PATTERN OF THYROID AUTOANTIBODIES, ESSENTIAL AND TOXIC TRACE ELEMENTS IN VARIOUS THYROID DISORDERS IN NIGERIA

by

NANCY AMALACHUKWU ABOH

Matriculation Number – 112382

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CERTIFICATION

I certify that this original Ph. D research work was carried out by ABOH, Nancy Amalachukwu (matric number - 112382) in Endocrinology and Micronutrients unit Laboratory, Department of Chemical Pathology, Faculty of Basic Medical Sciences, College of Medicine, University of Ibadan, Nigeria.

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SUPERVISOR

John I. ANETOR, Ph.D. (Ib), MIBMS (UK), FIMLS (Nig.), FRSC (UK)

Professor of Chemical Pathology

University of Ibadan

Ibadan, Nigeria

DEDICATION

To

The ALMIGHTY GOD, the fountain of wisdom, knowledge and everlasting joy. For His infinite Love, Mercy & Favour; without whom the completion may not have been a Reality.

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ABSTRACT

Thyroid disorders are metabolic diseases where autoimmunity has been identified as a major cause. Alteration of balance between certain essential (Copper, Iron, Selenium, Zinc) and toxic (Arsenic, Lead, Cadmium, Nickel) trace elements has a strong role in the pathogenesis of auto-immune thyroid disease but this has received little attention. The imbalance in various thyroid disorders in Nigerian patients was investigated.

Two hundred and seventy three (52 males, 221 females) consenting age-matched subjects of 18 to 65 years were consecutively enrolled based on clinical symptoms and thyroid function tests from University College Hospital, Ibadan and Lagos University Teaching Hospital, Lagos,Nigeria. They were divided into four groups: Simple Non-Toxic Goitre (SNTG, n=43), hyperthyroidism thyroidism (HYPERTH, n=79), hypothyroidism (HYPOTH, n=15) and controls (n=136). Socio-demographic characteristics and anthropometric indices were obtained using a structured question-naire. Spot urine samples were collected for determination of iodine status (IOD) using colori-metric method. Blood (10mL) was collected and the plasma was used for determination of Thyroid Function Tests (TFTs) and trace elements. The TFTs: Total Triiodothyronine (T_3), Total Thyroxine (T_4), Free Triiodothyronine (FT_3), Free Thyroxine (FT_4), Thyroid Stimulating Hormone (TSH), thyroglobulin, autoantibodies (Antithyroid Peroxidase, TPOAb and Antithyroglobulin, TgAb) were determined using Enzyme - Linked Immunosorbent Assay (ELISA). Trace elements were determined by Atomic Absorption Spectrophotometer (AAS). Data were analysed using Student's t-test, ANOVA and Pearson's correlation at $p = 0.05$.

Among subjects with thyroid disorders, 40.1% and 24.8% were positive for TPOAb and TgAb when compared with controls (8.8% and 5.9%), respectively. Antithyroglobulin was positive in 60% (168.8±92.3 kiu/L) HYPOTH, 24.7% (123.8±22.8 kiu/L) HYPERTH and 14.0% (130.9 ± 35.0 kiu/L) SNTG. Significantly reduced levels of Cu (11.9±0.3, 11.8±0.2, 16.1±0.5µmol/L), Fe (20.1±0.5, 19.8±0.4, 15.0±0.3 µmol/L) and Se (0.5±0.01, 1.0±0.1, 0.5±0.03 µmol/L) relative

to the controls (16.1±0.2, 28.7±0.2, 3.8±0.03 µmol/l); and elevated levels of Cd (0.4±0.01, 0.4±0.01, 0.3±0.03 µmol/L), and Pb (1.2±0.03, 1.2±0.03, 1.2±0.04 µmol/L) were found in SNTG, HYPERTH and HYPOTH respectively, compared with controls **(**0.6±0.01, 0.1±0.01 µmol/L**)**. Thyroglobulin was significantly elevated in HYPERTH, SNTG and HYPO compared with controls, suggesting immune alteration. The FT_3 and FT_4 were significantly increased in SNTG (3.7±0.02 and 14.9±0.2 pmol/L), HYPERTH (4.4±0.02 and 27.2±0.4 pmol/L) but lowered in HYPOTH (1.1±0.03 and 7.4±0.1 pmol/L) compared with controls (3.6±0.1 pmol/L and 13.1±0.1 pmol/L). The level of TSH was significantly increased in HYPOTH but decreased in SNTG and HYPERTH compared with controls. Negative correlations were obtained for FT_4 and TSH (r = - 0.568); FT_3 and TPO (r= - 0.2); TgAb and Zn (r = - 0.3). Positive correlations were found between TPO and As (r = 0.3); IOD and TPO (r = 0.3).

Imbalances between essential and toxic trace elements and presence of autoantibodies were among the key mechanisms involved in the pathology of thyroid disorders. Early detection of trace element abnormalities will be useful in overall management of thyroid disorders.

Keywords: Trace elements, Iodine, Thyroglobulin, Thyroid hormones, Autoimmunity

Word count: 497

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ABBREVIATIONS / GLOSSARY

µg	Microgram
AACE	American Association of Clinical Endocrinologists
ACB	Association for Clinical Biochemistry
ACTH	Adrenocorticotrophic Hormone
ADCC	Antibody dependent cell mediated cytotoxicity
AHRQ	Agency for Healthcare Research and Quality
AIH	Amiodarone-Induced Hyperthyroidism
AITD	Autoimmune thyroid disease
ANS	8-Anilino-1-Napthalene-Sulphonic Acid
ATA	American Thyroid Association
ATD	Anti-Thyroid Drug Treatment
BTA	British Thyroid Association
BTF	British Thyroid Foundation
CDC	Centers for Disease Control and Prevention
CT	Calcitonin
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T- lymphocyte antigen-4
CV	% Coefficient of Variation
DISC	Death inducing signal complex
DR	Death receptor
DTC	Differentiated Thyroid Carcinoma
EQA	External Quality Assessment
FADD	Fas-associated protein with death domain
FAO	Food and Agricultural Organization of the United Nations
FasL	Ligand for Fas
FDH	Familial Dysalbuminemic Hyperthyroxinemia
FFA	Free fatty acids
FLICE	FADD-like interleukin-1-[β]-converting enzyme
FMTC	Familial Medullary Thyroid Carcinomas
FNA	Fine Needle Aspiration
FSH	Follicle Stimulating Hormone
FT_3	Free Tri-iodothyronine
FT_4	Free Thyroxine
GD	Graves' disease
GP	General Practitioner
HAMA	Human Anti-mouse Antibody
HCC	C-cell Hyperplasia
HCG	Human chorionic gonadotropin
HLA	Human leukocyte antigen
ICAM	Intercellular Adhesion Molecule
ICCIDD	International Council for Control of Iodine Deficiency Disorders
IDD	Iodine deficiency disorders
IFN	Interferon
IIH	Iodine-induced hyperthyroidism
IL	Interleukin
IMA	Immunometric Assay
IQC	Internal Quality Control
ITU	Intensive Therapy Unit

LFA	Lymphocyte function associated antigen
LH	Luteinising Hormone
LMP	Large multifunctional proteosome
L-T_4	Levothyroxine
MDL	Minimum Detection Limit
MEN	Multiple Endocrine Neoplasia
MI	The Micronutrient Initiative
MTC	Medullary Thyroid Cancer
mU/L	Milliunits per litre
NACB	National Academy of Clinical Biochemistry
nmol/L	Nanomoles per litre
NTI	Non-Thyroidal Illness
PBI	Protein-bound Iodine
Pg	Pentagastrins
pmol/L	Picomoles per litre
ppm	Parts per million
PRL	Prolactin
PTU	Propylthiouracil
RCP	Royal College of Physicians
RET	RET Proto-oncogene
rhTSH	Recombinant human TSH
RIA	Radioimmunoassay
RT_3	Reverse T_3
SAC	School-age children (6–12 years)
SHBG	Sex Hormone Binding Globulin
SNTG	Simple non-toxic goitre
TAP	Transport of antigen processing
TBAb/TSBAb	TSH Receptor Blocking Antibody
TBG	Thyroxine Binding Globulin
TBII	TSH Binding Inhibitory Immunoglobulins
TBPA	Thyroxine Binding Prealbumin
TFT	Thyroid function tests
TgAb	Thyroglobulin autoantibodies
TGP	Total Goitre Prevalence
TNF	Tumor necrosis factor
TNFR	TNR receptor
TPOAb	Thyroid peroxidase autoantibodies
TRAb	TSH Receptor Antibody
TSAb	Thyroid Stimulating Antibody
TSH	Thyroid stimulating hormone, (thyrotrophin)
TSHoma	TSH secreting pituitary adenoma
TSH-RAb	TSH receptor antibodies
TT_3	Total tri-iodothyronine
TT_4	Total thyroxine
TTR	Transthyretin
UI	Urinary iodine
UK NEQAS	UK National External Quality Assessment Schemes
UL	Upper limit
USI	Universal salt iodization
UTSH	Ultrasensitive Thyroid stimulating hormone

CHAPTER ONE

1.0. INTRODUCTION

Thyroid disorders are a group of metabolic diseases where autoimmunity has been identified as a major cause. The etiology of autoimmune disease (AID) has remained unclear. Predisposing genetic factors (Nagataki *et al.*, 1990) seem to be evident, but environmental factors (Schleusener *et al.*, 1991), imbalances in trace element status as well as increase in age (Liel & Barchana, 2001) have been shown conclusively to be linked to AID. These factors such as stress (Glaser & Kiecolt-Glaser, 1994), infections, trauma, smoking (Holt, 1987), drugs and nutrition, especially a deficient or an increased iodine supply (a major cofactor and stimulator for thyroid peroxidase (TPO) (Kharrazian, 2010) and which plays a key role in the manifestation of autoimmune diseases (Mariotti *et al.*, 1996) have all been thought to play a major role. A four to seven times increased prevalence is observed in females (Behman *et al.*, 1996), strongly implying gender and hormonal influences (Volpe, 1996). The alteration of balance between certain essential trace elements, such as Copper (Cu), Iron (Fe), Selenium (Se), Zinc (Zn) and toxic trace elements (Arsenic (As), Lead (Pb), Cadmium (Cd) Nickel (Ni) has also also been implicated in the pathogenesis of autoimmune thyroid disease (AITD). So far, no published studies have been presented which have addressed the influence of deficient or increased levels of trace elements, iodine intake on the prevalence of autoantibodies (e.g. anti-thyroid peroxidase (TPO) among others) and autoimmune thyroiditis in a prospective study in the Nigeria population.

