Effects of Subclinical Thyroid Dysfunctions on important Serum Lipids Values

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Dedication

To my parents,
My family,
My friends,
And all those I love.
Acknowledgement

I acknowledge everyone who helped me completes my steps in this field.

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Abstract

This study was carried out at the University of Khartoum, faculty of Veterinary Medicine, Department of Biochemistry. Samples were collected from patients visiting the Radiation and Isotopes Center, Khartoum (RICK), Radio-immunoassay laboratory. This work was carried out to estimate the effects of subclinical thyroid disorders on important blood lipids values.

The study included 120 participants, divided into three equal groups: subclinical hypothyroidism, subclinical hyperthyroidism, and control. There were obvious differences in serum levels of total cholesterol, low density lipoproteins- cholesterol (LDL-C), and triglycerides (TG) among the different groups.

Among subjects with subclinical hypothyroidism, there was a significant increase \((p < 0.001)\) in the levels of total cholesterol, when compared with the control. The LDL-C levels were also found to be high in the subclinical hypothyroidism group \((p < 0.03)\). In contrast, the subclinical hyperthyroidism group showed normal values for both total cholesterol and LDL-C. TG levels were found to be significantly higher among the subclinical hypothyroid \((p < 0.01)\) and subclinical hyperthyroid \((p < 0.01)\) subjects than the controls, while no differences in the levels of HDL-C among the three different groups were observed.

According to the obtained results, subclinical hypothyroidism can significantly increase the levels of most serum lipids including total cholesterol, LDL-C, and TG. However, subclinical hyperthyroidism does not appear to have such effect except for the TG levels.
ملخص الأطروحة

تمت هذه الدراسة في قسم الكيمياء الحيوية بكلية البيطرة، جامعة الخرطوم. وأجريت الدراسة لمعرفة أثر العوامل تحت السريرية في إفراز هرمونات الغدة الدرقية (subclinical dysfunctions) للبروتينات والبروتين ثلاثي الابد (T3, T4)، على مستوى بعض الدهون في الدم.

تم جمع العينات من المرضى الذين زاروا معمل قياس الهرمونات بمركز الطب النووي والعلاج بالأشعة (RICK). أجريت الدراسة على عدد 120 فرد من الإثاث، تم تقسيمهم حسب حالة المريضة إلى ثلاث مجموعات متساوية العدد: مجموعة تعاني من قصور تحت سريري في إفراز هرمونات الغدة الدرقية (subclinical hypothyroidism) ، مجموعتين تعاني من زيادة تحت سريرية في إفراز هرمونات الغدة الدرقية (controls) ، ومجموعة تحكمية (subclinical hypothyroidism).

أوضح دراسة أن هنالك فروقات واضحة بين المجموعات الثلاث عند قياس مستوى الكوليسترول والبروتين الثلاثي (Triglycerides) والبروتين الدهني (LDL-C) في الدم.

في المجموعة التي تعاني من قصور تحت سريري في إفراز هرمونات الغدة الدرقية، كان مستوى الكوليسترول في الدم بالمقارنة مع المجموعة المتحكمة محققة فروقاً معنوية واضحة (P < 0.001). وكذلك الحال بالنسبة لمستوى البروتين الدهني (LDL-C), (P < 0.03).

أما بالنسبة للمجموعة التي تعاني من زيادة تحت سريرية في إفراز هرمونات الغدة الدرقية، فإنها لم تسجل أي فروق معنوية في مستويات الكوليسترول والبروتين الدهني (LDL-C) 

سجلت مستوى الجلسيديات الثلاثي (Triglycerides) مستويات عالية بين أفراد المجموعتين على السواء مقارنة مع المجموعة المتحكمة. أما عند قياس البروتين الدهني (HDL-C) كنوز النتائج متشابهة في كل المجموعات الثلاث ولم تسجل فروقات واضحة.

إتراجاً على النتائج المتحصلة من الدراسة، يقترح أن القصور تحت السريري في إفراز هرمونات الغدة الدرقية له تأثير واضح في زيادة مستويات معظم الدهون في الدم والتي شمل الكوليسترول، البروتين الدهني (LDL-C) والبروتينات الثلاثي (Triglycerides). بينما اتضح أن الزيادة تحت السريرية في إفراز هرمونات الغدة الدرقية يمكن أن يقتصر تأثيرها فقط على مستوى الجلسيديات الثلاثي (Triglycerides).
# Chapter One

## Literature Review

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Introduction

Sudan was first reported as an endemic goiter area by Woodman in 1952, when different areas described in southern Sudan (Eltom et al. 1984). Kambal, (1967) completed an extensive survey comprising 17470 people in Dar Fur province (Western Sudan) and found that 57% were goiterous. In Khartoum 12.6% of 5566 subjects were found to have goiter. Eltom et al. (1984) showed that the incidence of endemic goiter among school children in Dar Fur was 85%. Endemicity also started to spread and a new focus was reported by Eltom et al. in central Sudan (Kosti). It is estimated that 14 million of the 25 million inhabitants of Sudan are at risk of having iodine deficiency and consequently thyroid problem. Irrespective of this high incidence of thyroid disease, the normal range for thyroid hormones was not established for the Sudanese population.

The thyroid gland maintains the level of metabolism in the tissues that is optimum for their normal function. Thyroid hormones are well known to stimulate the oxygen consumption of most of the cells in the body, helps to regulate lipid and carbohydrates metabolism, modulation of gonadotropin secretion by the pituitary and maintenance of proliferative cell growth and maturation in hair. In addition, thyroid hormones stimulate both sodium pump and glycolytic pathway leading to calorogenesis and oxidative phosphorylation in tissues such as liver, kidney, and muscle (Ganong, 1997).

Physiological concentrations exert both anabolic and catabolic effects promoting the normal metabolic turn-over, but higher
concentrations exert catabolic responses. Decreased thyroid hormones level (hypothyroidism) leads to poor resistance to cold, mental and physical slowing, muscle weakness, cramps and stiffness. In children, there is mental retardation, deaf mutism and other neurological abnormalities (cretinism). Excess thyroid hormones secretion (hyperthyroidism) leads to body wasting, nervousness, tachycardia, tremor, muscle weakness (thyrotoxic myopathy) and excess heat production (Ganong, 1997).

Subclinical thyroid dysfunction is a common clinical problem for which there are many controversial issues regarding screening, evaluation, and management. Subclinical hyperthyroidism and hypothyroidism have subtle clinical manifestations at most, and the importance of timely diagnosis and treatment continue to be contentious subjects of research studies, position papers, and editorials. Both Subclinical hyperthyroidism and subclinical hypothyroidism have many clinical implications that can be seriously harmful if not treated. These include: progression to overt hypo and hyperthyroidism, cardiovascular diseases, atria fibrillation, osteoporosis and bone fracture (Helfand, 2004).

Previous studies conducted on subclinical thyroid dysfunctions in Sudan, are lacking. The aim of this study was to estimate the relation of Subclinical hypo and hyperthyroidism to serum lipids, and to compare the findings with those reported in the literature. According to Tunbridge et al. (1977) and Hak et al. (2000), subclinical disorders are found to be common and twice as often in
women as in men, so only women were chosen to participate in the present study.
1.1. The Thyroid gland

The thyroid gland was first described by Galen and was named "glandluae thyroidaeae" by Wharton in (1656). While Harrington (1935) reviewed the many older opinion concerning the function of this gland (Hardman and Limbird, 2001). It is a flat—appearing bilobed, pink structure, lying on either side of trachea lateral and inferior to the thyroid cartilage. The thyroid is the largest endocrine gland in man weighing about 20 grams in adults. The thyroid is so called because of its shield-shaped configuration (Merck, 2005).

The principal hormones of the thyroid gland are iodine containing amino acids derivatives of primary 3,5,3,5-L-tetraiodothyronine (thyroxine T4) and a lesser quantity of 3,5,3-L-triiodothyronine (T3). In addition, the parafollicular cells of human thyroid gland secrete calcitonin, which is important in calcium homeostasis (Merck, 2005).

1.2. Thyroid hormones synthesis

The major steps in the synthesis, storage, release, and interconversion of thyroid hormones are the following: (1) the uptake of iodide ion by the gland; (2) the oxidation of iodide and iodination of tyrosyl residues in thyroglobulin; (3) Coupling of iodothyrosine residues within thyroglobulin to generate the iodothyronines; (4) the
proteolysis of thyroglobulin, with release of free iodothyronines and iodotyrosines; (5) the conversion of T4 to T3 in peripheral tissues as well as in the thyroid, with conversion and reuse of librated iodide (Hardman and Limbird, 2001).

1.3. Iodide transport (the iodide trap)

Thyroid hormones are unique in that they require the trace element iodine for biologic activity. A complex mechanism has evolved to acquire and retain this crucial element and to convert it into a form suitable for incorporation into organic compound. The thyroid is able to concentrate I\(^-\) against a strong electrochemical gradient which is an energy-dependant process and is linked to the ATPase-dependant Na\(^+\)/K\(^+\) pump which in turn controlled primarily by TSH (Murray et al., 2003). I\(^-\) is transported across the basement membrane of the thyroid cell by an intrinsic membrane molecule called the Na\(^+\)/I\(^-\) symporter (NIS). The NIS derives its energy from Na\(^+\)/K\(^+\), ATPase, which derives the transport process. This active transport system allows the human thyroid gland to maintain a concentration of free iodide 30-40 times that in plasma (Williams, 2001). The transport mechanism is frequently called the "iodide- trapping mechanism" or "iodide pump". The pump is an example of secondary active transport (Ganong, 1997). A very small amount of iodide enters the thyroid by diffusion. Also any intracellular I\(^-\) that is not incorporated into monoiodotyrosine (MIT) or diiodtyrosine (DIT) is free to leave by this mechanism (Murray et al., 2003).

