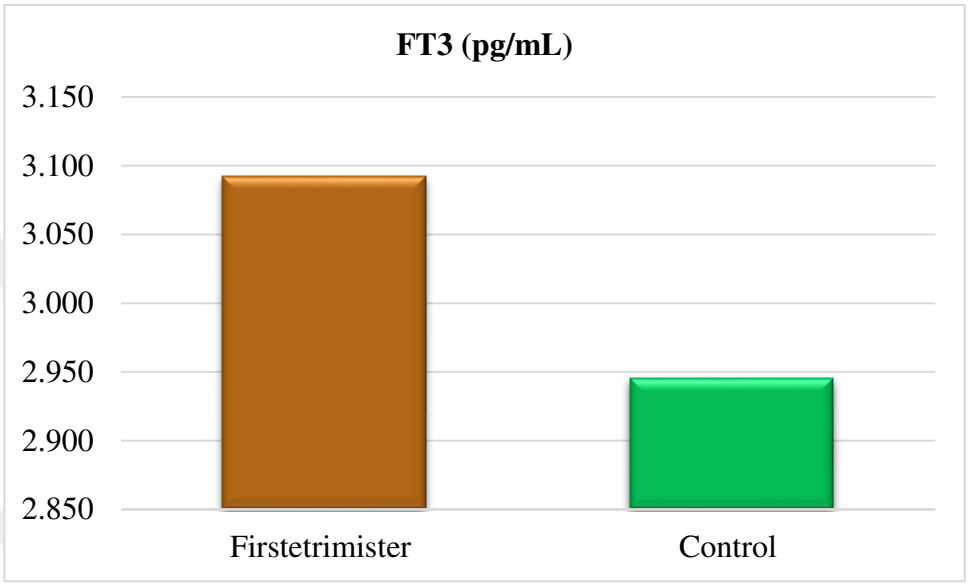

Figure 4.2 Comparison of FT3 level in study group with control group

4.4 Free Thyroxin (FT4)

The results showed the mean ± SD of FT4 in the first trimester was (1.735 ± 0.287), this result was closely than the mean ± SD (1.192 ± 0.296) ng/dL of the control group. Also, the present study shows not a significant difference (P=0.391) for the level of FT4 in control and patients (Table 4.1 and Figure 4.3).

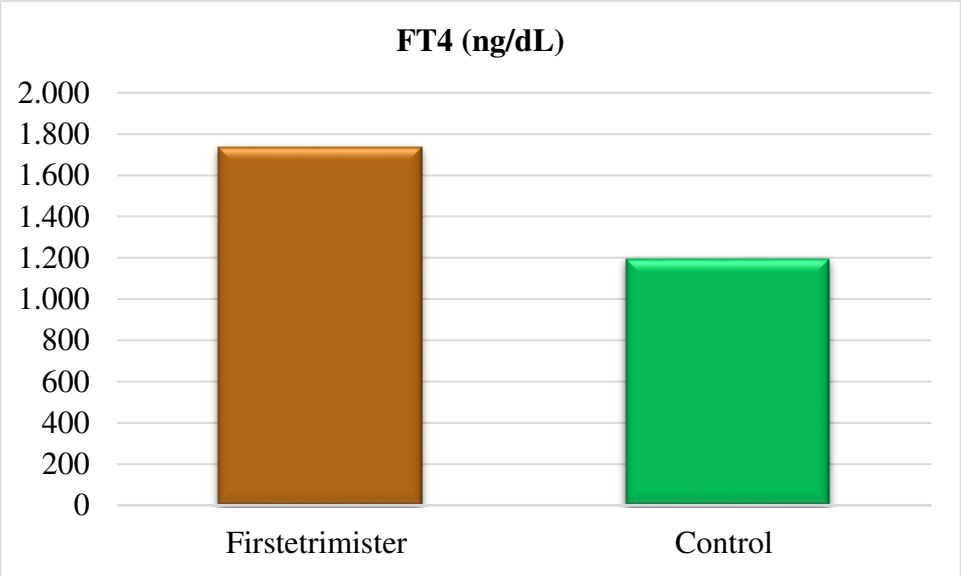

Figure 4.3 Comparison of FT4 level in study group with control group

Table 4.1 Comparing the levels of TSH and thyroid hormones between expectant women in their first trimester and their controls

Parameters	Firstetrimister (Mean ± SD) n= 120	Control (Mean ± SD) n= 45	P-value
TSH (µLU/ML)	4.417 ± 0.613	1.889 ± 0.222	0.046*
FT3 (pg/mL)	3.092 ± 0.463	2.945 ± 0.603	0.628
FT4 (ng/dL)	1.735 ± 0.287	1.192 ± 0.296	0.391

Subclinical hypothyroidism and subclinical hyperthyroidism are two prevalent clinical conditions that cover modest thyroid malfunction. There is debate about whether diagnostic tests and potential treatments are necessary because the clinical significance of minor thyroid over-and under activity is unknown (Biondi and Cooper 2008).

About 5-8 percent of all pregnancies have subclinical thyroid dysfunction, which is linked to an increased risk of unfavorable outcomes like miscarriage, premature birth, and subpar infant neurodevelopment. The proportion of pregnant women who have subclinical illness who continue to have it in the third trimester is unknown (Fan *et al.* 2019).

Detecting maternal thyroid insufficiency is of utmost importance because, during the majority of the first trimester, the fetus relies entirely on the mother's thyroid hormone supply and requires it until delivery. The numerous physiological changes that impact the amounts of thyroid-related analytes make it difficult to assess thyroid function during pregnancy.

During pregnancy, as estradiol levels gradually increase, there are notable rises in thyroxine-binding globulin (TBG) and total thyroxine (T4) compared to the nonpregnant state. Simultaneously, in the second half of the first trimester, increasing plasma human chorionic gonadotropin (hCG) concentrations cause a temporary decrease in thyroid-stimulating hormone (TSH). Finally, as gestational age increases, free T4 concentrations gradually decrease. In light of these factors, it is crucial to interpret thyroid function test findings using trimester-specific references (Geno and Nerenz 2022).