Since scanty data are available in the literature on the prevalence and pattern of anti-TPO antibodies in adults without an increased risk for autoimmunity; therefore, it was decided that certain trace element status, iodine excretion as well as the prevalence of autoantibodies in subjects with thyroid disorders be assessed.

The micronutrient deficiencies which are of greatest public health significance include: iron and iodine (Chakravarty and Ghosh, 2000) among others. Micronutrient malnutrition is a major global health concern (Hongo, 2003) currently because deficiency of trace elements in the body is associated with ill health and diseases such as thyroid disorders. Incredibly, over 2 billion people, mostly poor women, infants, children and pregnant women (Batra and Seth, 2002) in the world, are iron and iodine deficient among others. Infants deserve a lot of concern because they need adequate micronutrients to

maintain normal growth and development (Rush, 2000). The most devastating consequence of these deficiencies include goitre, hypothyroidism, poor learning capacity, reduced psychomotor skills (Batra and Seth, 2002), increased risk of stillbirths, abortions, impairment in cognitive performance, lowered work capacity, lowered immunity to infections, pregnancy complications, infant deaths and growth abnormalities (Ames, 2010) among others.

This study focused on thyroid disorders in Nigeria, a developing country since the statistics of malnutrition in these countries are high. One third of the total population in developing countries is reported to be affected by mineral deficiency and therefore more vulnerable to infections and impairment of physical and psycho-intellectual development (WHO, 2005). Certain Substances introduced with food, such as thiocyanate and isoflavones or certain herbal preparations, can interfere with micronutrients and influence thyroid function (Triggiani *et al.*, 2009). Iodine deficiency, which usually leads to thyroid disorders, has several important health consequences or multiple adverse effects on human growth, and development that together are called iodine deficiency disorders (IDD) (Hetzel, 1993) due to inadequate thyroid hormone production. It is the collective name for endemic goitre and endemic cretinism (Das *et al.*, 1998; Dunn, 2001), a major worldwide problem especially during pregnancy and childhood (Dunn, 2001); a threat to the social and economic development of countries. Iodine deficiency disorders are important public health problems and are a good model to study the effects of other trace elements whose actions in many human metabolisms have been underestimated (Thilly *et al.*, 2007). In areas of endemic goitre, micronutrient status can be an important determinant of iodine and thyroid metabolism (Zimmermann & Kohrle, 2002). Globally, it is estimated that about 2 billion individuals have an insufficient iodine intake (Tulchinsky, 2010); South Asia and sub-Saharan Africa are particularly affected (Zimmermann, 2009). It is however also known that about 50% of Europe remains mildly iodine deficient, and iodine intakes in other industrialized countries, including the United States and Australia, have fallen in recent years (Zimmermann, 2008). Assessment methods include urinary iodine concentration, goitre, newborn thyroid-stimulating hormone, and blood thyroglobulin.

Goitre, which has a multifactorial origin (Thilly *et al.*, 2007) is the chief consequence of iodine deficiency, resulting from either low iodine intake or ingestion of goitrogens. Goitre is conveniently referred to as endemic when it occurs in more than

10% of the population in a defined area (Delange, 2002). A total goitre rate (TGR) of 5% or more is the recommended cut-off point to indicate a public health problem (UNICEF/ICCIDD/WHO, 1992). This recommendation is based on the observation that goitre prevalence rates between 5% and 10% may be associated with a range of abnormalities including inadequate urinary iodine (UI) excretion or subnormal levels among adults, children and neonates (Zimmermann *et al.,* 2003). Iodine deficiency is the main preventable cause of brain damage in children and constitutes a universal public-health concern (WHO, 2001b; deBenoist *et al.,* 2003). This was the primary motivation in the worldwide drive to eliminate IDD (Andersson *et al.,* 2005). Although cretinism is the most extreme manifestation, the more subtle degrees of mental impairment leading to poor school performance, reduced intellectual ability and impaired work capacity are of considerably greater significance (Caventure *et al.,* 1998 & Andersson *et al.,* 2005). Iodine deficient women may give birth to babies with severe mental and neurological impairment. If this deficiency occurs during infancy or childhood, it causes irreversible mental retardation, growth failure, speech and hearing defects, among others (Zimmermann *et al.,* 2004). Even a mild deficiency may cause a low intellectual capacity. The prevalence of endemic goitre varies with the severity of iodine deficiency (El-Mougi *et al.,* 2004). Lack of iodine in the diet is not the only factor, which may lead to development of goitre; a large number of vegetables such as cabbage, brussels sprouts, cauliflower, rutabaga, soybeans and the brassica family (thioglycosides producing) among others have goitre producing qualities if consumed over long periods (Paynter *et al.,* 1988) because they interfere with iodine metabolism. Naturally occurring goitrogens include cyanoglycosides found in several staple foods in the tropics, namely cassava, maize, bamboo shoots, and sweet potatoes (Dillon *et al.,* 1999; Lezama *et al.,* 2001). Linamarin, the predominant cyanogenic glucoside in cassava is metabolized to thiocyanate which may cross the human placenta and affect the thyroid of the foetus, which in turn can block thyroidal uptake of iodine (Biassoni *et al.,* 1998) by inhibiting the trapping of iodine by the placenta (Kochupillai & Pandav; 1987); flavonoides from millet (e.g pearl millet), a staple food in Sudan are also known to have antithyroid activity (Elnour *et al.,* 1998) due to the presence of goitrogenic substances (Gaitan, 1989). Thiocyanate overload results from cassava consumption. Cigarette smoke as well acts on the thyroid by numerous paths, but among the thousands of compounds in cigarette smoke is thiocyanate (Fukayama et al., 1992), a metabolite of cyanide (with a half-life >

6 days) which competes with iodide for trapping by the sodium iodide symporter and for oxidation by the TPO; it inhibits iodide transport and organification as well as increases the release of iodide from the thyroid. Iodine deficient areas (without sufficient iodine supplementation) are the most at risk of goitrogenic compounds, including thiocyanate causing goitre, while 2,3-hydroxypyridine a cigarette smoke toxin, reduces iodothyronine deiodinase activity, thereby elevating thyroxine concentration due to reduced thyroxine deiodination (Petersen et al., 2009). Experimental and epidemiological studies have shown that thiocyanate overload aggravates the severity of iodine deficiency and worsens its outcome (Contempre et al., 2004).

Studies of malnourished individuals in endemic areas show alteration in thyroid morphology and function, suggesting that malnutrition has a goitrogenic effect (Brix & Hegedus, 2000; Lezama *et al.*, 2001) water, particularly from shallow or polluted streams and wells contain cyanoglycosides that are transformed to thiocyanate, after absorption (Geelhoed, 1999). Certain sulfur-containing onion volatiles are also goitrogenic (thiourea and thiouracil) (Cowan *et al.,* 1967). All of these substances interfere with the thyroidal iodide intake. Excessive intake of iodine may itself cause goitre (Lauberg *et al.,* 2010). The global salt iodization program has resulted in a reduction in goitre prevalence. The persistence of goitre in some areas with adequate iodine prophylaxis and the unequal geographic distribution of goitre in iodine deficient areas suggest the existence of other goitrogenic factors (Brix & Hegedus, 2000).

Report from the WHO Global database on Iodine Deficiency, 2004, by Andersson *et al.*, (2005), show that about 2 billion people is estimated globally, living in about 118 countries with insufficient iodine intake (Delange, 1998). The virtual elimination of IDD by the year 2000, as pledged by most world governments and international organizations during the world summit for children in 1990, has not yet been achieved (Toteja *et al.*, 2004; Andersson *et al.,* 2005). Dunn (1996) described seven major errors that occur during iodine supplementation programs and offered suggestions for their avoidance in order to achieve the goal of sustainable elimination of iodine deficiency. They include: Unreliable assessment of iodine deficiency, poor iodine supplementation plan, exclusion of relevant stakeholders, inadequate education, insufficient monitoring, cost and nonsustainability. Deficiencies of selenium (Vanderpas *et al.,* 1993) and iron (Zimmermann *et al.,* 2000) can act in concert with iodine deficiency to impair thyroid metabolism and modify the response to prophylactic iodine (Zimmermann *et al.,* 2002).

There is a cumulative effect of exposure to thiocyanate (from the breakdown of cyanogenic glycosides in food plants), resulting in thyroid toxicity, including goitre and cretinism (Nahrstedt, 1993).