Two thirds of the absorbed iodine is excreted in the urine within 2 to 3 days after ingestion, and may also be lost in the faeces, sweat
and milk. Most of this iodine comes from the breakdown of thyroid hormones (Williams, 2001).

1.4. Thyroglobulin

Thyroglobulin is a large glycoprotein molecule containing 5496 amino acids. It contains about 140 tyrosyl residues and about 10% carbohydrate. Thyroglobulin is unique among body proteins in its content of iodinated amino acids (Taurgo, 1978). The greatest part of iodine in the thyroid glands of animals on adequate iodine intake exists in the form of thyroglobulin. The iodothyrosines "T4 and T3 precursor" form the most abundant iodinated amino acid components of thyroglobulin.

1.5. Proteolysis of thyroglobulin and thyroid hormone secretion

At the cell-colloid interface, colloid is engulfed into a colloid vesicle by a process of macropinocytosis or micropinocytosis and is absorbed into the thyroid cell. The lysosomes then fuse with the colloid vesicle and hydrolysis of thyroglobulin occurs, releasing T4, T3, DIT, MIT, peptide fragments, and amino acids. T3 and T4 are released into the circulation, while DIT and MIT are deiodinated by intrathyroidal deiodinase and the I⁻ is conserved and mostly reutilized for hormone synthesis although a small amount leaks out of the thyroid into the body pool. A small amount of unhydrolysed thyroglobulin is also released from the thyroid cell (Williams, 2001).
1.6. Transport of thyroid hormones

Thyroid hormones are transported in serum bound to carrier proteins. There are three major thyroid hormones transport proteins: thyroxine-binding globulins (TBG); thyroxine-binding prealbumin (TBPA); and albumin. T4 and T3 have a high affinity for TBPA which allow it to carry about 70% of the circulating thyroid hormones. When fully saturated, TBG can carry about 20 μg of T4/dl. TBG binds about 10% of the circulating T4. Its affinity for T3 is about 10 fold lower than for T4, so that it mostly carries T4. Albumin has one strong binding site for T4 and T3 and several weaker ones. Because of its high concentration in serum, albumin carries about 15% of circulating T4 and T3 (Williams, 2001).

1.7. Metabolism of thyroid hormones

The daily secretion of the normal thyroid gland is about 100 nmol of T4, about 5 nmol of T3, and less than 5 nmol of metabolically inactive reverseT3 (rT3) (Williams, 2001).

T4 and T3 are deiodinated mainly in the liver, the kidney, and many other tissues. Two different enzymes are involved, 5′-deiodinase catalyzing the formation of T3, which is three to eight times more potent than T4, and 5-diodinase catalyzing the formation of r-T3, which is metabolically inert. T3 and r-T3 are then converted to various diiodothyronines (Ganong, 1997). Monodeiodination of the outer ring of thyroxine is a "step up" process; increasing the metabolic activity of the resultant compound, while monodeiodination of the inner ring is a "step down" or inactivation process. Further deiodination of the molecule abolishes normal activity.
Most peripheral target tissues utilize T3 that is derived from the circulating hormone. Notable exceptions are the brain and pituitary, for which local generation of T3 is a major source for the intracellular hormone (Hardman and Limbird, 2001).

1.8. Thyroid stimulating hormone

Thyroid stimulating hormone (TSH), or thyrotropin, is a glycoprotein synthesized and secreted by the thyrotrophs of the anterior pituitary gland. TSH is a primary factor controlling thyroid cell growth and thyroid hormones synthesis and secretion. It achieves this effect by binding to specific TSH receptor (TSH-R) on the thyroid cell membrane and activating both the G protein-adenyl cyclase-cAMP and the phospholipase C signaling systems (Williams, 2001).

The serum level of TSH is about 0.4-4.0 mU/L; it is increased in hypothyroidism and decreased in hyperthyroidism. TSH secretion is controlled by a negative feedback mechanism modulated by the circulating level of free T4 and free T3 and by conversion of T4 to T3 in the pituitary thyrotropic cells. Increased levels of free thyroid hormones (T4 and T3) inhibit TSH secretion from the pituitary, whereas decreased levels of T4 and T3 result in an increased TSH release from the pituitary. TSH secretion is also influenced by thyrotropin-releasing hormone (TRH), a 3-amino acid peptide synthesized in the hypothalamus. The precise regulation of TRH synthesis and release is not clear, although thyroid hormones do play a role (Merck, 2005).

Changes in thyroid cell morphology and size are found to be one of the most obvious effects of TSH in the thyrocytes. Also TSH stimulates all phases of iodide metabolism, from increased iodide
uptake and transport to increased iodination of thyroglobuline and increased secretion of thyroid hormones. Other effects of TSH include increase in mRNA for thyroglobulin and thyroperoxidase, with increase in incorporation of I\(^{-}\) into MIT, DIT, T4, and T3. TSH has still other effects on the thyroid gland, including stimulation of glucose uptake, oxygen consumption, CO\(_2\) production, and increase in glucose oxidation. Also there is accelerated turn over of phospholipids and stimulation of synthesis of purine and pyrimidine precursors, with increased synthesis of DNA and RNA (Williams, 2001).

1.9. Effects of thyroid hormones

It is likely that all cells in the body are targets for thyroid hormones. While not strictly necessary for life, thyroid hormones have profound effects on many "big time" physiologic processes, such as development, growth and metabolism. Some of the widespread effects of thyroid hormones in the body are secondary to stimulation of O\(_2\) consumption (calorigenic action), although the hormones also affect and regulate lipid metabolism, and increase the absorption of carbohydrates from the intestine (Hardman and Limbird, 2001).

1.9.1. Calorigenic action

A characteristic response of homoeothermic animals to thyroid hormone is increased oxygen consumption of almost all metabolically active tissues. The exceptions are the brain, tests, uterus, lymph nodes, spleen, and anterior pituitary. Indeed 30% to 40% of the thyroid hormone-dependant increase in oxygen consumption can be attributed to stimulation of cardiac contractility. At one time it was erroneously
believed that thyroid hormone uncoupled mitochondria oxidative phosphorylation. Thyroid hormones-dependant lipogenesis may constitute a quantitatively important energy sink. Further, thyroid hormones induce expression of several lipogenic enzymes.

Although the entire picture is not clear, there appears to be an integrated thyroid hormone response program for regulating the set-point of energy expenditure of maintaining the metabolic machinery necessary to sustain it (Hardman and Limbird, 2001).

1.9.2. Effects on growth

Thyroid hormones are essential for normal growth and skeletal metabolism. In hypothyroid children, bone growth is slowed and epiphyseal closure delayed. Not surprisingly, the growth-promoting effect of thyroid hormones is intimately intertwined with that of growth hormone. In the absence of thyroid hormones, growth hormone secretion is also depressed and thyroid hormones potentiate the effect of growth hormone on tissues (Ganong, 1997).

1.9.3. Effects on lipids metabolism

Alterations of the lipid profile are well known phenomena in thyroid dysfunction. Thyroid hormones regulate lipid metabolism through various mechanisms. Increased thyroid hormone levels stimulate fat mobilization, leading to increased concentrations of fatty acids in plasma. They also enhance oxidation of fatty acids in many tissues. Finally, plasma concentrations of cholesterol and triglycerides are inversely correlated with thyroid hormone levels (Spandrio et al., 1993).
It has been known for over fifty years that an increase in thyroid activity reduces the level of cholesterol in blood whereas a decrease in thyroid activity increases it. The blood cholesterol level represents the balance between ingestion and formation in one hand and excretion and utilization on the other. The action of the thyroid gland on cholesterol metabolism is complex. It stimulates the formation of cholesterol by the liver, but has greater effect on increasing the excretion of cholesterol in the bile, and increasing the entry of cholesterol into cells. The net result is that the blood level falls. Also it was clearly approved that the decrease in plasma cholesterol concentration is due to increased formation of LDL receptors in the liver, resulting in increased hepatic removal of cholesterol from the circulation (Ganong, 1997).

In hypothyroidism, the number of LDL-receptors is decreased and results in increased serum total cholesterol and LDL-cholesterol (Chiat, et al., 1979; Thompson, et al. 1987). However, Gross et al., (1987) reported that hyper-cholesterolaemia associated with hypothyroidism is due partly to increased HDL-cholesterol concentration. Also the hepatic excretion of cholesterol and its conversion to bile acids are decreased and improve by thyroxine administration.