The results disagree with Sawant and his team (2003) who stated that SOD activity was decreased in both hyperthyroidism and hypothyroidism, but more in hyperthyroidism (Sawant *et al.* 2003). Also, the results agree with Sawant and his team (2011) who found TSH was elevated in the patients with hypothyroidism compared to control group, while T4 and T3 were significantly lower in the patients than in control group (Sawant *et al.* 2011). Iddah and Macharia (2013) indicated that when TSH stimulates the thyroid cell, TG is endocytosed and hydrolyzed in lysosomes releasing T3 and T4 (Iddah and Macharia 2013).

4.5 APL and Anti-thyroid Hormones

The data presented in Table 4.2 and Figure 4.4 indicate the mean levels of Anti TPO, Anti TG, and anti-phospholipid antibodies in the serum of pregnant women and the control groups of patients. The mean ± SD of ANTI-TG (19.325 ± 1.9816) ng/mL, these results considered a higher than mean ± SD (16.588 ± 0.9915) of control group and with no significant.

Also, the result shows high significant between the ANTI-TPO (0.05) and APL (0.01) in compared with control group. ANTI-TPO (83.665±17.565) IU/mL and anti-phospholipid (7.087 ± 0.842) U/mL all were higher than of control group.

Table 4.2 A comparison of APL and anti-thyroid hormone levels between first-trimester pregnant women and their control expectant counterparts

Parameters	**First trimester (Mean ± SD) n= 120**	**Control (Mean ± SD) n= 45**	**P-value**
APL (U/mL)	7.087 ± 0.8426	4.762 ± 0.3437	0.045*
ANTI-TPO (IU/mL)	83.665± 17.565	4.595 ± 0.379	0.013 **
ANTI-TG (ng/mI)	19.325 ± 1.9816	16.588 ± 0.9915	0.549

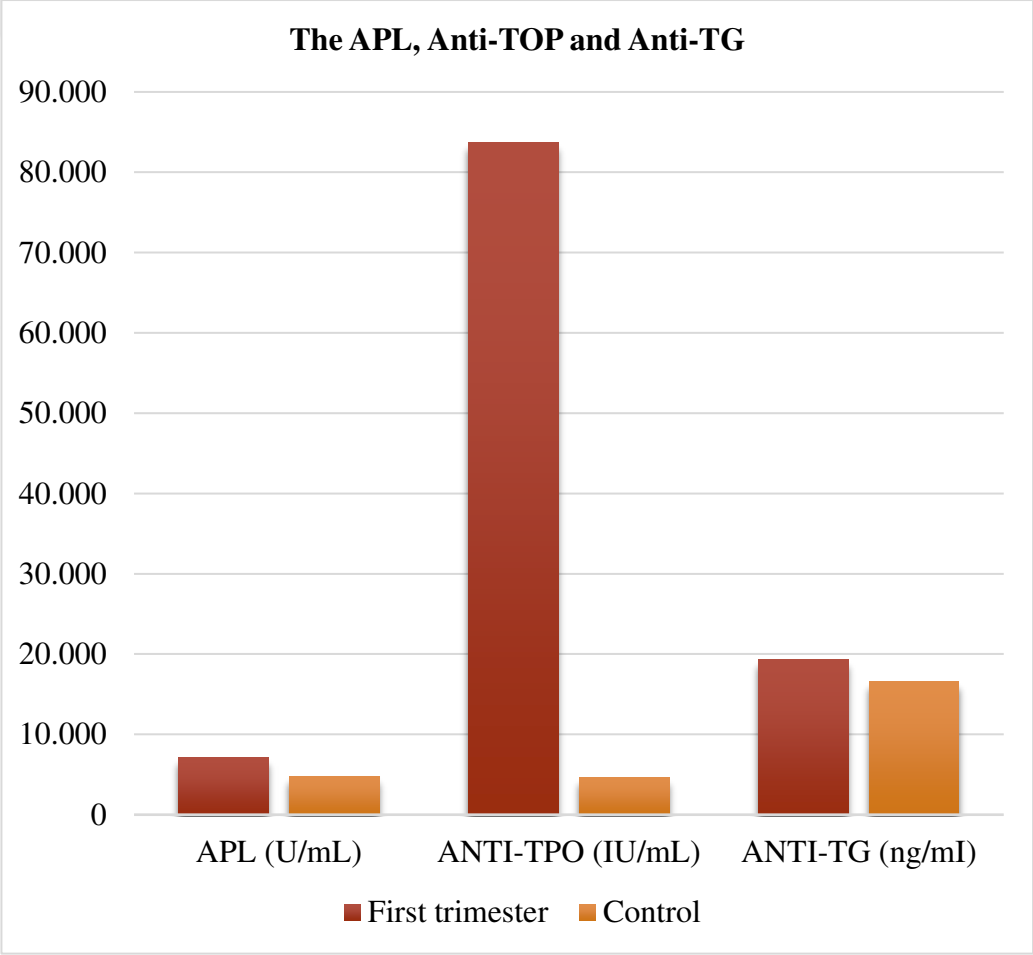

Figure 4.4 Comparison of the APL, Anti-TOP and Anti-TG level in study group with control group

Our study found that the risk of miscarriage was significantly higher among pregnant women who were diagnosed with autoimmune thyroid disease (AITD) in the first trimester of their pregnancies compared to those who were not diagnosed with AITD. This result aligns with the study conducted by Glinoer *et al.* (1994).

Another research study performed by (Triggianese *et al.* 2021), it has been shown that, compared to the control group, pregnant women who experienced recurrent miscarriage had a prevalence of thyroid autoimmunity of (37.8%), which was statistically higher (P = 0.01) than the control group.