Cyanide is metabolized in mammalian systems by one major route and several minor routes. The major route of metabolism for hydrogen cyanide and cyanides is detoxification in the liver by the mitochondrial enzyme rhodanese, which catalyses the transfer of the sulfane sulfur of thiosulfate to the cyanide ion to form thiocyanate (see fig.1). About 80% of cyanide is detoxified by this route. The rate-limiting step is the amount of thiosulfate. While rhodanese is present in the mitochondria of all tissues, the species and tissue distributions of rhodanese are highly variable. Although cyanide can interact with substances such as methaemoglobin in the bloodstream, the majority of cyanide metabolism occurs within the tissues. Iodine deficiency and goitre, hypothyroidism and cretinism are endemic in many areas of Africa. Several surveys in the endemic areas have demonstrated that there is also a strong cor- relation between cassava consumption and the thyroid effects (Abuye *et al*., 1998).

Knowledge of the impact of iodine deficiency on intellectual development and the resulting costs to society, including delayed socio-economic development, has played a significant role in mobilizing scientists, public health administrators and political leaders the world over to deal effectively with iodine deficiency disorders (IDD) (Pandav, 1996). Over 800 million are classified undernourished worldwide; the number of people affected by micronutrient deficiency disorders (MDD) is 2.5 times that size (about 2 billion people). This "hidden hunger" affects those who may appear well nourished and are actually micronutrient deficient (Anetor, 2001). While most forms of micronutrient deficiency disorders, MDD have been eradicated in industrialized countries; the developing world is yet to experience relief from the consequential effects of these deficiencies, particularly lack of iodine, iron and zinc among others which are recognized global public health problems (WHO/UNICEF/ICCIDD, 1994 and WHO/UNICEF, 1995). It is likely that other micronutrient deficiencies are also highly prevalent, at least seasonally. These deficiencies are mostly prevalent amid poverty, environmental deprivation and social disparity (Underwood, 1993; Sommer and West, 1996). These disorders take their toll on pregnant and lactating women, infants and young children whose needs are elevated to support rapid growth, overcome unphysiological losses from frequent infections and overcome some physiological losses associated with the woman's

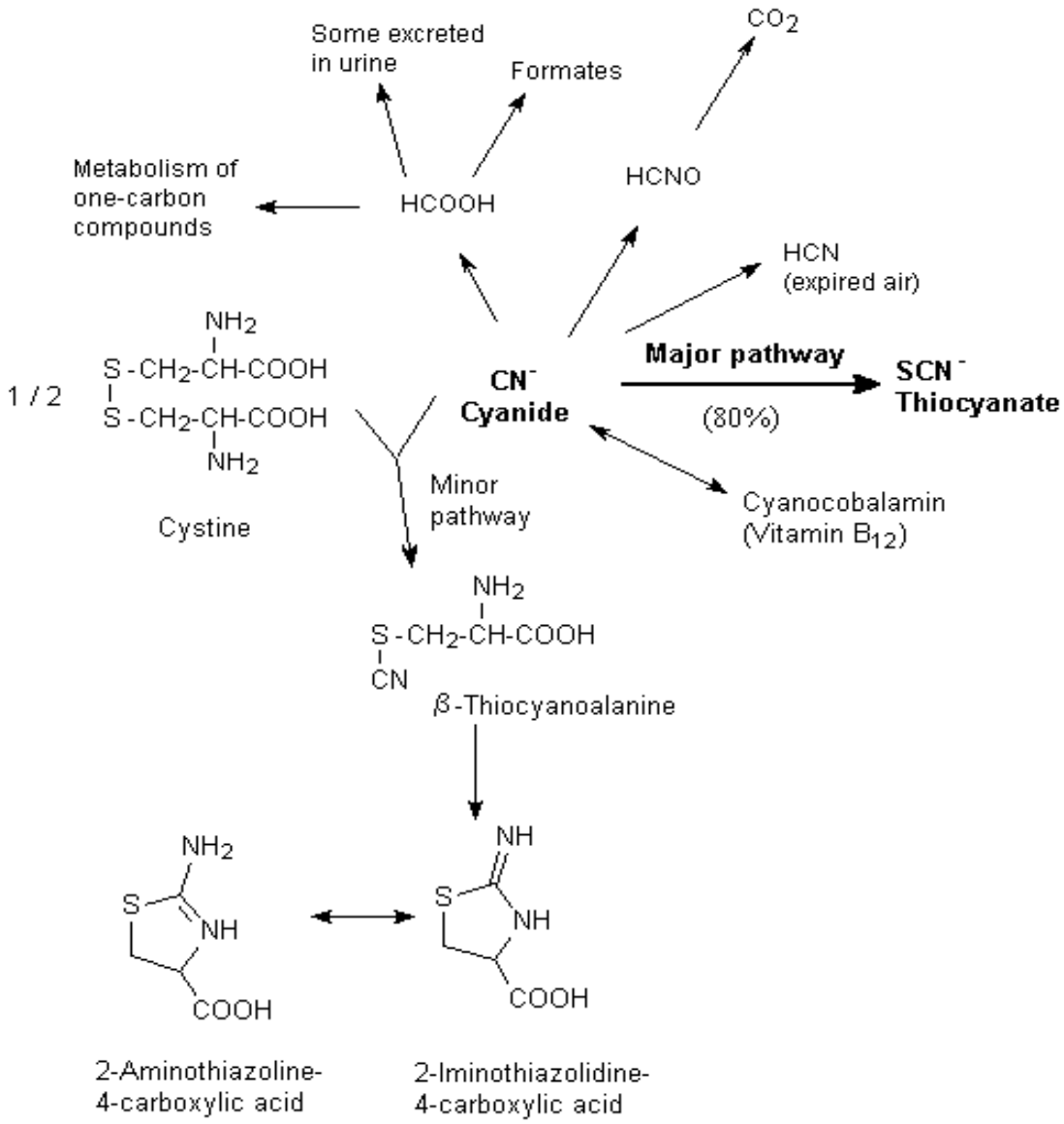


Fig. 1.0 Basic processes involved in the metabolism of cyanide (ATSDR, 1997)

fertile years (Underwood, 1998; Viteri, 1998).Severe deficiency of iodine occurs in mountainous areas and in frequently flooded areas, low lying interior planes where over the years, iodine has been washed out of the soil (Hetzel, 1996). These may have associated increased susceptibility to disease itself, a consequence of climate change.

Increased susceptibilities to communicable diseases, and those provoked by water or insect borne vectors are additional risks encountered by malnourished individuals. Much of what is known about the effects of these micronutrients on thyroid metabolism has been learned from studies involving single nutrient deficiencies (Ruz *et al.,* 1998). Nevertheless, two- and three-way nutrient interactions may have distinct manifestations in thyroid disorder or malfunction. With the notable exception of the iodine-selenium interaction, which has received much attention, few studies of two nutrient deficiencies have been reported. Migration of populations from rural to urban centers and the expansion of major metropolitan areas have had a significant and adverse impact on the quality of life of these citizens. Urbanization and the resultant burden on limited national resources is a major contributory factor to micronutrient deficiency and malnutrition (Iyengar and Nair, 2000). The major health concern with excess iodine ingestion is thyroid disorders, primarily hypothyroidism with or without iodine – induced goitre (Backer and Hollowell, 2000). A review of the human trials on the safety of iodine ingestion indicates that neither the maximum recommended dietary dose (2mg/day) nor the maximum recommended duration of use (3 weeks) has a firm basis. Experts suggest that much higher amounts are usually tolerated without problems (Braverman, 1994; Dunn, 1998). Other common deficiencies of micronutrients such as iron, selenium, and possibly zinc (plays a role in DNA and RNA as well as protein synthesis eg. thyroglobulin) may interact with iodine nutrition and thyroid function (Hess, 2010). Randomised controlled intervention trials in iodine and iron deficient populations have shown that providing iron along with iodine results in greater improvements in thyroid function and volume than providing iodine alone. Studies by Dillon and Milliez, (2000) on reproductive failure in women living in iodine deficient areas of West Africa, Dakar showed that reproductive failure was associated with low iodine status with severe iodine deficiency increasing the risk. Report from the study carried out by Beaufrere *et al.*, (2000) showed that the thyroid gland is very sensitive to iodine deficiency in newborns and infants because of its very low iodine content. Iodine deficiency during pregnancy increases the risk of neonatal transient hypothyroidism with a high recall rate in programs

of systematic screening for congenital hypothyroidism. Several randomized trials have examined the impact of iodine supplementation on the cognitive performance of children but their results are equivocal and methodological problems limit their interpretation (Huda *et al.,* 2001; Qian *et al.,* 2005). Despite numerous studies of the effect of selenium on iodine and thyroid metabolism in animals, most published randomised controlled intervention trials in human populations failed to confirm an impact of selenium supplementation on thyroid metabolism. Some evidence is available on the interactions between iodine and zinc metabolism (Hess, 2010).

Thyroid disruption may be caused by a variety of mechanisms, as different elements and chemicals interfere with the hypothalamic–pituitary–thyroid (H-P-T) axis at various levels. Mechanisms of action may involve the sodium–iodide symporter, thyroid peroxidase enzyme, receptors for thyroid hormones (THs) or TSH, transport proteins or cellular uptake mechanisms. The peripheral metabolism of the THs can be modulated through effects on iodothyronine deiodinases dependent on selenium or hepatic enzymes. Even small changes in thyroid homeostasis may adversely affect human health, and especially fetal neurological development may be vulnerable (Boas *et al.,* 2006). The roles of iron, zinc and copper in the thyroid gland are less well defined but sub – or supra optimal dietary intakes of these elements can adversely affect thyroid hormone metabolism (Arthur and Beckett, 1999); the recognition that autoimmunity is a major cause of thyroid dysfunction has led to the development of tests for thyroid antibodies; (Swain *et al.,* 2000) even though the etiology of autoimmune disease (AID) has remained unclear. Previous reports indicate that the alteration of balance between certain essential trace elements and toxicants has a strong role in the pathogenesis of AITD, thyroid antibody testing is not routinely available in developing countries; few studies have measured thyroid antibodies in Africans; the significance of thyroid autoimmunity in an African setting is thus unclear, hence the study to examine the relationship of essential and toxic trace elements to thyroid autoimmunity.