A study carried out by Duntas and Leonidas, (2002) showed that hyperthyroidism exhibits an enhanced excretion of cholesterol and increased turnover of LDL resulting in a decreased total and LDL-cholesterol, whereas HDL is decreased or not affected. Also, the composition and the transport of lipoproteins are seriously disturbed in thyroid diseases. Rassoul, et al., (1988) postulated that clinical manifestation of hypothyroidism lead to changes of plasma
lipoproteins, which are characterized by elevated LDL-cholesterol, an increase of the ratio of LDL-cholesterol/ HDL-cholesterol. On the contrary, patients with hyperthyroidism showed low lipoproteins levels.

Triglycerides output is decreased by thyroid hormones, while hypothyroidism is often accompanied by hypertriglyceridaemia as reported by Muller and Seiz, (1984) and Gomo and Ascot, (1994). Also it was found that in hypothyroidism; the catabolism of VLDL-cholesterol by lipoprotein lipase is also impaired resulting in an increased serum TG (Abrams, et al 1981). The degradation of TG contributes to the enhanced thermogenesis in hyperthyroid patients (Muller and Seiz, 1984).

Dullaart et al (1990) studied the activity of cholesteryl-ester transfer protein (CETP). He found that the thyroid hormones are involved in the regulation of (CETP) activity and that may play a role in the alterations in HDL-cholesterol lipids observed in hypothyroidism. Also Duntas and Leonidas, (2002) reported that, there is a marked increase in HDL levels in hyperthyroid patients due to increased activity of (CETP) and hepatic lipase (HL) which are regulated by thyroid hormones. He suggested that the low activity of (CETP) and more specifically of (HL), results in reduced transport of cholesteryl esters from HDL to VLDL and IDL and reduces transport of HDL2 to HDL3.

From all reported literature, it is clear that hypothyroidism and hyperthyroidism have opposite effects on plasma lipids and apolipoproteins.
1.9.4. Effects on carbohydrate metabolism

Thyroid hormones increase the rate of absorption of carbohydrate from the gastrointestinal tract, an action that is probably independent of their calorigenic action (Ganong, 1997).

Thyroid hormones stimulate almost all aspects of carbohydrate metabolism, including enhancement of insulin-dependent entry of glucose into cells and increased gluconeogenesis and glycogenolysis to generate free glucose. Several studies have shown that thyroid hormone is associated with glucose intolerance resulting from decreased glucose stimulated insulin secretion. This defect in insulin secretion is believed to result from an increase in the rate of apoptosis (programmed cell death) of pancreatic beta cells as a direct effect of thyroid hormone excess. This process is reversible, since when thyroid hormone is withdrawn the rate of beta cell replication increases until homeostasis returns (Jorns, et al, 2002).

1.9.5. Effects on protein metabolism

Thyroid hormones increase protein synthesis in virtually every body tissue ($T_3$ within the cell binds to discrete nuclear receptors and influences the formation of mRNA). Both protein synthesis and degradation of protein are increased in most patients with thyrotoxicosis. The increased degradation of protein comes to a greater extent than the former, as a result there is a net degradation of tissue protein. This is evident in negative nitrogen balance, loss of weight, muscle wasting, weakness, and mild hypoalbuminemia (Greenspan and Gardner, 2004).
1.9.6. Effects on vitamin metabolism

Thyroid hormones increase the demand for coenzymes and the vitamins from which they are derived. In hyperthyroidism, the requirement for water-soluble vitamins, such as thiamine, riboflavin, vitamin B12, and vitamin C, is increased and their tissue concentrations are reduced. The conversion of some water-soluble vitamins to the coenzymes form may be impaired, possibly as a result of defective energy transfer. On the other hand, the synthesis of some coenzymes from vitamins requires thyroid hormones. The metabolism of fat-soluble vitamins is also influenced by thyroid hormones. They are required for the synthesis of vitamin A from carotene and for the conversion of vitamin A to retinal. Moreover, it was found that vitamins D and E appear to be deficient in hyperthyroid animals (Williams, 2001).

1.9.7. Effects on heart and skeletal muscle

Thyroid hormones increase the number and affinity of B-adrenergic receptors in the heart and consequently increase its sensitivity to the inotropic and chronotropic effects of chatecholeamines. They also affect the type of myosin found in cardiac muscle and consequently this affect ATPase activity.

Muscle weakness occurs in most patients with hyperthyroidism. This may be due in part to increased protein catabolism. Thyroid hormones affect the expression of the myosin heavy chain (MHC) gene in skeletal as well as cardiac muscle. However, the effects produced are complex and their relation to myopathy is not
established. Hypothyroidism is also associated with muscle weakness, cramps, and stiffness (Ganong, 1997).

1.9.8. Effects on nervous system

In hypothyroidism, mentation is slow and the cerebral spinal fluid (CSF) protein level elevated. Thyroid hormones reverse the change and large doses cause rapid mentation, irritability, and restlessness.

Some of the effects of thyroid hormones on the brain are probably secondary to increased responsiveness to catecholamines, with consequent increased activation of the reticular activating system. In addition, thyroid hormones have marked effects on brain development; the parts of the CNS most affected are the cerebral cortex and the basal ganglia. Consequently, thyroid hormone deficiency during development causes mental retardation, motor rigidity, and deaf-mutism. Thyroid hormones also exert effects on the peripheral nervous system. The reaction time of stretch reflexes is shortened in hyperthyroidism and prolonged in hypothyroidism (Ganong, 1997).

1.10. Thyroid dysfunctions

The most thyroid disorders include: euthyroid goiter, euthyroid sick syndrome, hyperthyroidism, hypothyroidism, thyroiditis, subclinical hypothyroidism, subclinical hyperthyroidism, and thyroid cancers. Discussion of the synthesis and physiology of thyroid hormones and of the laboratory testing of thyroid function are
prerequisites to a thorough understanding of these disorders (Merck, 2005).

1.10.1. Subclinical hypothyroidism

Subclinical hypothyroidism is a term used for a condition in which there are small elevations in thyroid stimulating hormone (TSH), yet, normal circulating levels of thyroid hormones (William and William, 2004). This biochemical state has been given a variety of other names, including mild thyroid failure, as well as compensated, early, late, mild, minimally symptomatic, and pre-clinical hypothyroidism (Arem and Escalante, 1996). The term "subclinical" may not be strictly correct, since some of patients may have clinical symptoms, but no better terms are more appropriate (Adlen, 1998).

The world prevalence of subclinical hypothyroidism ranges from 1-10 percent; the highest age-and sex- specific rates are in women older than 60 years of age approaching 20 percent in some reports (Tunbridge et al., 1977, Canaris et al., 2002). In the Whickham survey, TSH levels above 6 mIU/L were approximately three times more common in females than males and occurred more frequently in females over 45 years of age. (Tunbridge et al., 1977).

1.10.1.1. Causes

Subclinical hypothyroidism is caused by the same disorders of the thyroid gland as those that cause overt hypothyroidism such as chronic autoimmune thyroiditis, treated Graves' disease, congenital, hypopituitarism, iodine deficiency, radioactive therapy & antithyroid drugs. Chief among these is chronic autoimmune thyroiditis
Hashimoto's disease), which is commonly associated with increased titers of antithyroid antibodies (Adlen, 1998). Medications such as lithium and aminodarone, sulfonyl ureas and ethioamide can interfere with thyroid hormone production or release and secondary result in a slight elevation of (TSH) (Kek et al., 2003).

1.10.1.2. Clinical implications

Clinical manifestations of subclinical hypothyroidism include abnormal lipid metabolism, cardiac dysfunction, and several cross-sectional studies have suggested that it confers an elevated risk of atherosclerosis and coronary heart disease. However, neither of these associations has been confirmed by others (Imaizumi et al., 2004).

1.10.1.2.1. Serum Lipids

The relationship between mild thyroid failure and reversible elevation in serum lipid levels has been widely investigated, but, the findings remain controversial.

In patients with full-blown hypothyroidism, serum levels of triglycerides (TG), total cholesterol and low-density lipoproteins (LDL) cholesterol are elevated. In patients with subclinical hypothyroidism, not surprisingly, the same changes are present but are less marked and less consistent. This pattern of lipid abnormalities, of course, is important because it is a risk factor for atherosclerotic cardiovascular disease (Adlen, 1998).

Several cross-sectional studies have demonstrated that serum levels of total cholesterol and LDL cholesterol are higher in patients with subclinical hypothyroidism than in euthyroid controls (Cooper, 2001). It is believed that is resulted from impaired metabolism due to
a decreased thyroid function (Langer et al., 2003). In other similar studies, however, the observed differences between euthyroid and mild hypothyroid individuals have not been significant (Geul et al., 1993, Prale et al., 1992). Several authors showed a significant reduction of such lipid level after restoration of euthyroid state with thyroxine substitution treatment (Langer et al., 2003).

1.10.1.2.2. Cardiac effects

Cardiac changes are evident in subclinical hypothyroidism. These include impairment of left ventricular diastolic function at rest (affecting the relaxation of the ventricle and hence ventricular filling), reduced LV systolic function, prolongation of pre-ejection time, and lastly intrinsic myocardial contractility (Kek et al., 2003). It has been demonstrated in the Rotterdam study that subclinical hypothyroidism is a strong indicator risk for atherosclerosis and myocardial infarction (Hak et al., 2002). Impairment of endothelium-dependent vasodilatation, a harbinger of atherosclerosis, has been detected in patients with subclinical hypothyroidism (Lekais et al., 1997). In view of clear structural and biological cardiovascular risks associated with the presence of subclinical hypothyroidism, treatment of this condition would be expected to provide protection against the development of cardiovascular disease, although there have been no long term outcome studies published to date (Kek et al., 2003).