4.6 Body Mass Index

According to the division of the body mass of women in aimed group for study (First trimester group) into normal weight (BMI = 18.5-24.9) and over weight (BMI = 25-29.9), there was a significant difference ($p \leq 0.001$) between the body mass index, where the mean ± SD body mass of pregnant women with normal weight is (23.895 ± 2.454) and the mean ± SD body mass of overweight is (26.738 ± 3.964). Also the taple shows the mean ± SD of abortion in over weight (1.900 ± 0.834) is highly of normal weight that mean ± SD (1.815 ± 0.827) with no a significant as shown in the Table 4.3.

Table 4.3 Mean and SD of abortion in women of normal weight and overweight

	Groups	**N**	**Mean ± SD**	**P – value**
BMI	Normal weight	85	23.895 ± 2.454	0.000
	Over weight	35	26.738 ± 3.964	
Aboratıon	Normal weight	85	1.815 ± 0.827	0.524
	Over weight	35	1.900 ± 0.834	

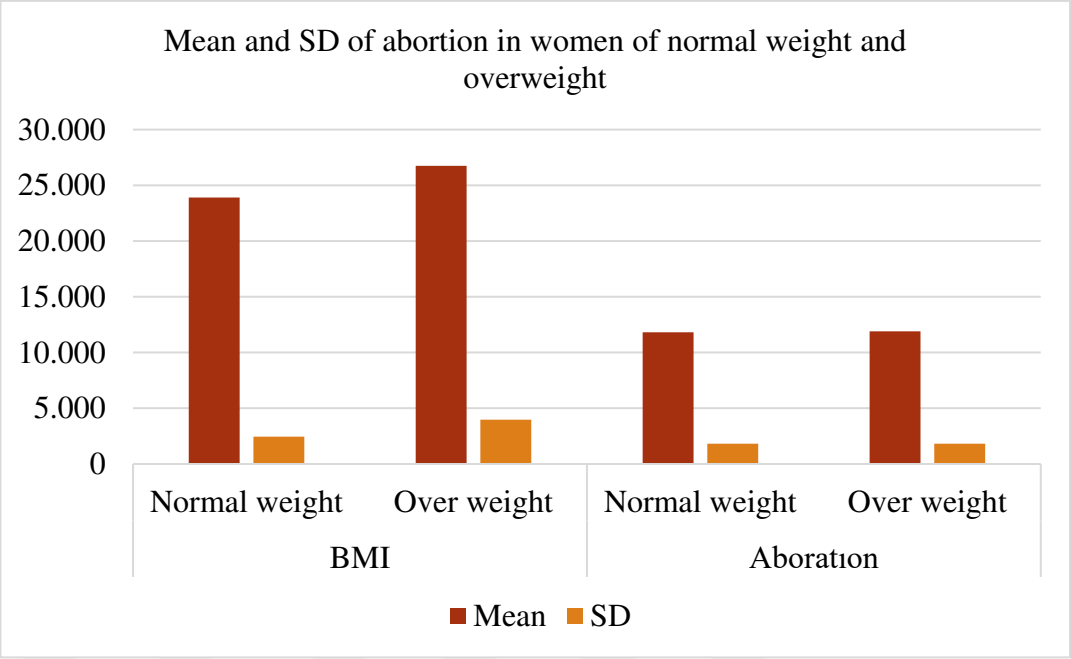

Figure 4.5 Comparison of mean and SD of abortion in women of normal weight and overweight in study group with control group

Our research found that women with a higher than normal body mass index (BMI) were more likely to experience a spontaneous abortion. As in studying (Zhang *et al.* 2010) persons with a BMI of 24 kg/m^2 or greater had significantly higher odds of developing recurrent miscarriage compared to their counterparts.

4.7 Gene Expression

4.7.1 RNA extraction

Successful extraction of total RNA was achieved from various samples, with RNA concentrations ranging from 62 to 288 ng/µL. Aseptic procedures are essential for obtaining a high concentration of total RNA, which is highly dependent on the extraction conditions. It is advised that TRIzol be used for total RNA extraction from blood samples. (Kang *et al.* 2011).

4.7.2 Gene expression of heat shock protein (*MIR146A*) by RT-PCR

Following RT-PCR analysis and normalization to the expression of a housekeeping gene, we were able to find the MIR146A gene in these samples. The 2-ΔΔCt approach of comparing threshold cycles (CT) was used to quantify the relative changes in expression levels. Amplification and detection of the MIR146A genes were conducted using the SYBR Green qRT-PCR method.

In hyperthyroidism, the positive results revealed amplification at CT (threshold cycle) values within the range of 16.38-17.04 for the housekeeping gene and 28.86-29.42 for the MIR146A gene (Figure 4.6).