RATIONALE FOR STUDY

There is an increasing prevalence of thyroid disorders especially among women (Laurberg *et al.,* 2005; Ogbera *et al.,* 2007; Okosieme *et al.,* 2007). Brundtland, 2002 stated that over 2billion individuals are at risk of thyroid disorders worldwide. Certain trace elements (Cu, Zn, Fe, Se, I, As, Cd, Pb & Ni) and autoantibodies have

been implicated in thyroid dysfunction (Al-Sayer *et al.*, 2004). Despite several studies conducted in Nigeria on thyroid disorders (Famuyiwa & Bello 1990; Ojule, 1994; Ojule & Osotimehin, 1998, Okosieme *et al.*, 2007) data still remain scanty and limited on the interaction between essential and toxic trace elements, autoantibodies and hormonal profile in patients with thyroid disorders.

1.2. AIM OF THE STUDY

The major aim of this study is to examine the relationship and interaction between essential and toxic trace elements (Copper, Iron, Zinc, Selenium, Arsenic, Cadmium, Nickel, Lead, Iodine) levels and autoantibodies status of patients with thyroid disorders.

1.3 SPECIFIC OBJECTIVES OF THE STUDY:

1. To determine the plasma levels of the trace elements (Cu, Fe, Zn, Se, As, Cd, Ni, Pb) status and urinary iodine levels of patients with thyroid disorders.
2. To correlate the plasma levels of the trace elements (Cu, Fe, Zn, Se, As, Cd, Ni, Pb) and urinary iodine in patients with thyroid disorders.
3. To understand the possible interactions between trace elements (essential and toxic metals), autoantibodies and hormonal profiles in individuals with various forms of thyroid disorders.
4. To determine the plasma thyroid hormonal (Free and total Thyroxine, triiodothyronine - FT_4, FT_3, T_4, T_3, and thyroid stimulating hormone - TSH, autoantibodies: thyroglobulin antibodies and antigens (TgAb, TgAg), thyroid peroxidase antibodies (TPO Ab) and prevalence in patients with thyroid disorders.
5. To identify the possible contributions and effect of autoantibodies, trace elements (Cu, Fe, Zn, Se, As, Cd, Ni, Pb), urinary iodine, and hormonal interactions in simple non-toxic goitre, toxic nodular goitre, hyperthyroidism, hypothyroidism, thyroid cancers

1.4 Significance of Study

1. A comprehensive evaluation of the biochemical and biophysical indices in patients with thyroid disorders – hyperthyroidism, hypothyroidism, toxic goitres, thyroid cancers, simple multinodular goitre, may improve the currently limited understanding and assessment of thyroid dysfunction in Nigeria; shed new light on the understanding of

trace elements and other factors that best correlate with thyroid dysfunction and the need for early detection, management which will help to prevent irreversible damages. These may contribute or enhance prospective studies or clinical interventions as well as aid in the reduction of incidence of thyroid dysfunction.

2. The identification of the required trace element ratio as regards the specific trace elements and autoantibodies as well as their relationships will help establish their role as a causative/contributory factor in thyroid dysfunction. It may also stimulate research interest in other possible causes of unexplained thyroid dysfunction. Correlations among trace elements/toxicants and autoantibodies may reveal other unknown underlying factors

3. Furthermore, knowledge of the presence of autoantibodies and a reoccurrence of thyroid dysfunction may help explain the rise in thyroid disorders. Identification of the specific biochemical or specific trace elements status as well as auto antibodies will aid in the diagnosis and treatment of thyroid disorders.

4. The study will also enhance the understanding of the danger to human health of micronutrient deficiency in the face of nutritional inadequacy; thus, eliminating or reducing the morbidity and or mortality associated with these disorders. Increased foetal loss and IQ deficits in infants are associated with mothers who had undiagnosed or inadequately treated hypothyroidism during pregnancy (BTA, 2006).

1.5 Research Hypothesis:

Toxic and altered levels of essential trace elements and autoantibodies provoke autoimmune responses and interactions which may contribute or predispose individuals to thyroid disorders.

1.6 Null Hypothesis (H_1)

Selected trace elements and urinary iodine as well as autoantibody levels in patients with thyroid disorders are significantly different from subjects without thyroid disorders.

CHAPTER TWO

LITERATURE REVIEW

2.1 THE THYROID GLAND:

The thyroid gland is the largest organ specialized for endocrine function in the human body. Its function is to secrete a sufficient amount of thyroid hormones, primarily 3,5,3',5'-L- tetraiodothyronine thyroxine (T_4) and a lesser quantity of 3,5,3'-L-triiodothyronine (T_3) (Greenspan,1991). In addition, the thyroid gland secretes small amounts of biologically inactive and minute quantities of monoiodothyrosine (MIT) and diiodothyrosine (DIT) which are precursors of T_3 and T_4 (Whitley, 1989).

The secretory units of the thyroid gland are follicles, which consist of an outer layer of epithelial cells. These cells rest on a basement membrane and enclose an amorphous material called colloid (Whitley, 1989). Colloid is mainly composed of thyroglobulin (an iodinated glycoprotein) and small quantities of iodinated thyroalbumin. The follicles are embedded in stromal tissue, which contains blood vessels and autonomic nerve fibres. Increased activity of the gland is charactrised by a decrease in the quantity of colloid with subsequent reduction of follicular volume. The thyroid gland also contains another type of cell known as parafollicular or C-cells (Whitley, 1989). These cells have been shown to produce the polypeptide hormone calcitonin.The weight of the thyroid gland in the normal individual, as determined by ultrasonic examination, varies depending on dietary iodine intake, age and body weight, but in adults is approximately 10-20g (Greenspan, 1991) upward growth of the thyroid gland is limited by the attachment of the sternothroid muscle to the thyroid cartilage, however, posterior and downward growth is unhampered, so that thyroid enlargement, or goitre, will frequently extend posteriorly and inferiorly, or even substernally. The thyroid gland is unique among the endocrine glands for its dependence on an essential micronutrient, iodine, for normal hormone production (Boyages, 1993). The phylogenic association of thyroid gland and the gastrointestinal tract is evident in several functional respects (Williams, 2000). Thus the salivary and gastric glands, like the thyroid, are capable of concentrating iodide in their secretions, although iodide transport in these sites is not responsive to stimulation by TSH (thyroid stimulating Hormone).

The Hypothalamic-Pituitary-Thyroid Axis (HPTA) is the neuroendocrine system that regulates the production and secretion of thyroid hormones (Bishop *et al.*, 2000). The

secretion of the thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3) by the thyroid gland is stimulated by TSH (Earlson & Hershman, 1975). This peptide hormone exerts its effect by binding to a specific receptor on the thyroid cell membrane (Amir *et al*; 1973); binding of TSH activates the enzyme, adenylcyclase, in the thyroid cell, which coverts intracellular ATP to cyclic AMP. Cyclic AMP then initiates a series of biochemical events which ultimately results in stimulation of thyroid hormone production.Thyrotropin secretion, in turn is stimulated by thyrotropin-releasing hormone (TRH). In 1969, the structure of TRH was shown to be pyroglutamyl-histidyl prolinamide, and this tripeptide was synthesized by several groups (Folkers *et al.,* 1970). The TRH is produced by several hypothalamic nuclei and is secreted into the hypophyseal portal verous system which carries blood from the hypothalamus to the anterior pitiatary. In the pituitary gland, TRH interacts with cell membrane receptors on the thyrotroph, resulting sequentially in activation of adenyl cyclase, increased production of cyclic AMP, and finally enhanced secretion of TSH (Labrie *et al.,* 1972; Poirer *et al.,* 1972). Feedback control of TSH secretion appears to operate at both the pituitary and hypothalamic levels. At the pituitary level, low levels of circulating thyroid hormones result in enhanced TSH secretion, while elevated concentrations of thyroid hormone suppress TSH secretion (May and Donabedian, 1973).

2.1.1 Thyroid Hormone Synthesis and Release

The synthesis of T_4 and T_3 by the thyroid gland involves six major steps:

(1). Active transport of iodide (I^-) across the basement membrane into the thyroidal cell (i.e. trapping of iodine). Iodide (I^-) is transported across the basement membrane of the thyroid cell by an active energy – requiring process that is dependent upon Na^+ - K^+ ATPase. This active transport system allows the human thyroid gland (follicular cells) to maintain a concentration of free iodide 30 – 40 times that in plasma. The thyroiodide trap is markedly stimulated by TSH and by TSH receptor stimulating antibody (TSH – R Ab [stim] found in Graves' disease). It is saturable with large amounts of Iodide (I^-) and inhibited by ions such as ClO^-_4 (perchlorate), SCN^- (thiocyanate), NO_3^- (nitrate) and TcO_4- (pertechnetate). Once in the thyroid, iodide is oxidised to elemental iodine by the enzyme thyroid peroxidase (Saller, 1998). This iodide transport to the follicles is the rate-limiting step for protein iodination and hormone synthesis (Tietz, 1999). The oxidation and organic binding of iodide to thyroglobulin is blocked by thiourylenes, sulfonamides