1.10.1.2.3. Somatic and neuromuscular effects

Patients with subclinical hypothyroidism can have subtle clinical manifestation and non-specific symptomatology such as dry
skin, cold intolerance, constipation, and easy fatigability. In addition patients with muscular symptoms have mitochondrial oxidative dysfunction with significant lactate increment during exercise. Misiunas et al., (1995), also demonstrated the presence of subclinical polyneuropathy of probable axonal origin in patients with subclinical hypothyroidism. Subclinical hypothyroidism subjects reported significantly more total symptoms than euthyroid individual in the Colorado study (Canaris et al., 2000) and these symptoms do improve with L-T4 therapy. Prospective studies suggest that patients with mild thyroid failure have a higher prevalence of somatic symptoms, mood disorders, cognitive dysfunction, and atypical responses to standard psychiatric therapeutic interventions (Ayala et al., 2002). The lifetime frequency of depression is significantly higher in patients with subclinical hypothyroidism compared with patients with normal thyroid function, suggesting that subclinical hypothyroidism lowers the threshold for depression (Lekais et al., 1997).

1.10.1.3. Treatment options

To date there is still no consensus regarding the treatment of subclinical hypothyroidism. Evidence seems to indicate that subclinical hypothyroidism represent the mildest form of thyroid hormone deficiency and may be associated with adverse consequences. Some authors have taken the approach of treating patients with subclinical hypothyroidism with view to overall symptomatic improvement, lowering effect of lipoprotein fractions and prevention of progression of cardiac abnormalities (Kek et al., 2003).
Treatment is similar to that recommended in patients with overt hypothyroidism. Levothyroxine is the agent of choice, rather than a preparation containing T₃, since T₃ has short half-life and requires multiple daily doses to maintain blood levels in the normal range. Levothyroxine, however, has a long half-life and is partially converted to T₃ in the body, resulting in a constant physiologic blood level of both T₄ and T₃ with a single dose (Roti et al., 1993). The goal of therapy is to maintain TSH levels within the normal biological range and usually a small dose of Levothyroxine is sufficient. Special caution should be exercised in patients with ischemic heart disease. In these cases, a more conservative approach to starting therapy is indicated to prevent dysrhythmias, worsening angina, or even precipitation of myocardial infarction (Kek et al., 2003).

1.10.2. Subclinical hyperthyroidism

Subclinical hyperthyroidism is defined as persistently suppressed serum TSH with normal thyroxine and triiodothyronine in patients who do not have symptoms (Kek et al., 2003).

While the diagnostic criteria and treatment modalities for overt hyperthyroidism are well known, the literature on assessment and treatment of patients with subclinical hyperthyroidism is markedly less extensive. The precise pathophysiology, natural history, risks and long-term outcome of subclinical hyperthyroidism are unknown. (Diane and Kenneth, 2002).

The prevalence of subclinical hyperthyroidism varies amongst the reports. To date, there is no definitive information about the incidence of subclinical hyperthyroidism in the general population. Its
prevalence ranges from 0.6 to 16% depending on diagnostic criteria, the sensitivity of the methods used to measure serum TSH and iodine intake. Moreover, the reported concentrations, prevalence of subclinical hyperthyroidism is affected by the investigator’s definition of the lower limit of the normal range for TSH being 0.7% when the TSH cut-off point was 0.1mU/l and 2.1% with a TSH cut-off of 0.3 mU/l (Biondi et al., 2005). Prevalence of subclinical hyperthyroidism was reported to be 10% in Wickham Survey (Tunbridage et al., 1977) and 12% in the Framingham Study (Utiger, 1994). More recently, the Colorado Thyroid Disease Prevalence Study involving 25,862 subjects showed a prevalence of 2.1% (Canaris, et al., 2000). Hollowel et al., (2002) reported a population representative study of 17,353 people aged 12 and above and found that the prevalence of subclinical hyperthyroidism was only 0.7%.

1.10.2.1. Causes

Most patients with subclinical hyperthyroidism are ambulatory outpatients who are otherwise relatively healthy or have stable, chronic medical conditions. Abnormalities in the TSH remain for months or years in the absence of overt clinical symptoms (Diane and Kenneth, 2002).

The most common causes of subclinical hyperthyroidism are excessive thyroid hormone therapy. The causes can be endogenous as Graves' (early in its course) or it can be caused by thyroid conditions such as autonomous adenoma, multinodular goiter, or thyroiditis, or due to nonthyroidal conditions euthyroid sick syndrome, acute psychiatric disease, pituitary and hypothalamic disorders, pregnancy
and drugs: thyroxine dopamine, glucocorticoids, aspirin and furosemide (Kek et al., 2003). In patients with toxic adenoma or multinodular goiter, subclinical hyperthyroidism is usually a slowly progressive disorder and may last several years before being diagnosed (Biondi et al., 2005).

1.10.2.2. Clinical implications

Increasingly, it is recognized that subclinical hyperthyroidism is not merely a biochemical abnormality dissociated from the clinical manifestations and squeal of thyrotoxicosis. In fact, clinical features of thyrotoxicosis can be identified in subclinical hyperthyroidism albeit of insufficient severity to cause major symptoms. The clinical significance of subclinical hyperthyroidism thus relates to three risk factors: progression to overt hyperthyroidism, cardiac effect, skeletal effects (Kek et al., 2003).

1.10.2.2.1. Cardiac effects

Patients with subclinical hyperthyroidism are at increased risk for cardiac abnormalities. The data on cardiac effects come largely from studies of patients on L-T4 suppressive therapy. TSH secretion for a period one to nine years showed a number of significant cardiovascular changes: 1) increased heart rate, 2) increased incidence of atria arrhythmia such as atria premature beats, 3) increased left ventricular mass, left ventricular posterior wall and interventricular systolic function. Also many studies proved that subclinical hyperthyroidism is a risk factor for the development of atrial fibrillation (Kek et al., 2003).
1.10.2.2.2. Skeletal effects

The issue of increased bone loss in patients with subclinical hyperthyroidism has also been studied, although the published reports are generally small studies that were not controlled, prospective, long term, or double blinded. Premenopausal women with subclinical hyperthyroidism do not appear to be at increased risk of bone loss; whereas two, meta analysis concluded that postmenopausal women with hyperthyroidism may be at increased risk of bone loss. An analysis of 1,250 subjects enrolled in 41 studies revealed that in postmenopausal women, suppressive thyroid hormone therapy was associated with significant bone loss in the trail similarly led to the conclusion that postmenopausal women had enhanced bone loss when taking suppressive dose of thyroid hormone (Diane and Kenneth, 2002).

1.10.2.2.3. Progression to overt hyperthyroidism

Results of long-term studies suggest that subclinical hyperthyroidism may develop into overt disease at a rate of at least 1 to 3 percent per year. In the Whickham Survey where 2,779 subjects were followed up for 20 years, subclinical hyperthyroidism subjects with initial suppressed but detectable TSH levels tend to return to normal on follow-up, while those with undetectable TSH remained unchanged with small risk of progression to frank hyperthyroidism (Vanderpump et al., 1995). The rate of progression to overt hypothyroidism has been estimated to be 5% per year with subjects with autonomous thyroid adenoma and nodular goiter (Wjersinga,
1995). Therefore, the likelihood of progression of subclinical hyperthyroidism to overt hypothyroidism is small.

### 1.10.2.2.4. Other clinical features

With the exception of one study, subclinical hyperthyroidism is associated with relevant signs and symptoms of thyroid hormone excess, and with impaired quality of life. With different kinds of questionnaires formulated to investigate the psychophysical effects of thyroid hormone, patients with subclinical hyperthyroidism, whether exogenous or endogenous, were found to have a higher prevalence of palpitations, tremor, heat intolerance, sweating, nervousness, anxiety, reduced feeling of well-being, fear, hostility, and inability to concentrate. Noteworthy, in a retrospective study a near threefold increased risk of dementia and Alzheimer’s disease was found in patients with subclinical hyperthyroidism (Biondi et al., 2005).

### 1.10.2.3. Treatment Options

Treatment modalities in patients with subclinical hyperthyroidism have not been studied for long-term, and alternative treatment options have not been compared in controlled clinical studies. As has been mentioned, few data are available to guide clinical decision regarding the treatment of endogenous subclinical thyrotoxicosis. A decision regarding the treatment of subclinical hyperthyroidism has based on evidence-based research in problematic because prospective controlled studies comparing different therapies do not exist. Therefore, individual studied, case report, and personal clinical experience must serve as parameters for assessing patients
with subclinical hyperthyroidism. These patients could be treated with either antithyroid agents, surgery, or radioactive iodine or, alternatively, these patients could simply be monitored periodically (Burman et al., 1995).

The American Thyroid Association recommends that serum TSH concentration screening be instituted at age 35 years in both men and women and be repeated every five years (Burman et al., 1995), while the American Association of Clinical Endocrinology had recommended treatment of patients with symptoms of hyperthyroidism, atrial fibrillation, or unexplained weight loss and also women osteopenia (Levy et al., 1990).
Chapter Two

Materials and methods

2.1. Statistical survey:

Before samples collection a general statistical study was carried out of Sudanese subjects visited the SAEC laboratories during 1994-1996. The study focused on the variation in sex type, age, subject's status, whether they were new cases or under treatment and their thyroid status.