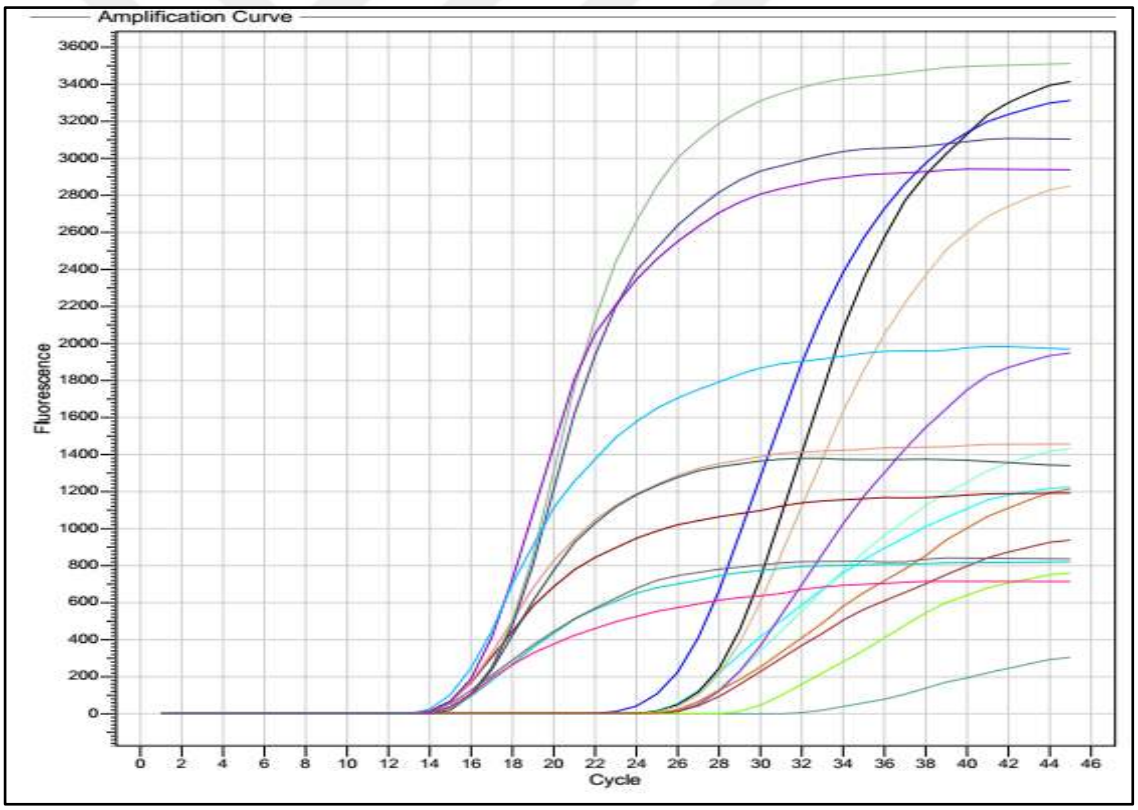

Figure 4.6 Curve of cycling of *MIR146A* gene and housekeeping gene

The melting temperature (Tm) values obtained for the sample's hyperthyroidism patients, and control; the melting temperature was in the range 81.64, 82.04, 81.94, 81.44, 81.04, 81.34, 82.54, 82.64, 82.34, 82.44 ºC.

Respectively for housekeeping gene and the melting temperature of *MIR146A* was in the range of 81.24, 81.44, 81.54, 81.94, 81.24 ºC respectively (Figure 4.7).

The expression of microRNA itself or in conjunction with multi-chaperone complexes acts as a significant repressor of heat shock transcription factor. In research of Zuehlke and his friends (2015) elucidate in some disease instances the elevated expression of *MIR146A* potentially could serve as a prognostic indicator (Zuehlke *et al.* 2015).

Furthermore, Calderwood (2018) reported that a significant upregulation of numerous microRNAs, including MIR146A, MIR146B, MIR148A, and miR-146a-5p, has been observed in various cancer types such as breast, prostate, lung, and melanoma. This increased expression of microRNAs is strongly linked to poor patient outcomes (Calderwood 2018). MicroRNA itself is found to be overexpressed in thyroid cancer, as observed in the study conducted by Soudry *et al.* (2017). Moreover, the increased expression of microRNA is associated with poor patient survival. This provides a strong rationale for inhibiting microRNA function as an innovative therapeutic approach in the treatment of certain subtypes of thyroid cancer, as suggested by Lettini *et al.* (2020).