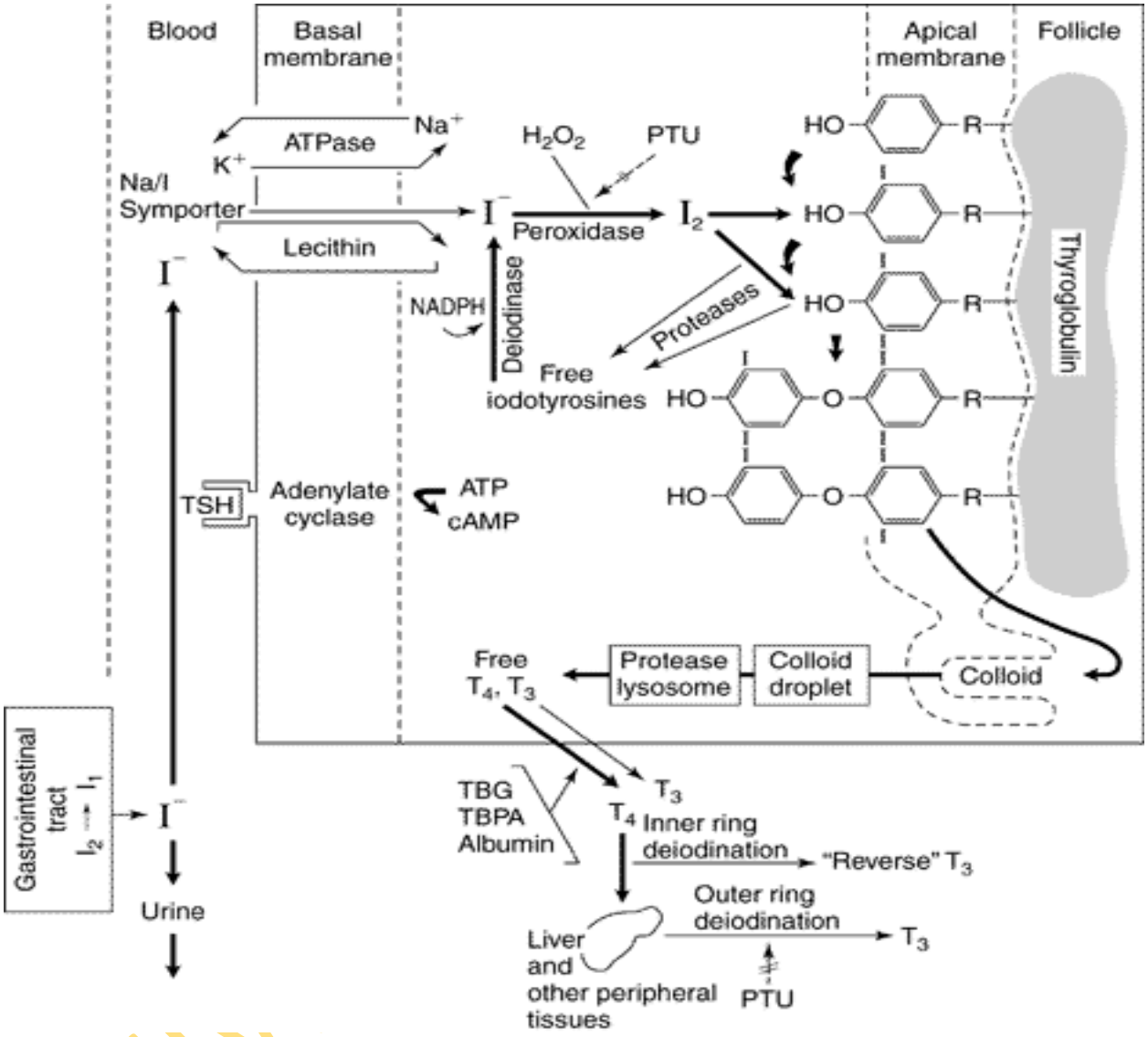


Fig. 2.0: The metabolism of iodine, emphasizing formation and secretion of the thyroid hormones

(modified from Bergers, Quinn JL: Thyroid function. In Tietz NW (ed): Fundamentals of Clinical Chemistry, 2nd ed, p. 585, Philadelphia, WB Saunders, 1976)

and high concentrations of iodine. Some of these inhibitors have clinical utility – Potassium perchlorate and potassium thiocyanate have been used to treat iodide – induced hyperthyroidism. Both discharge iodide (I^-) from the trap and prevent further I^- uptake. Potassium perchlorate has also been used clinically with ^{123}I to demonstrate organification defects in the thyroid gland; it will displace and allow the discharge of nonorganified iodide (I^-) from the iodide trap (Larsen *et al.*, 1998).

This ability of perchlorate to inhibit iodide transport allows its use in the so-called perchlorate – discharge test for detection of defects in the thyroid organic – binding mechanism, and uptake of the radio-active anion pertechnetate by the gland makes it suitable for thyroid imaging by radioscan.

(2.). Oxidation of iodide and iodination of Tyrosyl residues in thyroglobulin.

$$H_2O_2 + 2\,I^- + 2H^+ \xrightarrow{\text{Iodine Peroxidase}} 2 \text{ active I} + 2\,H_2O$$

(3). Coupling of Iodotyrosol Residues in Thyroglobulin.

(4). Proteolysis of Thyroglobulin and Thyroid Hormone secretion

(5). Deiodination of iodotyrosines within the thyroid cell, with conservation and reuse of the liberated iodide.

(6). Intrathyroidal 5'- deiodination of T_4 to T_3

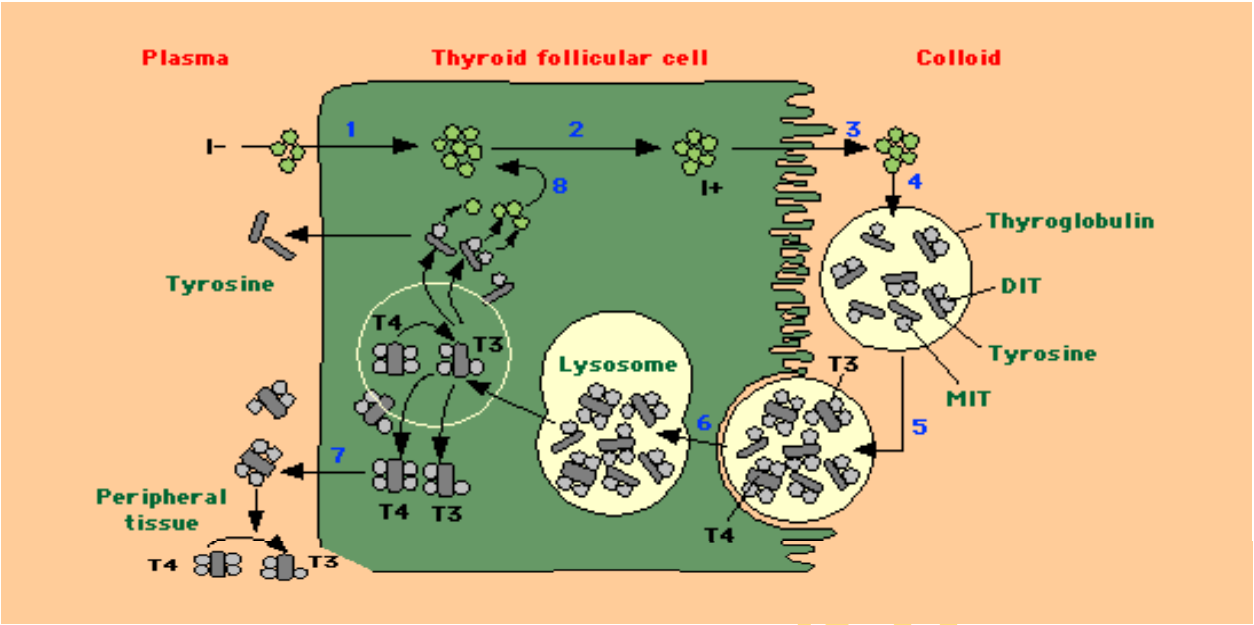


Fig. 2.1: Summary of Thyroid Hormone Biosynthesis (with emphasis on the autoantibody targets) (Modified from American Medicine, Scientiific American, New York, 1995)

Autoantibody targets in thyroid hormone biosynthesis include:

1. Location of Na – I symporter,

4. Thyroglobulin,

6. TPO or microsomal antibody Ab, *TSH receptor

2.2 NUTRIENTS

They can be described as organic and inorganic substances required by the body for the maintenance of the physicochemical and metabolic processes essential to life. They may be broadly classified as macro (major) or micro (trace) nutrients or minerals. The macro- nutrients are required in amounts greater than 100 mg/dl and the micro-nutrients are required in amounts less than 100 mg/dl (Murray *et al.*, 2000). Every form of living matter requires these inorganic elements for their normal life processes (Hays and Swenson, 1985; Ozcan, 2003). The macro-minerals include calcium, phosphorus, sodium and chloride, while the micro-elements include iron, copper, cobalt, potassium, magnesium, iodine, zinc, manganese, molybdenum, fluoride, chromium, selenium and sulfur (Eruvbetine, 2003). The third categories is the ultra trace elements or nutrients and include boron, silicon, arsenic and nickel which have been found in animals and are believed to be essential for these animals. Evidence for requirements and essentialness of others like cadmium, lead, tin, lithium and vanadium is weak (Albion Research Notes, 1996). The mineral elements are separate entities from the other essential nutrients like proteins, fats, carbohydrates, and vitamins.