2.2. Subjects

This study involved 120 women, aged between 35-65 years, and attended the Radiation and Isotopes Center, Khartoum (RICK), Radio-immunoassay laboratory, during February, 2005 to August, 2006, suspected as cases of thyroid dysfunction. Subjects were divided into three equal groups: euthyroid as control, subclinical hypothyroid and subclinical hyperthyroid subjects. Subclinical hyperthyroidism describes conditions characterized by a low thyroid stimulating hormone (TSH) and normal levels of circulating thyroid hormones (thyroxine and triiodothyronine) (Helfland, 2004). Subclinical hypothyroidism is a condition in which there are small elevations in thyroid stimulating hormone, yet normal circulating levels of thyroid hormones (William and William, 2004). In the present study subjects that considered as subclinical hypothyroid cases had serum TSH levels between (6.0-10 mIU/l) and euthyroid values for T3 and T4. While,
subjects that considered as subclinical hyperthyroid had serum TSH levels between (0.1-0.3 mIU/l) and euthyroid values for T3 and T4.

2.3. Samples and sampling techniques

Blood samples (5 ml) were collected from the cubital vein after overnight fasting. The blood samples were allowed to clot at room temperature and then centrifuged at 4000 r.p.m to obtain the serum. The clear serum was taken immediately for analysis or stored at -20°C for further use.

2.4. Laboratory methods

The levels of TSH, T4, and T3 were estimated by using Radioimmunoassay (RIA) technique, while commercial enzymatic methods were used for determination of total cholesterol, triglycerides, LDL-cholesterol, and HDL-cholesterol.

2.4.1. Radioimmunoassay (RIA)

(RIA) kits are all supplied by the Department of Isotopes, China Institute of Atomic Energy, 1994. Each Kit has specific code: IMK-437, IMK-438, IMK-432 for T4, T3, and TSH respectively.

2.4.1.1. T4 kits

These kits consisted of 6 vials of lyophilized standards for T4 to give different concentrations when reconstituted by 1 ml of distilled water. (0.0, 26, 52, 104, 208, 416 nmol/l) thyroxin. One vial of T4-I\(^{125}\) tracer and T4 antibodies coupled to magnetic particles, and one vial for quality control (QC) sample.
2.4.1.2. T3 kit

Included 6 vial of triiodothyronine, standard solution in different concentrations, (0.0, 0.5, 1.0, 2.0, 4.0, 8.0 nmol/l) triiodothyronine.
One vial contains T3-I\textsuperscript{125} tracer, one vial Anti-T3 antibody, and one vial of (QC) sample.

2.4.1.3. Thyroid stimulating hormone (TSH) kit

Contains 7 vial standard solution of TSH with known concentration as follow: A. 0.0 mIU/L  B. 0.23 mIU/L. C. 1.0 mIU/L. D. 3.0 mIU/L. E. 10.0 mIU/L. F. 20.0 mIU/L. G. 80.0 mIU/L.
One vial contain anti-TSH antibodies coupled to magnetic particles,(solid phase separation system), one vial of tracer (labeled anti TSH with I\textsuperscript{125}, one vial wash Buffer, 3 vial of QC samples A, B, C with different ranges.

Equipments
1. Adjustable micropipettes (10-200µl) with disposable tips.
2. Polystyrene test tubes (disposable).
3. Vortex mixer (single and multi-tubes).
5. Water bath
6. Incubator
7. Centrifuge
Assay procedure

2.4.1.4. T4

Sufficient (polystyrene) test tubes were labeled in duplicates and arranged in assay rack, and then 25 µl were pipetted into each tube of the standards, quality control sample and patient's sample. And 250 µl anti T4 antibody were added to each tube, and mixed well, to the STD and QC and samples 250 µl of tracer were added.

After well mixing the tubes were incubated at 37 °C for 45 minutes, then the rack was placed in the magnetic base for 10 minutes, to separate the bound fraction free from the free fractions by decanting the supernatant. Lastly, each tube was counted in the gamma counter to evaluate the gamma emission per minutes, and binding percent was plotted vs. the concentration, to get standard calibration curve, and from the curve obtained the concentration of thyroxin in the patient's samples was evaluated. This method is bioassay method, (Radioimmunoassay), using radio active isotope of iodine. (I\(^{125}\)) which is gamma emitter.

2.4.1.5. T3

Sufficient number of test tubes were labeled and in duplicate arranged in assay rack. 25 µl of standard solutions, QC samples and patients sample were added to each target tube. 250 µl of T3- I\(^{125}\) tracer and 100 µl anti-T3 antibody were added to each tube and mixed. After mixing, the tubes are incubated at 37 °C for one hour and then vortexed well and centrifuged to separate bound fraction,(liquid phase separation system) the supernatant was decanted and then each tube was placed in gamma counter. The principle of the assay is the same as that for T4.
2.4.1.6. TSH

Sufficient test tubes were labeled and arranged in assay rack in duplicates. 100 µl of STD and QC and samples were pipetted in target tubes and 25 µl tracer (anti TSH labeled by $^{125}$I) were added to each tube and vortexed gently, and then incubated at 37°C in the incubator for one hour. 250 µl of anti TSH (antibody coupled to magnetic particles) were added to each tube and mixed well and incubated at 25°C for one hour. Then the racks were placed in the magnetic separator for 10 minutes and the supernatant was separated by decantation.

**Washing step**

In this step first the concentrated wash buffer was diluted by adding water (1:9), and then 500 µl of the diluent was added to each tube and then vortexed well and then placed again in the magnetic base and allowed to stand for 10 minutes. Then the supernatant was decanted and drained thoroughly on adsorbent paper. The wash step was repeated again. All the tubes were counted in the gamma counter, to evaluate the concentration of TSH in the patient sample.

The quantitative analysis of TSH is achieved by the above method, which is immunoradiometric method. It is non-competitive method in which the radioactive compound (tracer) is TSH antibody. There are two antibodies which react with the TSH in the analyte to get a sandwich complex.

**Calculation of results**

The results in these two methods are obtained by using computerized method in which a software provided from International Atomic Energy Agency (IAEA) was used.
The count per minute of each tube was counted in the Gamma counter, to get the emission all tubes in one minute including the patients samples, the standard curve was plotted, the concentration of T3, T4, TSH standards in the X axis Vs. the bound percentage (the count of each standard over the total count B/T%) in the y axis.

\[ \frac{B_0/T}{\%} = \frac{B_0 \text{ (cpm)} - \text{NSB (cpm)}}{T \text{ (cpm)} - \text{Background count (cpm)}} \times 100 \]

\[ \% \text{Bound} = \frac{B - \text{NSB}}{B_0 - \text{NSB}} \times 100 \]

Where:
- NSB: Non specific binding counts.
- B0: Average zero standard count.
- B: Average standard (or sample) counts.

Then concentration of each sample was calculated from the standard curve (normal interpolation).

2.4.2. Estimation of serum lipids

**Equipments and reagents**

1. Polystyrene test tubes (disposable).
2. Vortex mixer (single and multi-tubes).
3. Multidose micropipette. (Eppendorfe).25µl and 250 µl,
5. Spectrophotometer (Biosystem 305- BTS), with 500 nm filter (490-510).
• All kits and reagent were supplied by (Biosystems) company, for reagents and instruments. Methods and procedures are applied according to the instruction described in the kits manuals. Each Kit has specific code: 11505 (1 X 200 ml), 11648, 11579, and 11528 for Cholesterol, HDL-C, LDL-C, and TG respectively.

2.4.2.2. Estimation of total cholesterol

Principle of the method

In the presence of Cholesterol esterase, the cholesterol esters in the sample are hydrolyzed to cholesterol and free fatty acids. The cholesterol produced is oxidized by Cholesterol oxidase to cholestenone and hydrogen peroxide. Hydrogen peroxide is detected by a chromogenic oxygen acceptor, phenol- ampyrone, in the presence of Peroxidase. The red quinine formed is proportional to the amount of cholesterol present in the sample. (Deeg, and Ziegenhorn 1983).

\[
\begin{align*}
\text{Cholesterol ester + H}_2\text{O} & \xrightarrow{\text{CHE}} \text{Cholesterol + fatty acid} \\
\text{Cholesterol + 1/2 O}_2 + \text{H}_2 & \xrightarrow{\text{CHOD}} \text{Cholesterol + H}_2\text{O}_2 \\
2\text{H}_2\text{O}_2 + \text{Aminoantipyrine + Phenol} & \xrightarrow{\text{POD}} \text{Quinoniemine +4H}_2\text{O}
\end{align*}
\]

Contents and composition of reagents

1- Reagent-1 (Buffer): PIPES PH 6.9 90 mmol/l, phenol 26 mmol/l
2- Reagent-2 (Enzymes): Cholesterol esterase (CHE) 300 U/ml, Cholesterol oxidase (CHOD) 300 U/L, Peroxidase (POD) 1250 U/ml, 4-aminoantipyrine (4-AP) 0.4 mmol/l, pH 7.0.

3- Cholesterol standard 200 mg/dl.