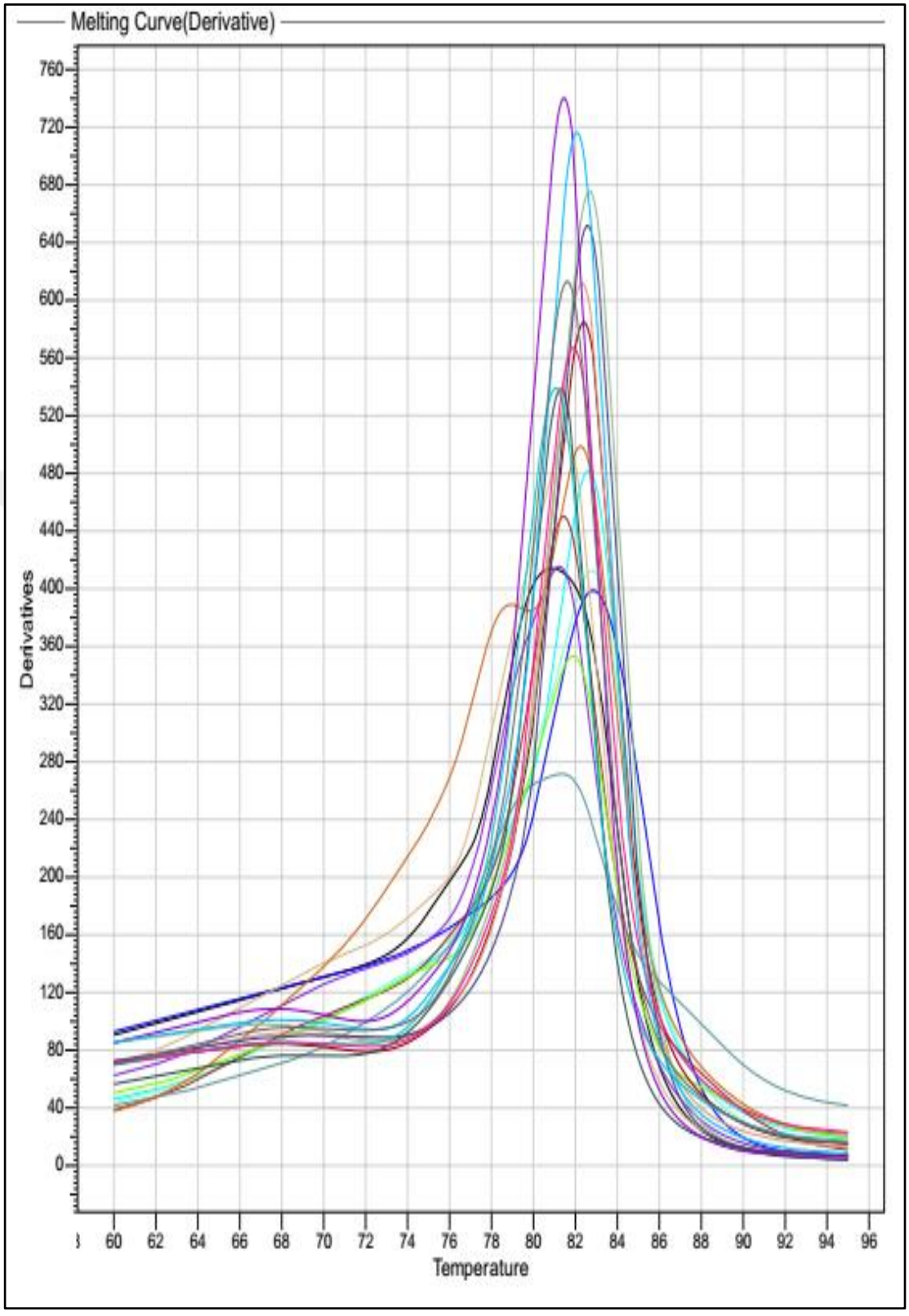

Figure 4.7 Curve of melting *micRNA146A* gene and housekeeping gene

The results disagree with Graham and his team (2009) who observed that *micRNA146A* expression was depressed after 4 days of thyroxine treatment in short-day birds (Graham *et al.* 2009). The findings from Wallin and his team's study in 1992 demonstrate that the expression of HSP90 mRNA is associated with the level of cellular differentiation (Wallin *et al.* 1992).

The production of microRNA in endothelial cells can cause the protein to become surface-localized and can also stimulate an increase in the level of HSP90 surface expression on these cells. As a consequence of this, HSP90 might now be considered a possible target for autoimmune responses. Therefore, HSPs (Heat Shock Proteins) have a variety of tasks, and these roles can be either beneficial or detrimental, depending on how they are involved in different cellular pathways (Szyller and Bil-Lula 2021).

The production of important antioxidant enzymes including SOD (Superoxide Dismutase) and CAT (Catalase) is stimulated by heat shock protein 90. In response to the stress factors that are present under stressful settings, Kruger et al. 2019 discovered that there was an increase in the activity of CAT and SOD as well as the expression of microRNA.

5. CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

1- Thyroid hormones (FT3, FT4) and TSH have a connection with oxidative stress and antioxidant status in patients with hypo- and hyperthyroidism.
2- The *micRNA146A* genes can use as a risk indicator in the diagnoses of thyroid disorders.
3- Real-time PCR is a significant tool to determine the gene expression of *micRNA146A* genes as risk factors could be signified as a powerful molecular tool to study the relationship between these genes and thyroid disorders.

5.2 Recommendations

1- Assessment of levels urinary iodine in hypothyroidism and hyperthyroidism patients in relation with thyroid function.
2- Other genes related to micRNA such as *MIR146B* and *MIR148* genes should be studied in thyroid disorders.

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APPENDICES

APPENDIX 1. The consolidated report, included the experimental name for the gene and some other details

APPENDIX 2. The consolidated report, this page included the table plate and samples

APPENDIX 3. The consolidated report, this page included the amplification curve and analysis results of samples

APPENDIX 4. The consolidated report, this page included analysis results in addition to the relative plot

APPENDIX 5. The consolidated report, this page included the plot of the relative analysis results

APPENDIX 6. The consolidated report, this page included the melting curve (Dervative) ans melting analysis results

APPENDIX 7. The consolidated report, this page included the melting analysis results

APPENDIX 1. The consolidated report, included the experimental name for the gene and some other details

Consolidated Report

Experiment Name: miRNA-146a
Experiment Type: Relative
User Name: admin
File Name: C:\Users\Excellence\Downloads\Telegram Desktop\miRNA-146a Mohammed hassan.fqd
Run Time: 2022/06/07 15:46:15 - 2022/06/07 17:35:34
Gain: F1:9
Reference Sample:

Run Program

Hold Stage

Target	Incubation Time	Rate	Sampling
95	60	4	☐

PCR Stage Cycles:45

Target	Incubation Time	Rate	2nd Temp.	Step Size	Step Delay	Grad Temp.	Grad Range	Sampling
95	15	4						☐
60	30	4						☑

Melting Stage Incubation Time:20

Target	Step	Melting
95	0.3	☐
60	0.3	☐
95	0.3	☑

Detectors

Detector	Endogenous Control	Reporter	Color	Master Mix	Primer	Probe	Supplies	Batch Number
mi		SYBR						

Plot Plate

Table Plate

#	Well	Assay Item	Property	Dye	Std. Con.	Sample ID
1	A01	mi	Unknown	SYBR		C1
2	A02	mi	Unknown	SYBR		C2
3	A03	mi	Unknown	SYBR		C3
4	A04	mi	Unknown	SYBR		C4
5	A05	mi	Unknown	SYBR		T1
6	A06	mi	Unknown	SYBR		T2
7	A07	mi	Unknown	SYBR		T3
8	A08	mi	Unknown	SYBR		T4
9	A09	mi	Unknown	SYBR		T5
10	A10	mi	Unknown	SYBR		T6
25	B01	mi	Unknown	SYBR		H.KC1
26	B02	mi	Unknown	SYBR		H.KC2
27	B03	mi	Unknown	SYBR		H.KC3
28	B04	mi	Unknown	SYBR		H.KC4

APPENDIX 2. The consolidated report, this page included the table plate and samples

Consolidated Report

Table Plate

#	Well	Assay Item	Property	Dye	Std. Con.	Sample ID
29	B05	mi	Unknown	SYBR		H.KT1
30	B06	mi	Unknown	SYBR		H.KT2
31	B07	mi	Unknown	SYBR		H.KT3
32	B08	mi	Unknown	SYBR		H.KT4
33	B09	mi	Unknown	SYBR		H.KT5
34	B10	mi	Unknown	SYBR		H.KT6