2.2.1 MICRONUTRIENS

Micronutrients are the collective term applied to essential vitamins and minerals (WHO, 2003) and comprise of organic (vitamins) and inorganic (dietary minerals) which although required by the body in small quantities, are vital to health, for disease prevention and wellbeing. Vitamins are organic compounds that are required in minute quantities to sustain life and prevent disease (Kraemer *et al.*, 2010). They play a central part in metabolism and in the maintenance of tissue function (Shenkin, 2006). Minerals, as with vitamins, are indispensible (essential) in small amounts to ensure that the body grows, develop, and stay healthy; they are found in a wide range of foods. The body uses minerals to perform many different functions — from building blood and strong bones to transmitting nerve impulses and maintaining a normal heartbeat. Many micronutrients are involved in specific biochemical pathways and have dedicated functions in the body, but they are also interconnected in complex metabolic networks such as oxidative-reductive, inflammatory pathways and hormonal regulation, in which the overarching function is to optimise health (van Ommen *et al.*, 2008). Precise regulation of these processes is required to maintain the balance between optimal health and early onset of (diet-related)

disease. Micronutrients act as cofactors in metabolic pathways (riboflavin, vitamin B_{12}), and also in enzymes such as superoxide dismutase and glutathione peroxidase (GP_X), that are involved in defense mechanisms (Se, Zn, Cu, Fe). They have roles as chemical antioxidants (vitamins C, E), and some essential fatty acids are associated with protection against inflammation (EPA, DHA, ALA). Other micronutrients are involved in hormonal regulation, including iodine, which is essential for the biosynthesis of thyroxine (T_4) involved in metabolic regulation.

Inorganic micronutrients can be classified into two groups:

a) Bulk minerals: C, H, O, N, S, Ca, P, K, Na, Cl and

b) Essential trace elements: Cu, Fe, Mg, Zn, Mn, Mo, Co, Cr, I, F, Se.

Micronutrient deficiencies have been associated with a series of pathologies such as hypertension (Chiplonkar *et al.*, 2004), colon and prostate cancer (Gross, 2005) breast cancer, cardiovascular diseases (CVD) and osteoporosis. Yet, in many cases, our current knowledge does not allow us to make a causal link with the deficiency, although it may be described as a predisposing risk for the onset of the disease. It is largely accepted that a disturbance of metabolic homeostasis, oxidation and/or inflammation is a trigger for these diseases, and data linking modulation of these processes in (pre-) diseased states to micronutrients have been published (Devaraj *et al.*, 2007; 2008)

2.2.2 MICRONUTRIENT INTERACTIONS:

The interaction of micronutrients can be viewed in various ways. Deficiencies of the various micronutrients under discussion interact according to their geographical setting and according to how the micronutrients are metabolized; example iron and iodine deficiencies often occur in countries in which poverty limits dietary sources and in which geography limits the composition of food that normally would contain these micronutrients (Levin *et al.*, 1993). Other components in foods may inhibit the use of dietary micronutrients, such as phytates in some plants, which inhibit iron absorption (Levin *et al.*, 1993). Iron deficiency can have adverse effects on the thyroid metabolism by influencing the response to supplemental iodine in the areas of endemic goitre. Zimmermann *et al.*, (2000) in their study on the response of goitrous children with iron deficiency anemia to oral iodine supplementation showed that the therapeutic response to oral iodine was impaired in these children, suggesting that iron supplementation may

improve efficacy of this intervention. Another study showed that the severe selenium deficiency partially blunts the thyroid response to iodine supplementation. A point worthy of note was the variation in the female results only, obtained for the values of FT_4 (plasma free thyroxine) which was thought to be a sex-linked hormonal response to concomitant selenium and iodine deficiencies (Zagrodzki *et al.,* 2000). Selenium-dependent enzymes also have several modifying effects on the immune system (Gartner *et al.,* 2002). Some principal essential and non essential trace elements are going to be discussed in relation to their nutritional bioavailability and their importance to health in relation to disorders of the thyroid gland.

In this context, the proposed study will focus on trace element and autoantibody interactions between thyroid function/disorders. It will also compare the various levels of trace element on the patients and in normal subjects. It is envisaged that the results will offer explanations to some consequences of deficiencies in micronutrients and offer suggestions to eradicate this problem of thyroid disorder.

2.3 TRACE ELEMENTS

They are dietary minerals needed by the human body in very small quantities (generally less than 100ng per day) as opposed to macrominerals which are required in larger quantities (Wikipedia encyclopedia). Milne, 1999 described trace elements as elements which occur in human and animal tissues in milligram per kilogram amount or less. Many vitamins or vitamin metabolites are required to play an active part within complex biochemical reactions for example, riboflavin (FMN, FAD) and niacin (NAD, NADP) in energy metabolism, folic acid in one-carbon transfer (Powers, 2003; van Ommen *et al.,* 2008). Much research has been carried out concerning the role of minerals in the body, but in many cases, difficulties in investigating their individual effects has been expressed because, intake is often and vegetables contain several minerals (Sandstead *et al.,* 2000). Micro minerals from inorganic sources such as heavy metals cannot be used by the body as they tend to build up in the tissues. Trace elements can either be essential or non essential. Essential trace elements are those that are required by an organism to maintain normal complex metabolism (e.g. metallo proteins) which are required in enzymatic activities and can play structural role in connective tissues or cell membranes. Nonessential trace elements are considered toxic and are not required for physiologic

processes. Without essential elements the organism cannot complete its life cycle or achieve normal healthy growth.

Heavy metals are natural constituents of the earth's crust, but indiscriminate human activities have drastically altered their geochemical cycles and biochemical balance (Joseph *et al.*, 2012); they constitute a heterogeneous group of elements widely varied in their chemical properties and biological functions. Heavy metals are chemical elements with a specific gravity (Lide, 1992) and occur in environment from natural processes and anthropogenic activities. (Connell *et al.*, 1999; Franca *et al.*, 2005) Heavy metal such as iron, chromium, nickel commonly known as trace elements play an important role in biological systems, yet they may become highly toxic when present in high concentrations (Ibok *et al.*, 1989). Cadmium, mercury and lead are biologically non-essential, but are important metals for industrial applications. Prolonged exposure to heavy metals such as lead can cause deleterious health effects in plants, fishes and humans.

2.3.1 ESSENTIAL ELEMENTS:

An element is considered essential when a deficient intake produces an impairment of function, and physiological amounts of only that element prevent or alleviate the impairment (Milne, 1999). To be considered essential, a trace element must fulfill the criteria postulated by Cotzias (1967); its concentration in the species must be relatively constant and withdrawal produces similar structural and physiological abnormalities in different species, which are prevented or reversed by addition of the element.

**Essential to man: Chromium, Cobalt, Copper, Fluorine, Iodine, Iron, Manganese, Molybdenum, Selenium, and Zinc

**Probably essential to man: Arsenic, Lithium, Nickel, Silicon and Vanadium

**Some evidence of essentiality in some animals: Bromine, Cadmium, Lead, Tin.

2.3.2 ESSENTIAL TRACE ELEMENTS AND THE THYROID GLAND

2.4 IODINE

Iodine is an important trace element that is essential for the maintenance of normal thyroid function where it is required for the synthesis of the thyroid hormones, L-triiodothyronine (T_3) and L-thyroxine (T_4) (also called 3,5,3', 5'- tetraiodothyronine).

The major role of iodine in nutrition arises from the important part played by the thyroid hormones in the growth and development of human and animals (WHO, 1996). Most of the iodine used in the synthesis of the calorigenic thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3) is obtained from dietary source (Vagenakis and Braverman, 1975). The T_3 and T_4 are responsible for regulating cellular oxidation and hence have a key role in influencing cellular metabolism and metabolic rate.The recommended daily intake (RDI) for iodine varies for individuals. The RDI for adults' ranges from 100-150 µg/day, with intakes of 150-290 µg/day recommended for pregnant and lactating women. Intakes of 90 µg/day are recommended for young children.

In stable iodine intake situation, the amount of iodine excreted in the urine correlates well with the iodine intake and serves as an estimate of iodine intake (Vermiglio *et al.*, 1992). The stomach and the upper small intestine rapidly absorb iodine as either one or two chemical forms: iodine or iodate. Iodate is reduced to iodide, which is transported in the blood to the thyroid gland, where an active transport mechanism pumps it into the thyroid cell. About 60ug of iodine needs to be trapped per day to maintain an adequate thyroxine level; the efficiency of the trapping mechanism is regulated with the help of TSH depending on the availability of iodine and help of TSH depending on the availability of iodine and the gland's activity. In the gland, iodine is oxidized to iodine, which is bound to tyrosine to form mono-and diioodotyrosine. These are coupled to form triiodothymine (T_3) and thyroxine (T_4) in the thyroid epithelial cells. Thyroxin is then stored in colloid follicles bound to thyroglobulin. When needed, the TSH will stimulate the proteolysis of thyroglobulin in the thyroid cells to release thyroxine into the blood stream (Hetzel, 1989; Passmore and Eastwood, 1986)

A feedback system regulates iodine metabolism through the hypothalamus. The level of thyroxine (T_4) in the blood regulates metabolism. Decreasing T_4 levels lead to increasing TSH levels, which serves to increase thyroid, iodine uptake as well as the production and release of more T_4 and T_3 into the bloodstream. A high TSH levels, the thyroid will prefentially produce the biological more active T_3. Conversely, as thyroid hormone levels rise, TSH secretion falls. Sustained high TSH levels stimulate an increase in the size and number of follicular cells, an increase in vascularization and consequently thyroid hypertrophy which leads to better iodine capture. At some stage of hypertrophy the thyroid is regarded a "goitre". In addition, persistent stimulation may also cause the formation of thyroid nodules (Hetzel, 1989). Thyroid hormones regulate the metabolism

of target organs by entering the cells of the peripheral tissues and binding to the nuclear chromation via a thyroid hormone receptor protein, which in turn affects transcription (Baniahmad *et al.,* 1992). The T_3 is much more potent than T_4. Thyroxine is therefore generally transformed to T_3 prior to biological action with the help of the selenium containing enzyme 5'- deiodinase (Kohrle, 1999). This explains the links between iodine and selenium deficiency (Untoro *et al*, 1999). A study by Zimmerman *et al.,* 2000 suggests that concurrent iron deficiency anaemia impairs the therapeutic repose to iodine supplementation, possibly mediated via decreases T_4 and T_3 conversion or through decreased thyroperoxidase activity impairing iodide organification.