**Procedure**

1- The working reagent was prepared by dissolving one vial of Reagent-2 enzymes in one bottle of reagent-1 Buffer.

2- The working reagent was brought to room temperature.

3- Into labeled test tubes the following were pipetted:

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard (ST)</th>
<th>Sample (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol standard</td>
<td></td>
<td>10 µl</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>10 µl</td>
</tr>
<tr>
<td>Working reagent</td>
<td>1.0ml</td>
<td>1.0ml</td>
<td>1.0ml</td>
</tr>
</tbody>
</table>

4- The tubes were incubated for 10 minutes at room temperature (16-25 C).

5- The absorbance (A) of the standard and the samples were measured at 500 nm against the blank.

**Calculations**

Cholesterol (mg/dl) = \[ \frac{A_S}{A_T} \times 200 \]
2.4.2.3. Estimation of high-density lipoprotein Cholesterol (HDL-C)

Principle of the method

Very low-density lipoprotein (VLDL) and low density lipoprotein (LDL) in the sample are precipitated with phosphotungstate and magnesium ions. The supernatant contains high-density lipoproteins (HDL). The HDL-cholesterol is then spectrophotometrically measured by means of the coupled reactions as described in total cholesterol in page 32.

Contents and composition of reagents

1- Reagent B: 50 ml, phosphotungstate 0.4 mmol/L, magnesium chloride 20 m mol/L

2- HDL-cholesterol standard 3 ml cholesterol 40 mg/dL

3- Cholesterol Kit (Biosystem code. 11505, 11506, 11539)

Procedure

Precipitation

1- Into labeled test tubes the following were pipetted:

<table>
<thead>
<tr>
<th>Sample</th>
<th>0.2 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent B</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

2- The tubes were let to stand for 10 minutes at room temperature.

3- Centrifuged at minimum of 4000 r.p.m for 10 minutes.

4- The supernatant was collected carefully.

Colorimetry
1- The cholesterol reagent brought to room temperature.

2- Into labeled test tubes the following were pipetted:

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard (ST)</th>
<th>Sample (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>100 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HDL-cholesterol standard</td>
<td>-</td>
<td>100 µl</td>
<td>-</td>
</tr>
<tr>
<td>Sample supernatant</td>
<td>-</td>
<td>-</td>
<td>100 µl</td>
</tr>
<tr>
<td>Cholesterol standard</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

3- The tubes were incubated for 10 minutes at 37°C.

4- The absorbance (A) of the sample and the standard were measured at 500 nm against the blank.

**Calculations**

\[
\text{HDL-cholesterol (mg/dl)} = \frac{A_S}{A_{ST}} \times 3.5 \times 40
\]

**2.4.2.4. Estimation of low- density lipoprotein cholesterol (LDL-C)**

**Principle of the method**

Low- density lipoprotein (LDL) in the sample is precipitated with poly vinyl sulphate. Their concentration is calculated from the difference between the serum total cholesterol in the supernatant after centrifugation. The cholesterol is spectrophotometrically measured by means of the coupled reactions as described in total cholesterol in page 32.
Contents and composition of reagents

1- Reagent B: 10 ml polyvinyl sulphate 3 g/L, polyethylene glycol 3 g/L.
2- Cholesterol kit (Biosystem code 11505, 11506, 11539).

Procedure

Precipitation

1- Into labeled test tubes the following were pipetted:

<table>
<thead>
<tr>
<th>Sample</th>
<th>0.2 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent B</td>
<td>0.1 ml</td>
</tr>
</tbody>
</table>

2- The tubes were let to stand for 10 minutes at room temperature.
3- Centrifuged at minimum of 4000 r.p.m for 10 minutes.
4- The supernatant was collected carefully.

Colorimetry

1- The cholesterol reagent was brought to room temperature.

2- Into labeled test tubes the following were pipetted:

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard (ST)</th>
<th>Sample (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>20 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cholesterol standard</td>
<td>-</td>
<td>20 µl</td>
<td>-</td>
</tr>
<tr>
<td>Sample supernatant</td>
<td>-</td>
<td>-</td>
<td>20 µl</td>
</tr>
<tr>
<td>Cholesterol reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>
3- The tubes were mixed and incubated at room temperature for 30 minutes.

4- The absorbance (A) of the sample and the standard were measured at 500 nm against the blank.

**Calculations**

The dilution factor of the sample in the precipitation is 1.5 and the concentration of the standard is 200 mg/dL according to the following formula is;

\[
\text{cholesterol in the supernatant (mg/dl)} = \frac{A_s \times 1.5 \times 200}{A_{st}}
\]

\[
\text{LDL- cholesterol} = \text{Total- cholesterol} - \text{Cholesterol in the supernatant.}
\]

**2.4.2.5. Estimation of triglycerides**

**Principle of the method**

Triglycerides in the sample originate, by means of the coupled reactions described below, a colored complex that can be measured by spectrophotometry (Fossati and Prencipe, 1982).

\[
\text{Triglycerides} + \text{H}_2\text{O} \xrightarrow{\text{lipase}} \text{Glycerol} + \text{Fatty acids}
\]

\[
\text{Glycerol} + 3\text{ATP} \xrightarrow{\text{glycerol kinase}} \text{Glycerol-3-p} + 3\text{ADP}
\]

\[
\text{Glycrtol-3-p} + \text{O}_2 \xrightarrow{\text{G3p-oxidase}} \text{Dihydroxyacetone-p} + \text{H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + 4\text{-Aminoantipyrine} + 4\text{-cholesterol peroxidase} \rightarrow \text{Quinoneimine} + 4\text{H}_2\text{O}
\]
Contents and composition of reagents

1- Reagent A. PIPES 45mmol/L, magnesium chloride 5mmol/L, 4-chlorophenol 6mmol/L, lipase > 100 U/ml, glycerol kinase > 1.5 U/ml, glycerol-3-phosphate oxidase > 4 U/ml, peroxidase > 0.8 U/ml, 4-aminoantipyrine 0.75mmol/L, ATP 0.9mmol/L, PH 7.5.

2- Triglycerides standard. Glycerol equivalent to 200 mg/dl triolein.

Procedure

1- The Reagent was brought to room temperature.

2- Into labeled test tubes the following were pipetted:

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard (ST)</th>
<th>Sample (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglycerides standard</td>
<td>-</td>
<td>10mL</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>10mL</td>
</tr>
<tr>
<td>Reagent</td>
<td>1.0mL</td>
<td>1.0mL</td>
<td>1.0mL</td>
</tr>
</tbody>
</table>

3- The tubes were incubated for 15 minutes at room temperature.

4- The absorbance (A) of the standard and sample was measured at 500 nm against the blank.

Calculations

\[
\text{triglycerides (mg/dL)} = \frac{\text{AS}}{\text{ASt}} \times 200
\]

2.5. Statistical analysis

Statistical analysis was done using the Statistical Package for Social Sciences (SPSS 10.1). Different statistical methods were used as appropriate. Mean ± SD was determined for quantitative data and frequency for categorical variables.
Chapter Three
Results

3.1 Data from statistical survey

Data collected from the survey study was analyzed using statistical package for social sciences (spss 10.1). The total number of subjects visited RIA Lab, SAEC during (1994-1996) were about 4268 subjects. 3644 subjects visited the RIA Lab for the first time, while a number of 624 were reported as subjects under treatment (Appendices, I and II). Males represent 538 of the whole subjects, 85.13% of them were new cases and 14.87% were under treatment (Appendix, III). In contrast, 3730 out of the total number were females, 85.42% of them were new cases while 14.58% under treatment (Appendix, IV). The mean age among both males and females was found to be similar (32.0 + 17.35, 30.8 + 11.84) respectively (Appendix, V).

(Appendix, VI) shows the incidence of hypothyroidism and hyperthyroidism among subjects. 426 of the total number of males were considered as euthyroid with percentage of 79.18%, while 69 males were found to have hyperthyroidism with percentage of 12.82% and 43 subjects with a percentage of 8.0% were found to have hypothyroidism, (Appendix, VII). Among females, 3139 were considered as euthyroid with percentage of 84.15%, while 414 females were found to have hyperthyroidism with percentage of 11.10% and 220 subjects with a percentage of 5.15% were found to have hypothyroidism, (Appendix, VIII).
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<thead>
<tr>
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<th>Normal range</th>
<th>References</th>
</tr>
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<tr>
<td>1</td>
<td>Serum total thyroxine</td>
<td>50-150 nmole/l</td>
<td>Edward (1980)</td>
</tr>
<tr>
<td>2</td>
<td>Serum total triiodothyronine</td>
<td>0.8-3.0 nmole/l</td>
<td>Edward (1980)</td>
</tr>
<tr>
<td>3</td>
<td>Serum total thyroid stimulation hormone</td>
<td>0.4-4.0 mIU/ml</td>
<td>Edward (1980)</td>
</tr>
<tr>
<td>4</td>
<td>Serum total cholesterol</td>
<td>Desirable up to 200mg/dl</td>
<td>Richmond (1973)</td>
</tr>
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<td></td>
<td></td>
<td>Suspect &gt;220mg/dl</td>
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<td></td>
<td></td>
<td>High risk &gt;240 mg/dl</td>
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</tr>
<tr>
<td>5</td>
<td>Serum high density lipoprotein cholesterol</td>
<td>Female &lt; 65 mg/dl</td>
<td>Assmam (1979)</td>
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<tr>
<td>6</td>
<td>Serum low density lipoprotein cholesterol</td>
<td>&lt;150 mg/dl</td>
<td>Friedewald et al. (1972)</td>
</tr>
<tr>
<td>7</td>
<td>Serum triglycerides</td>
<td>35-160 mg/dl</td>
<td>Young and Pestonar (1975)</td>
</tr>
</tbody>
</table>
3.2 Thyroid hormones and TSH in the study groups

In the present study, one hundred and twenty women were selected. Forty women fit the criteria for subclinical hypothyroidism, equal number considered as subclinical hyperthyroidism cases, and the left 40 were euthyroid and selected as controls. The normal range of all parameters measured is presented in table (1).