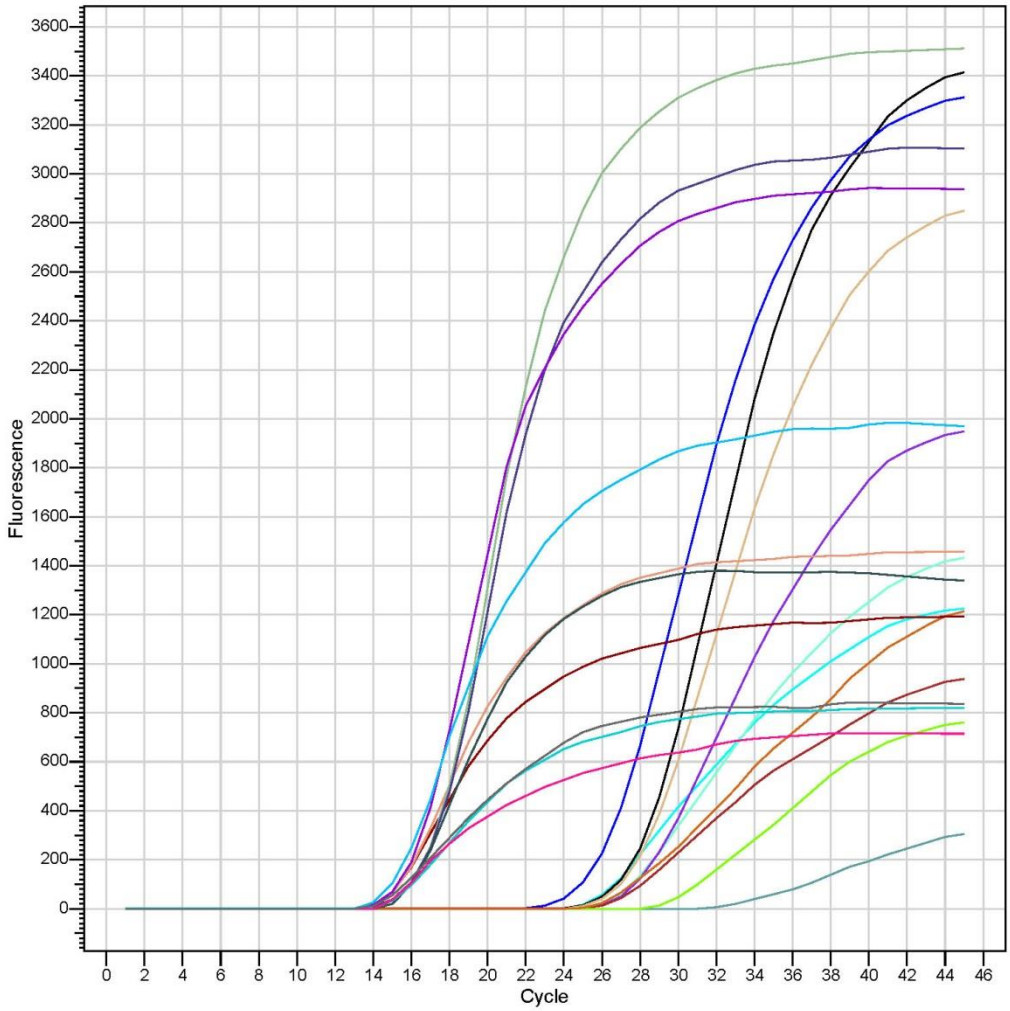
Samples

Sample Id	Color	Sample Name	Name	Sex	Age	Case No.	Outpatient No.	Bed No.	Hospital No.	Nationality	Sampling Time	Doctor	Dept.	Submitting Date
C1											2022/06/07			2022/06/07
C2											2022/06/07			2022/06/07
C3											2022/06/07			2022/06/07
C4											2022/06/07			2022/06/07
T1											2022/06/07			2022/06/07
T2											2022/06/07			2022/06/07
T3											2022/06/07			2022/06/07
T4											2022/06/07			2022/06/07
T5											2022/06/07			2022/06/07
T6											2022/06/07			2022/06/07
H.KC1											2022/06/07			2022/06/07
H.KC2											2022/06/07			2022/06/07
H.KC3											2022/06/07			2022/06/07
H.KC4											2022/06/07			2022/06/07
H.KT1											2022/06/07			2022/06/07
H.KT2											2022/06/07			2022/06/07
H.KT3											2022/06/07			2022/06/07
H.KT4											2022/06/07			2022/06/07
H.KT5											2022/06/07			2022/06/07
H.KT6											2022/06/07			2022/06/07

APPENDIX 3. The consolidated report, this page included the amplification curve and analysis results of samples

Consolidated Report

Amplification Curve



Quan. Analysis Result

#	Well	Assay Item	Property	Dye	Ct	Ct Aver.	Ct SD	Cal. Con.	Con. Aver.	Con. SD	Sample ID
1	A01	mi	Unknown	SYBR	27.93	27.93	0				C1
2	A02	mi	Unknown	SYBR	28.84	28.84	0				C2
3	A03	mi	Unknown	SYBR	27.79	27.79	0				C3
4	A04	mi	Unknown	SYBR	25.91	25.91	0				C4
5	A05	mi	Unknown	SYBR	28.86	28.86	0				T1
6	A06	mi	Unknown	SYBR	29.76	29.76	0				T2
7	A07	mi	Unknown	SYBR	27.97	27.97	0				T3