Absorption: Inorganic iodine is >97% absorbed from the gastrointestinal tract, generally as iodide. Although some absorption occurs in the stomach, the small intestine appears to be the principal site of absorption in both humans and rats (Small *et al.*, 1961). The mechanism by which iodide is transported across the intestinal epithelium is not known. Gastrointestinal absorption appears to be similar in children, adolescents and adults, although absorption in infants may be lower than in children and adults (ATSDR, 2001).

Distribution: Once absorbed, iodide enters the circulation and is distributed throughout the extracellular fluid where it is taken up by those tissues with specialized transport mechanisms for iodide (Cavalieri, 1980). The human body contains about 10 – 15µg iodine in total, the majority of which (>90 %) is stored by the thyroid gland (Cavalieri, 1997). The concentration of iodine in serum is about 50 – 100 µg/L under normal circumstances, with about 5% being in the inorganic form as iodide and the remaining 95% consisting of various organic forms of iodine, principally protein complexes of the thyroid hormones. Other tissues that accumulate iodide include the salivary glands, gastric mucosa, choroid plexus, mammary glands, placenta, and sweat glands. The tissue distribution of iodide and organic iodine are very different and are interrelated by metabolic pathways that lead to the iodination and de-iodination of proteins and thyroid hormones.

The uptake of iodide by the thyroid gland is controlled by the thyroid-stimulating hormone (TSH), which is secreted from the anterior lobe of the pituitary gland. In addition to stimulating iodide transport from the blood into thyroid cells, TSH is also responsible for stimulating the oxidation of iodide to iodine, and iodine binding to tyrosine. Iodide taken up by the thyroid gland is used for the production of the thyroid

hormones, which are stored in the gland. Approximately 90% of the thyroid iodine content is in the organic form and includes iodinated tyrosine residues comprising the thyroid hormones T_4 and T_3, and their various synthesis intermediates and degradation products. Once requirements for thyroid hormone synthesis have been met, the thyroid does not accumulate more iodine and any excess is excreted in the urine (Bender and Bender, 1997). Iodide uptake into the thyroid gland is highly sensitive to iodide intake. At low intakes representing iodine deficiency, uptake of iodide into the thyroid gland is increased (Delange and Ermans, 1996). At very high intakes, iodide uptake into the thyroid gland decreases, primarily as a result of decreased iodothyronine synthesis (the Wolff-Chaikoff effect) and iodide transport into the gland (Nagataki & Yokoyama, 1996; Saller, 1998).

Metabolism: (Please see figure 2.0)

Excretion: All absorbed iodine is excreted primarily in the urine and faeces, but is also excreted in breast milk, exhaled air, sweat and tears (Cavalieri, 1997). Urinary excretion normally accounts for 97% of the elimination of absorbed iodine, while faecal excretion accounts for about 1-2% (Larsen *et al.*, 1998). The fraction of the absorbed iodide dose excreted in breast milk varies with functional status of the thyroid gland. A larger fraction of the absorbed dose is excreted in breast milk in the hypothyroid state compared to the hyperthyroid state. In the hypothyroid state, uptake of absorbed iodide into the thyroid gland is depressed, resulting in greater availability of the absorbed iodide for distribution to the mammary gland and breast milk.

Bioavailability: Some nutrients are known to compete with each other for absorption and bioavailability when they are consumed together in the same meal. There is no literature to suggest that iodine inhibits the bioavailability of any other nutrient; however the absorption of dietary iodine can be reduced by the calcium, magnesium and iron content in food and water (SCF, 2002). Goitrogens can affect the utilisation of dietary iodine in the body by interfering with the biosynthesis of the hormones T_3 and T_4. Heat from cooking will inactivate most of the goitrogens present in these vegetables. Of particular importance for Application is that iodine bioavailability can also be compromised by the consumption of soy flour. The digestive by-products of soy flour block the enterohepatic circulation of thyroxine (T_4) (ESCF, 2002), and therefore soy-based FSFYC may

inadvertently have the potential to affect iodine status. However, this effect is expected to be negligible as it is only the exclusive use of soy products (e.g. feeding of soy-based formulas to infants, who cannot receive nutrition from other sources) that has been known to adversely impact on iodine status, and not supplementary feeding as occurs with FSFYC.

2.4.1 Iodine Dietary Requirements: The long-term solution for the sustainable elimination of iodine deficiency would more likely take place through increasing the iodine content of the general food supply. Up to the early 1960s, the correction of IDD was almost exclusively focused on the administration of iodine in the form of solutions of potassium iodide or Lugol's solution. In spite of several effective salt iodization programmes in the developed world in the early 1920s, large preventive programmes were virtually untried in the developing world. Pilot studies using iodized oil as a source of long-lasting iodine supplementation at a population level were implemented in New Guinea in the early 1950s (McCullagh, 1963). Although there had been iodization trials using drinking water, bread and sweets, the most promising vehicle was clearly common table salt. By 1991 universal salt iodization, i.e., all salt for human and animal consumption, was identified as the preferred means of reaching populations, including those consuming salt produced by small-scale artisanal saltworks. Thus the focus shifted from treatment of cases to working with the salt industry to upgrade technologies and management. The application of salt iodization technology to developing country settings has progressed enormously during the past five years. Comprehensive technical guides are available (Mannar and Dunn, 1995; Sullivan *et al.*, 1995). Current recommendations made by WHO for dietary intake of iodine are shown in Table (Recommended Dietary Intakes of Iodine for Populations). These are slightly higher for three vulnerable groups (young infants, pregnant and breastfeeding women) than previous recommendations made in the USA (NAS, 1989). Delange (1993) has since recommended intakes of 90 µg iodine per day from birth to six years of age. The prevalence of goitre gives an idea of the past history of iodine nutrition at the population level.

2.4.2 Iodine and Thyroid Function

Nutritional iodine status is an important factor associated with thyroid dysfunction and thyroid autoimmunity (Papanastasiou *et al.*, 2007). The primary effects of excessive iodine ingestion are on the thyroid gland, the regulation of thyroid hormone production

and secretion. Adverse effects on the pituitary and adrenal glands are secondary to disorders of the thyroid gland. Excess iodine can result in goitre, hypothyroidism (with or without goitre), or hyperthyroidism (thyrotoxicosis). The effect produced depends on the current and previous iodine status of the individual and any current or previous thyroid dysfunction (WHO, 1989). For example, individuals exposed to low levels of iodine early in life may be prone to the development of iodine-induced hyperthyroidism if iodine exposure increases later in life. Those with underlying thyroid disease also respond more to increased iodine intake, and it also appears that females are more likely to respond to excess iodine than males.

2.4.3 Iodine and Thyroid malignancy

Several large-scale epidemiological studies have examined the relationship between iodine intake and thyroid cancer. The results of these studies suggest that an increased iodine intake may be a risk factor for thyroid cancer in certain populations, namely, populations residing in iodine deficient, endemic goitre regions (Franceschi, 1998; Franceschi & Dal Maso 1999). Not all of these studies have found an increased risk of cancer; however, a recurrent observation is an apparent shift in the histopathology towards a higher prevalence of papillary cancers, relative to follicular cancers, after increased iodine intake in otherwise iodine-deficient populations (Petterson *et al.,* 1991; 1996; Bakiri *et al.,* 1998). Two studies in particular found a significant excess of thyroid gland cancer in populations from endemic goitre regions whose diets had been supplemented to achieve approximate iodine intakes of 3.5 µg/kg bw/day (Harach & Williams 1995; Bacher-Stier *et al.,* 1997).

2.4.4 Iodine Poisoning/Toxicity: A large number of human experimental, clinical, and epidemiological studies on the effects of excess iodine on human health have been reported (WHO 1989; ATSDR 2001). The Joint FAO/WHO Expert Committee on Food Additives (JECFA)) and the Agency for Toxic Substances and Disease Registry concluded that there are three potential types of adverse response to excess iodine. The first is disturbance of thyroid activity, which may alter the size of the gland and/or affect the production of thyroid hormones (ATSDR, 2001). There is also evidence to indicate that iodine (or the lack of it) may alter the pattern of thyroid malignancy.

Table 2.2: Recommended Dietary Intakes of Iodine for Populations

Age range/state	Intake µg/d (WHO, 1996)	RDA µg/d(NAS, 1989)	
0-12 months	50	0-6 mths 6-12 mths	40 50
1-6 years	90	1-6 yr	70-90
7-12 years	120	7-10 yr	120
12 years to (and through) adulthood	150	150	
Pregnancy	200	175	

Source: WHO (1996b,) and NAS (1969,)

2.4.4.1 The toxic effects associated with stable iodine

Sources - The oceans are considered to be the most importance source of natural iodine. Iodine in seawater enters the air via aerosols or as a gas and from there is deposited onto soil, surface water and vegetation. The iodine content of foods is thus both reflective of background levels in the environment as well as processing technology and manufacturing practices. For example, the high iodine content of milk and dairy products has been attributed to the use of iodine-containing supplements in feed for dairy cattle, iodophore-based medications, teat dips and udder washes as well as iodophors used as sanitising agents in dairy processing establishments. The use of iodophors by the dairy industry has however become less commonplace, resulting in milk becoming a less important source of dietary iodine (Eastman, 1999). In addition to dietary sources, various mineral supplements and medical preparations can further increase iodine intake to a significant extent (WHO, 1989).