Serum concentration of thyroxin (T4), triiodothyronine (T3) and thyroid stimulating hormone (TSH) in subjects studied are shown in tables (2) and (3).

The mean levels of T4 in serum of the control group, subclinical hypothyroid and subclinical hyperthyroid subjects were found to be within the same range (94.28± 9.97, 89.70± 11.39, 103.30± 15.23) nmole/l respectively, (Fig. 1).

The same findings are reported for T3 levels (1.96± 0.470, 1.95± 0.45, and 2.19± 0.38) nmole/l respectively, (Fig. 2).

The normal mean level of TSH, when determined in this study, was found to be (1.60± 0.54 mIU/ml). Subclinical hypothyroid cases, showed significantly higher levels (P < 0.001) of (9.55± 2.69 mIU/ml) compared to controls. While there was a significantly lower level (P < 0.001) among subjects with subclinical hyperthyroidism subjects, of mean TSH equals (0.18± .08 mIU/ml) compared to the control, (Fig. 3).

3.3 Serum total cholesterol, triglycerides, LDL-C, and HDL-C

Serum lipid values for the all three study groups, showed that persons with subclinical hypothyroidism and subclinical hyperthyroidism were
Table (2): Serum concentrations of triiodothyronine (T3), Thyroxine (T4), and thyroid stimulating hormone (TSH) in subclinical hypothyroid and euthyroid subjects. Mean ± SD, N = 40

<table>
<thead>
<tr>
<th>subjects</th>
<th>parameters</th>
<th>T3 nmole/l</th>
<th>T4 nmole/l</th>
<th>TSH mIU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euthyroid subjects</td>
<td></td>
<td>01.96 ± 00.47</td>
<td>94.28 ± 09.97</td>
<td>01.60 ± 00.54</td>
</tr>
<tr>
<td>Subclinical Hypothyroid subjects</td>
<td></td>
<td>01.95 ± 00.45</td>
<td>89.70 ± 11.39</td>
<td>09.55 ± 02.69</td>
</tr>
</tbody>
</table>

- Means within the same column having different letters were significantly different, at (p < 0.001).
- N= Number of replicates.
Table (3): Serum concentrations of triiodothyronine (T3), Thyroxine (T4), and thyroid stimulating hormone (TSH) in subclinical hyperthyroid and euthyroid subjects. Mean ± SD, N = 40

<table>
<thead>
<tr>
<th>parameters</th>
<th>T3 nmole/l</th>
<th>T4 nmole/l</th>
<th>TSH mIU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euthyroid subjects</td>
<td>01.96 ± 0.47</td>
<td>94.28 ± 0.97</td>
<td>01.60 ± 0.54</td>
</tr>
<tr>
<td>Subclinical Hyperthyroid subjects</td>
<td>02.19 ± 0.38</td>
<td>103.30 ± 1.53</td>
<td>00.18 ± 0.08</td>
</tr>
</tbody>
</table>

- Means within the same column having different letters were significantly different, at \( p < 0.001 \).
- N= Number of replicates.
Fig (1): Mean T4 among different study groups

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Mean T4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euthyroid</td>
<td>94.27</td>
</tr>
<tr>
<td>Subhypo</td>
<td>89.7</td>
</tr>
<tr>
<td>Subhyper</td>
<td>103.3</td>
</tr>
</tbody>
</table>
Fig (2): Mean T3 among different study groups

- Euthyroid: 1.95
- Subhypo: 1.95
- Subhyper: 2.19
Fig (3): Mean TSH among study groups

<table>
<thead>
<tr>
<th>Study group</th>
<th>Mean TSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euthyroid</td>
<td>1.6</td>
</tr>
<tr>
<td>Subhypo</td>
<td>9.55</td>
</tr>
<tr>
<td>Subhyper</td>
<td>0.18</td>
</tr>
</tbody>
</table>
more likely to have higher levels in their average cholesterol (Ch), triglycerides (TG) levels, as well as low density lipoprotein-cholesterol (LDL-C) compared to euthyroid subjects. The mean concentrations of total cholesterol, TG, LDL-C, and HDL-C are presented in tables (4) and (5).

Subjects with subclinical hypothyroidism showed significantly higher levels of cholesterol ($P < 0.001$) compared to euthyroid subjects. The mean concentrations of cholesterol were $(172.68 \pm 31.0, 237.50 \pm 40.5)$ mg/dl in controls and subclinical hypothyroid subjects respectively, (Fig. 4).

The same findings were true for TG. In subclinical hypothyroid subjects the mean level was $(168.53 \pm 35.6)$ mg/dl and this is significantly higher ($P < 0.01$) than the euthyroid level which was only $(129.75 \pm 31.8)$ mg/dl, (Fig. 5).

When LDL-C levels were considered, again there was statistically significant relationship between subclinical hypothyroid cases and the controls ($P < 0.03$). The mean of the serum concentrations were $(166.25 \pm 41.3$ and $123.95 \pm 32.6)$ mg/dl respectively, (Fig. 6). There was no significant difference reported in the levels of HDL-C ($P < 0.5$). The mean of the serum levels among the controls and the subhypothyroid subjects were $(52.05 \pm 11.5, \text{ and } 52.20 \pm 9.6)$ mg/dl respectively, (Fig. 7).

When serum lipid values for the subclinical hyperthyroid subjects were compared with values of the control group, no statistically significant difference was found. The mean levels of cholesterol in subhyperthyroid subjects and the controls were $(172.15 \pm 29.7$ and $172.68 \pm 31.0$) mg/dl respectively, (Fig. 4). For LDL-C and HDL-C, the results were $(123.95 \pm 32.6, 115.28 \pm 29.4)$ mg/dl and $(52.05 + 11.5, 48.57 + 10.9)$ mg/dl for the controls and the subhyperthyroid subjects respectively, (Figs. 6, 7). The
only statistically significant difference, \((P < 0.01)\), was found for the TG levels when comparison was made between the subhyperthyroid and euthyroid subjects. The mean levels were \((165.00 \pm 27.3\) and \(129.75 \pm 31.8\)) mg/dl respectively, (Fig. 5).
Table (4): Serum concentrations of triglycerides (TG), total cholesterol (Ch), low density lipoproteins cholesterol (LDL-C), and high density lipoproteins cholesterol, (HDL-C) in subclinical hypothyroid and euthyroid subjects. Mean ± SD, N = 40

<table>
<thead>
<tr>
<th>parameters</th>
<th>TG mg/dl</th>
<th>Ch mg/dl</th>
<th>LDL-C mg/dl</th>
<th>HDL-C mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euthyroid subjects</td>
<td>129.75 ± 31.8</td>
<td>172.68 ± 31.0</td>
<td>123.95 ± 32.6</td>
<td>52.05 ± 11.5</td>
</tr>
<tr>
<td>Subclinical Hypothyroid subjects</td>
<td>168.53 ± 35.6</td>
<td>237.50 ± 40.5</td>
<td>166.25 ± 41.3</td>
<td>52.20 ± 9.6</td>
</tr>
</tbody>
</table>

- Means within the same column having different letters were significantly different, at \( p < 0.05 \).
- N= Number of replicates.
Table (4): Serum concentrations of triglycerides (TG), total cholesterol (Ch), low density lipoproteins cholesterol (LDL-C), and high density lipoproteins cholesterol, (HDL-C) in subclinical hyperthyroid and euthyroid subjects. Mean ± SD, N = 40

<table>
<thead>
<tr>
<th>parameters</th>
<th>TG mg/dl</th>
<th>Ch mg/dl</th>
<th>LDL-C mg/dl</th>
<th>HDL-C mg/dl</th>
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<tbody>
<tr>
<td>Euthyroid subjects</td>
<td>129.75 a ± 31.8</td>
<td>172.68 a ± 31.0</td>
<td>123.95 a ± 32.6</td>
<td>52.05 a ± 11.5</td>
</tr>
<tr>
<td>Subclinical Hyperthyroid subjects</td>
<td>165.00 b ± 27.3</td>
<td>172.15 a ± 29.7</td>
<td>115.28 a ± 29.4</td>
<td>48.57 a ± 10.9</td>
</tr>
</tbody>
</table>

- Means within the same column having different letters were significantly different, at ($p < 0.05$).
- N= Number of replicates.
Fig (4): Mean Cholesterol among different study groups

- Euthyroid: 12.78
- Subhypo: 13.87
- Subhyper: 13.07
Fig (5): Mean TG among different study groups

Study Group

- Euthyroid: 9.1
- Subhypo: 12.48
- Subhyper: 12.24
Fig (6): Mean LDL-C among different study groups

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Mean LDL-C</th>
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<tr>
<td>Euthyroid</td>
<td>9.62</td>
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<tr>
<td>Subhypo</td>
<td>10.6</td>
</tr>
<tr>
<td>Subhyper</td>
<td>9.65</td>
</tr>
</tbody>
</table>
Fig (7) : Mean HDL-C among different study groups

- **Euthyroid**: 51.73
- **Subhypo**: 52.2
- **Subhyper**: 48.58

Study Group
Chapter Four

Discussion

Recently, several reports have shown that lipid abnormalities are encountered in patients with subclinical thyroid dysfunction; which may be attributed to impaired thyroid function (Hak et al., 2002, William and William, 2004).