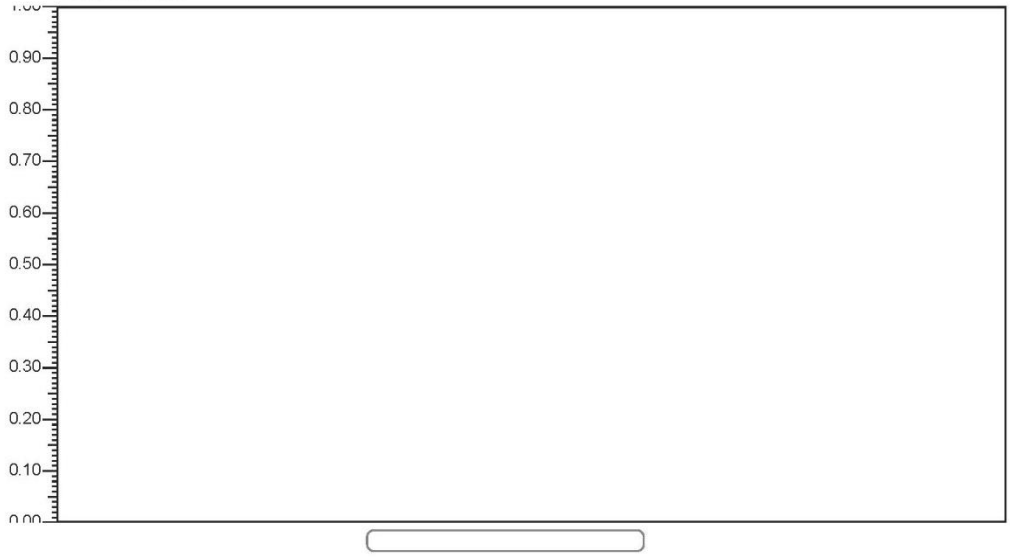
APPENDIX 4. The consolidated report, this page included analysis results in adition to the relative plot

Consolidated Report

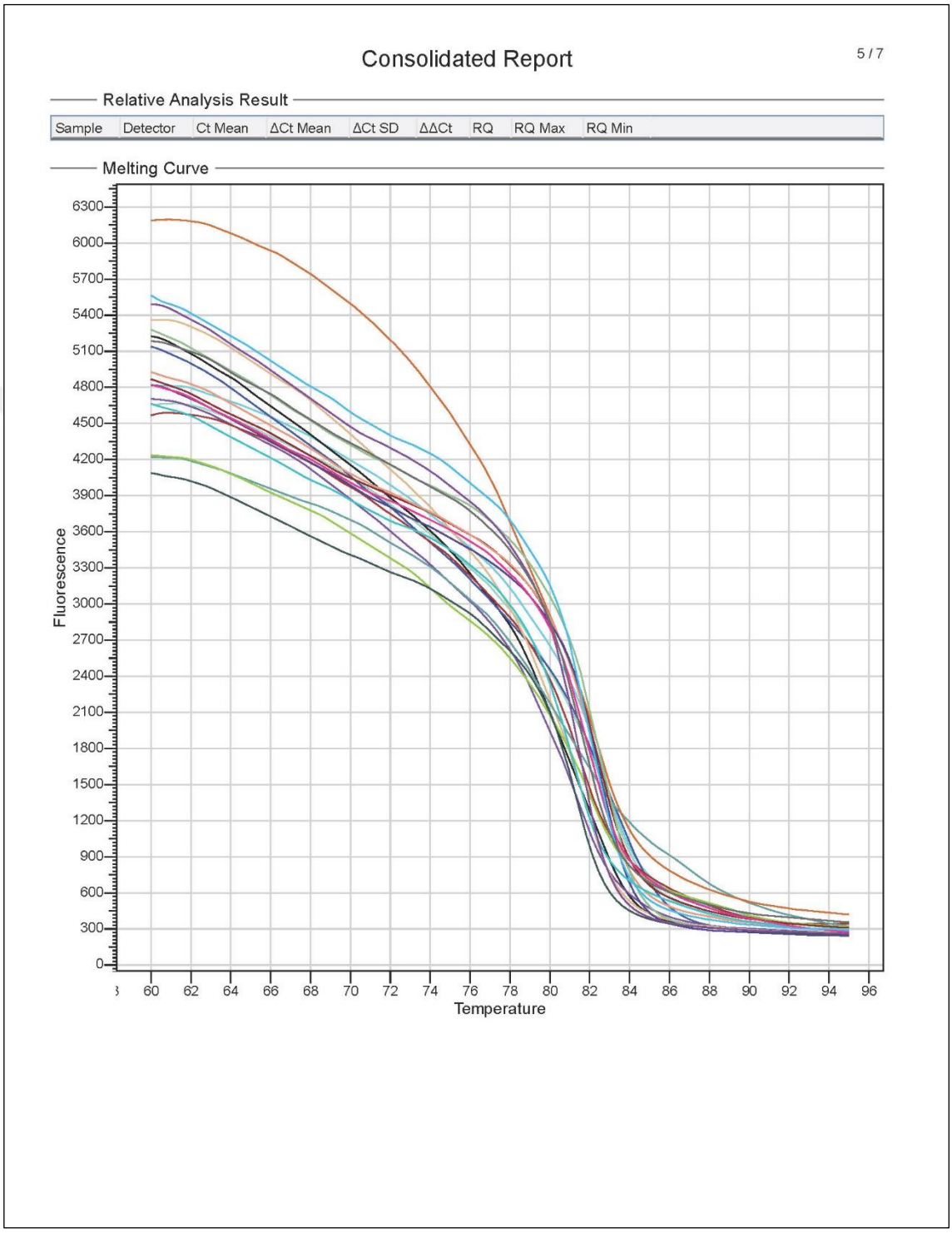
Quan. Analysis Result

#	Well	Assay Item	Property	Dye	Ct	Ct Aver.	Ct SD	Cal. Con.	Con. Aver.	Con. SD	Sample ID
8	A08	mi	Unknown	SYBR	40.63	40.63	0				T4
9	A09	mi	Unknown	SYBR	32.86	32.86	0				T5
10	A10	mi	Unknown	SYBR	29.42	29.42	0				T6
25	B01	mi	Unknown	SYBR	16.37	16.37	0				H.KC1
26	B02	mi	Unknown	SYBR	16.31	16.31	0				H.KC2
27	B03	mi	Unknown	SYBR	16.8	16.8	0				H.KC3
28	B04	mi	Unknown	SYBR	16.82	16.82	0				H.KC4
29	B05	mi	Unknown	SYBR	16.86	16.86	0				H.KT1
30	B06	mi	Unknown	SYBR	17.4	17.4	0				H.KT2
31	B07	mi	Unknown	SYBR	16.14	16.14	0				H.KT3
32	B08	mi	Unknown	SYBR	17.26	17.26	0				H.KT4
33	B09	mi	Unknown	SYBR	15.82	15.82	0				H.KT5
34	B10	mi	Unknown	SYBR	17.04	17.04	0				H.KT6

Relative Plot



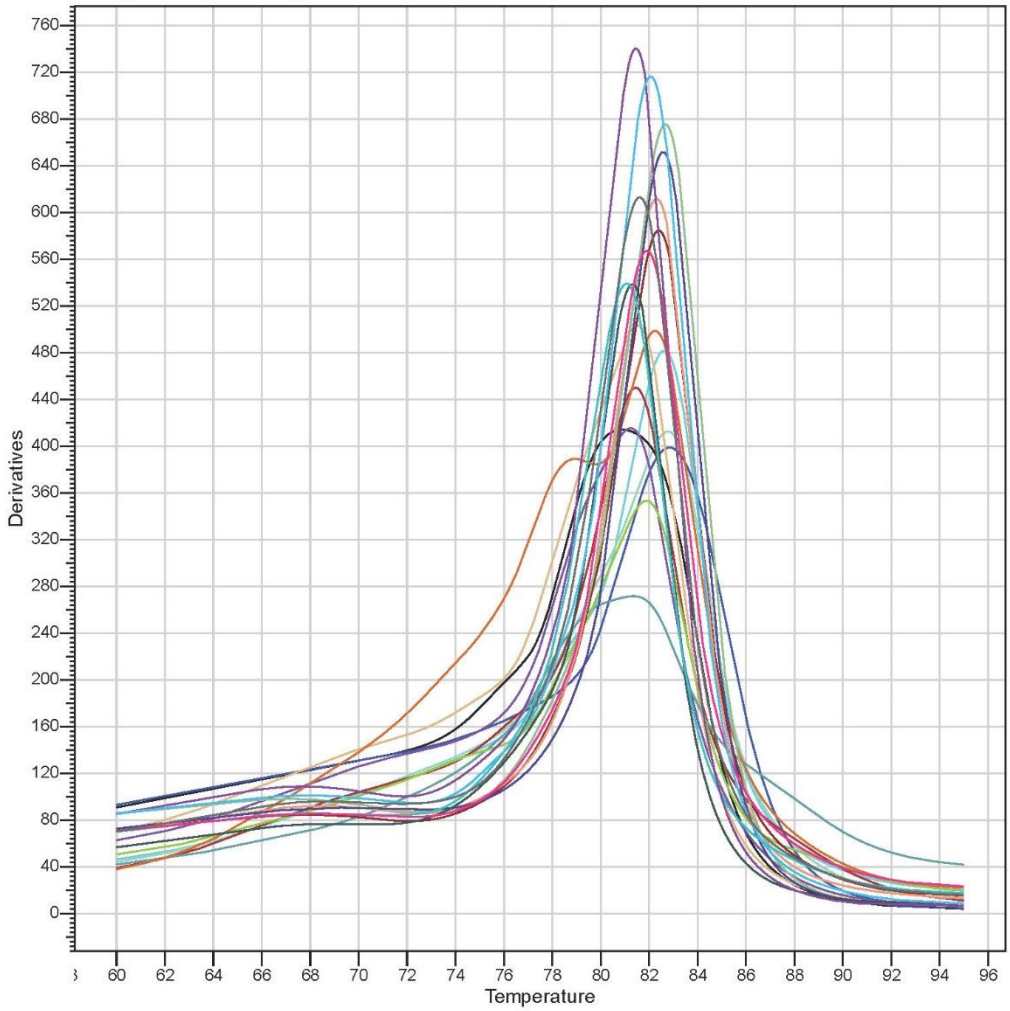
APPENDIX 5. The consolidated report, this page included the plot of the relative analysis results



APPENDIX 6. The consolidated report, this page included the melting curve (Dervative) ans melting analysis results

Consolidated Report

Melting Curve(Derivative)



Melting Analysis Result

#	Well	Assay Item	Property	Dye	Sample ID	Tm Count	Temp.1	Derivative1	Temp.2	Derivative2
1	A01	mi	Unknown	SYBR	C1	1	82.64	481.69		
2	A02	mi	Unknown	SYBR	C2	1	82.74	412.46		
3	A03	mi	Unknown	SYBR	C3	1	80.84	414.02		
4	A04	mi	Unknown	SYBR	C4	1	82.83	398.72		
5	A05	mi	Unknown	SYBR	T1	1	81.24	415.33		
6	A06	mi	Unknown	SYBR	T2	1	81.44	450.01		
7	A07	mi	Unknown	SYBR	T3	1	81.54	503.36		

APPENDIX 7. The consolidated report, this page included the melting analysis results

Consolidated Report

Melting Analysis Result

#	Well	Assay Item	Property	Dye	Sample ID	Tm Count	Temp.1	Derivative1	Temp.2	Derivative2	
8	A08	mi	Unknown	SYBR	T4	0					
9	A09	mi	Unknown	SYBR	T5	1	81.94	353.23			
10	A10	mi	Unknown	SYBR	T6	2	82.24	498.7	78.95	389.04	
25	B01	mi	Unknown	SYBR	H.KC1	1	82.44	584.61			
26	B02	mi	Unknown	SYBR	H.KC2	1	82.34	611.71			
27	B03	mi	Unknown	SYBR	H.KC3	1	82.64	675.31			
28	B04	mi	Unknown	SYBR	H.KC4	1	82.54	651.6			
29	B05	mi	Unknown	SYBR	H.KT1	1	81.34	538.44			
30	B06	mi	Unknown	SYBR	H.KT2	1	81.04	539.01			
31	B07	mi	Unknown	SYBR	H.KT3	1	81.44	740.33			
32	B08	mi	Unknown	SYBR	H.KT4	1	81.94	567.23			
33	B09	mi	Unknown	SYBR	H.KT5	1	82.04	716.31			
34	B10	mi	Unknown	SYBR	H.KT6	1	81.64	613.07			

CURRICULUM VITAE

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