4.1 Subclinical hypothyroidism

In this study subjects that considered as subclinical hypothyroid cases are obviously have elevated TSH levels and euthyroid values for T3 and T4 which match the definition of subclinical hypothyroidism. These findings are explained by Adlen, (1998), who assumed that the elevation of TSH levels reflects the sensitivity of the hypothalamic-pituitary axis to small decreases in circulating thyroid hormone; as the thyroid gland fails, the TSH level may rise above the upper limit of normal when the free T4 level has fallen only slightly and is still within the normal range.

The effect of subclinical hypothyroidism on serum lipid levels and cardiovascular disease remain surrounded by controversy. In the present study hypercholesterolemia was a common criterion among subclinical hypothyroid women. It is generally well known and repeatedly published that patients with subclinical hypothyroidism show increased levels of cholesterol (William and William, 2004). This is believed to be resulted from impaired metabolism due to a decreased thyroid function. The same results are found to be true for LDL-C and TG among the subclinical
hypothyroid subjects. However, the HDL-C levels were found to be within the normal range.

Recent cross-sectional study of 279 women older than 65 found that women who had a serum TSH greater than 5.5 mU/l had 13% higher LDL-cholesterol and 13% lower HDL-cholesterol than those with a normal serum TSH (0.1-5.5 mU/l), (Bauer et al, 1998). Also the Colorado study and others noted significantly elevated LDL cholesterol in subjects with subclinical hypothyroidism (Canaris et al., 2000, Kahaly, 2000). Some, cross-sectional studies have demonstrated that serum levels of total cholesterol and LDL-cholesterol are higher in patients with subclinical hypothyroidism than euthyroid controls as reported by Elder et al, (1990).

In some studies the relation between TSH and total cholesterol or LDL-C is inconsistent. In a survey carried out in (1977), the Wickham study, no reported relations were found between subclinical hypothyroidism and hyperlipidemia (Tunbridge et al., 1977). Also in the Rotterdam survey, a population- based cross sectional study, lipid levels were significantly lower among euthyroid women. They claim that, this might be an artifact of higher use of diet or other lipid-lowering therapy (Hak et al., 2002). Other several studies showed a significant reduction of euthyroid state with thyroxine substitutive treatment as reported by Langer et al, (2003).

The New Mexico Elder Healthy Survey, which was a fair quality study of randomly selected Medicare recipients, no differences founds in HDL-C and LDL-C levels between euthyroid subjects and those who had serum TSH greater than 10 mU/l (Lindeman et al., 1999).
Another large field surveys carried by Langer et al, (2003), focused on the evaluation of thyroid status of Slovak rural population demonstrated that there was no difference in the level of TG and cholesterol. They assumed that this interrelation resulted from very high cholesterol intake due to inappropriate general nutritional status of rural population resulting from the consumption of unhealthy foods.

Moreover, Williams and Williams, (2004), in a population-based study, concluded that subclinical hypothyroidism does not appear to be associated with abnormalities in serum cholesterol or TG levels when adjusted for confounding variables.

4.2 Subclinical hyperthyroidism

As mentioned before, the literature on assessment and treatment of patients with subclinical hyperthyroidism is markedly less extensive. The precise pathophysiology, natural history, prevalence, risks and long-term outcome of subclinical hyperthyroidism are unknown. Diane and Kenneth, (2002) reported that in the United States, subclinical hyperthyroidism became an entity that is increasingly recognized, probably of the aging of the Untied States population and the development of assays with enhanced TSH sensitivity.

In the present study subjects with subclinical hyperthyroidism were found to have normal serum T3 and T4 with TSH levels suppressed below the normal range. Diane and Kenneth, (2002), reported that, the decreased levels of subclinical hyperthyroidism is due to the sensitivity of the pituitary gland to respond to minor elevations in serum or tissue T4 and T3. Although the levels of T3 and T4 remain within the normal
range, minimal increases in these thyronines are sufficient to decrease the serum TSH (Diane and Kenneth, 2002).

The present study has detected no significant differences in total serum cholesterol levels between subjects with subclinical hyperthyroidism and euthyroid subjects. Also there was no significant effects found regarding the HDL-C or LDL-C levels. These results to some extent are not surprising, since many studies, concerning patients with overt hyperthyroidism, reported that hyperthyroidism is associated with decreased or normal concentrations of total cholesterol and the other serum lipoproteins and lipids (Prale et al., 1992, Arem et al., 1996, Duntas, and Leonidas, 2002). Moreover, Hansson, (1983) studied the effect of experimental hyperthyroidism on plasma lipoproteins and stated that, the decreased LDL-cholesterol concentrations in hyperthyroid patients may be due to a mechanism include both an increased number of LDL-receptors and enhanced transfer of cholesterol and phospholipids to the LDL sub-fraction with subsequent rapid uptake in the liver via hepatic lipase. Another study carried by Caparevic, et al (2000), included 55 elderly patients with subclinical hyperthyroidism, concluded that patients with subclinical hyperthyroidism tend to have low serum total cholesterol, LDL-cholesterol and HDL-cholesterol levels. Also they found a significant increase of serum cholesterol, LDL-cholesterol and HDL-cholesterol levels after treatment.

In the present study, the triglycerides values among subjects with subclinical hyperthyroidism and the controls are found to be different. The subclinical hyperthyroid subjects showed higher values compared to the controls. These findings were similar to those obtained by Davidson et al, (1988), who observed that concentrations of triglycerides were
higher in patients with subclinical hyperthyroidism compared to euthyroid subjects. They found this observation is not surprising as hyperthyroidism is known to exert widespread effect on hepatic triglycerides assembly and secretion. On the other hand, it was demonstrated by some authors that triglycerides metabolism is disturbed due to thyroid dysfunction but to smaller extent than that of plasma cholesterol, (Muller, 1984 and Barbagallo, 1995).

Relaying on these findings above, and the fact that thyroid hormones could increase lipolysis, it is fairly expected that the levels of serum lipids in patients with subclinical hyperthyroidism are decreased or at the normal range.

In this study, subjects with subclinical hyperthyroidism, to some extent, are found to be having normal serum lipids, suggesting that there might not be relationship between subclinical hyperthyroidism and lipids abnormalities.

Worthy to be noted, most of researchers and authors focused on other implications that related to subclinical hyperthyroidism. These include atrial fibrillation, osteoporosis, and cardiovascular diseases.
Conclusions

The present study is carried out to estimate the changes of serum lipids related to subclinical thyroid dysfunction in Sudanese females' subjects, compared to euthyroid individuals.

Subjects with subclinical hypothyroidism showed higher serum levels of cholesterol, LDL, and TG which are significantly different from the euthyroid subjects. These findings are found to be in agreement with studies conducted abroad among different populations in the United States and Europe.

Unfortunately, records on the levels of the thyroid hormones in euthyroid subjects and cases of thyroid dysfunctions are lacking in Sudan. Also there is no any kind of studies that focus on changes in the plasma lipids profile associated with subclinical thyroid dysfunctions and how this is affected by other factors such as age and sex.

Recommendations

Further multi-center randomized and population based studies are needed in our community to study the relation between subclinical thyroid dysfunctions and cardiovascular disease and other risk factors. Large-scale controlled intervention and outcome trials are also needed to assess the potential benefit of L–thyroxine treatment and anti thyroid medications in subjects with subclinical hypo and hyper thyroidism, so further recommendation regarding screening for the thyroid disease in the community will influenced by the results of these studies.
References


Appendices

Statistical Survey Results

(Tables and Figures)
Appendix (I)


<table>
<thead>
<tr>
<th>Sex</th>
<th>Under treatment</th>
<th>New cases</th>
<th>Total</th>
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<tr>
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<td>80</td>
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<td>Female</td>
<td>544</td>
<td>14.58</td>
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<tr>
<td>Total</td>
<td>624</td>
<td>14.71</td>
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Appendix (II)


3730
87%

538
13%

Males
Females
Appendix (III)
Appendix (IV)
Appendix (V)
Means ages among patients' under study

<table>
<thead>
<tr>
<th>Sex</th>
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Appendix (VI)


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<td>%</td>
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<td>3139</td>
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<td>Both</td>
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<td>83.53%</td>
<td>220</td>
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</table>
Appendix (VII)
The incidence of hypothyroidism and
hypothyroidism among males visited RIA Lab,

- Normal: 426 (79%)
- Hypo: 69 (13%)
- Hyper: 43 (8%)
Appendix (VIII)