2.4.5 Safe Limit for Oral Iodine Intake

A number of safe intake levels have been recommended as a result of reviews on the toxicity of excess iodine. The highest level of intake that has been found to be safe for the majority of the population is about 1000 µg iodine/day. This level was used by JECFA to establish a PTDI for iodine of 17 µg/kg bw from all sources (WHO 1989). Individuals with thyroid disorders or a long history of iodine deficiency, however, may respond adversely at levels of intake below the PTDI.

2.5 URINARY IODINE

The adequacy of dietary iodine is usually determined by the measurement of urinary excretion of iodine Lee *et al*, 1999. In the context of initiatives to achieve universal salt iodization, a number of population-based studies have addressed the question of which indicators best reflect improvement in iodine status and thyroid function (Buchinger *et al.*, 1997; Pardede *et al.*, 1998). Although thyroid stimulating hormone (TSH) (Sandell & Kolthoff, 1937) is widely used to screen neonates for congenital hypothyroidism, there have been doubts about its specificity in older children and adults when assessing hypothyroidism induced by iodine deficiency (WHO/UNICEF/ICCIDD, 1994). Several studies have shown that although urinary iodine concentrations and thyroid volumes were indicative of iodine deficiency in the populations studied both serum TSH and free thyroxine (FT_4) concentrations were within normal ranges (Benmiloud *et al.*, 1994; Pardede *et al.*, 1998). Thyroid volume, serum

thyroglobulin (Tg) concentration and urinary iodine concentration have all been suggested as useful indicators for measuring improvement in iodine status after iodine prophylaxis. However, all three have their own characteristics and limitations. Normal ranges for thyroid volume have been established (Delange *et al.*, 1997; WHO & ICCIDD, 1997), but this indicator reflects longstanding hypothyroidism and does not respond rapidly to changes in iodine status. Serum Tg concentration is thought to respond quickly to stimulation of the thyroid, increasing when iodine supply to the thyroid is depleted and returning to normal levels when the supply is sufficient. However, Tg immunoassays show large interlaboratory and interassay variability, which makes it difficult to establish a universal normal range and cut-off points for distinguishing between different degrees of iodine deficiency (Feldt-Rasmussen 1996; Feldt-Rasmussen *et al.*, 1996). Urinary iodine concentration is not a direct measure of thyroid function, but reflects recent iodine intake and thyroid hormone catabolism. Thus, population groups, even if currently found to be in the mildly deficient to normal range of urinary iodine concentration, may still experience serious functional consequences of iodine deficiency in the preceding period. The value 100µg/L, recommended as the minimal desirable population median, was chosen because 100 µg corresponds to the requirements for daily synthesis of thyroid hormones. It also corresponds to the level below which iodine stores in the thyroid start to decrease. Monitoring urinary iodine is the best way to assess current access of populations to iodine.

Iodine in urine generally occurs as the iodide ion (I^-). While iodine may be ingested in food or water in a variety of chemical forms, most of it is broken down to iodide in the gut and absorbed into the blood stream in that form. Virtually all of the iodide in the blood is either taken up by the thyroid and converted into thyroid hormone, or is excreted in the urine. The iodide trapped by the thyroid, which may approach 100% of ingested iodine in areas of iodine deficiency, is converted into the thyroid hormones (thyroxine and triiodothyronine) and secreted as hormone into the circulation. In target tissues (principally liver, kidney, muscle, and developing brain), iodine is removed from the thyroid hormones and returned to the circulation for eventual excretion by the kidney. Iodine may also escape the body in faeces and in breast milk. However, over 90% usually comes out in the urine, presumably as iodide. For example, thiocyanate in the urine can accelerate the Sandell-Kolthoff reaction, thus artifactually elevating the apparent iodide value. Thiocyanate is derived from the ingestion of cassava, which is consumed in many

parts of the world including those with iodine deficiency, and its interference with the Sandell-Kolthoff reaction in undigested urine can introduce serious errors in iodine estimation. Cigarette smoking is another frequent source of thiocyanate; heavy smokers (10 or more cigarettes per day) have urinary thiocyanate levels as high as those of cassava eaters. Other interfering substances have not been as well characterized, but most observers have noted that failure to incorporate a digestion or ashing step can artifactually raise or lower the apparent iodine concentration in the Sandell-Kolthoff reaction, presumably by failing to remove such substances. From these considerations, the safest course at present is to include an ashing or digestion step prior to colorimetric reaction for urinary iodine. Other methods have occasionally been employed for urinary iodine determination, but they are generally not practical for survey purposes. Ion specific electrodes can determine iodide in urine, but have been more difficult to apply. Neutron activation has also been used, as has gas liquid chromatography. While these approaches have specific use for research purposes, they do not as yet offer the likelihood of replacing simple methods based on the Sandell-Kolthoff reaction. An adequate dietary intake of iodine is required for normal thyroid gland hormone production and to maintain a euthyroid state. It follows therefore that the measurement of iodine intake from foodstuffs or medications has clinical relevance. In the clinical laboratory, iodine measurements are used primarily for epidemiological studies or for research (Hollowell *et al.*, 1998). To date, the major application of iodine analysis is to assess the dietary iodine intake of a given population (Delange *et al.*, 1995; Hollowell *et al.*, 1998). This is an issue of considerable importance, since it has been estimated that iodine deficiency disorders (IDD) potentially affect about 2.2 billion people throughout the world. As the majority of ingested iodine is excreted in the urine, the measurement of urinary iodine excretion (UI) provides an accurate approximation of dietary iodine intake (Dunn, 1998). In most circumstances the determination of UI provides little useful information on the long-term iodine status of an individual, since the results obtained merely reflect recent dietary iodine intake. However, measuring UI in a representative cohort of individuals from a specific population provides a useful index of the iodine level endemic to that region (Dunn, 1998; Knudsen *et al.*, 2000). Besides estimating the UI concentration in people, other applications of iodine measurements include determining iodine in milk, food products and drinking water (Aumont and Tressol, 1986; Unak *et al.*, 1999).

2.6 Selenium Biochemistry

More than 25 selenoproteins have been identified in human biochemistry (Lyn, 2001). The functions of selenium are believed to be carried out by the selenoproteins, in which selenium is specifically incorporated as the aminoacid selenocysteine (Behne & Kyriakopoulos, 2001). In addition to incorporation as selenocysteine, selenium can replace sulfur in methionine, forming selenomethionine. This compound can be incorporated non-specifically into proteins in place of methionine (Lyn, 2004). Finally, selenium can be tightly bound to certain proteins, known as selenium binding proteins to distinguish them from true selenoproteins (Behne & Kyriakopoulos, 2001).

2.6.1. Interactions between Selenium and Iodine: Selenium and iodine are two minerals which are critically important in the proper functioning of the thyroid. While the importance of iodine has been known a long time, the importance of selenium has only been discovered and explored since 1990. Much research is presently being conducted on the functions of these two minerals in thyroid function and it is becoming clear that there is an interaction between the two. Iodine has a seemingly simple role in the thyroid – it is incorporated into the thyroid hormone molecule. Iodine status is also influenced by selenium intakes. Although iodine is essential for the synthesis of thyroid hormones, selenium-dependent enzymes (iodothyronine deiodinases) are also required for the conversion of T_4 to the biologically active thyroid hormone triiodothyronine (T_3) (Goyens, 1987; Vanderpas, 1990). Selenium deficiency can thus exacerbate the thyroid complications of iodine toxicity, as the uptake of iodide by the thyroid gland increases in compensation to a reduced T_3 production (ATDSR, 2001). The potential for selenium deficiency to exacerbate iodine deficiency is of importance, as it could impact on the iodine status of young children (Thompson, 2004). Selenium deficiency results in diminution of selenium-containing antioxidative enzymes such as glutathione peroxidase, deiodinases and thioredoxine reductases (Birringer *et al.*, 2002; Zimmermann & Kohrle, 2002), leading to increased levels of reactive oxygen species. There is also some evidence that iodide itself may act as an antioxidant (Winkler *et al.*, 2000). Cretinism or myxeodematous cretinism which results from a severe iodine deficiency in pregnancy is also influenced by a selenium deficiency. Selenium is a cofactor for type 1 deiodinase enzymes which convert T_4, the thyroid prohormone into T_3, the cellularly active hormone and also convert T_3 intoT_2, thereby degrading it. It is also critical for the formation of

glutathione peroxidase (GSHPx or GPx), which protects the thyroid from oxidative stress (Zagrodzki *et al*., 2000). As part of GPx, selenium prevents lipids and fats from being peroxidized; this can be seen on the skin as 'age spots' or 'liver spots' (autopsies show that skin 'liver spots' are accompanied by similar spots of peroxidized fats in the liver). Therefore selenium protects all of the cellular membranes, which are made up of fats, from peroxidation. Peroxidation of cellular membranes reduces the ability of the membrane to pass nutrients including minerals and vitamins, so selenium deficiency is the first step towards developing the many problems caused by nutrient deficiencies. Selenium deficiency is associated with AITD perhaps as a result of increased inflammatory activity arising from decreased activity of selenium containing antioxidative enzymes such as glutathione peroxidase (Zimmermann & Kohrle, 2002), whereas increasing dietary selenium or administration of selenomethionine have also been reported to diminish TPO antibody levels (Gartner *et al.*, 2002; Duntas *et al.*, 2003). Selenium, an important antioxidant in the thyroid and involved in the metabolism of iodine-containing thyroid hormones, may play an interactive role in the development of these thyroid irregularities, and in turn, cardiovascular disease (Hoption Cann, 2006). Selenium is a cofactor for type 1 deiodinase, which converts T_4 to T_3. It is also critical for formation of glutathione peroxidase, which protects the thyroid from oxidative stress.

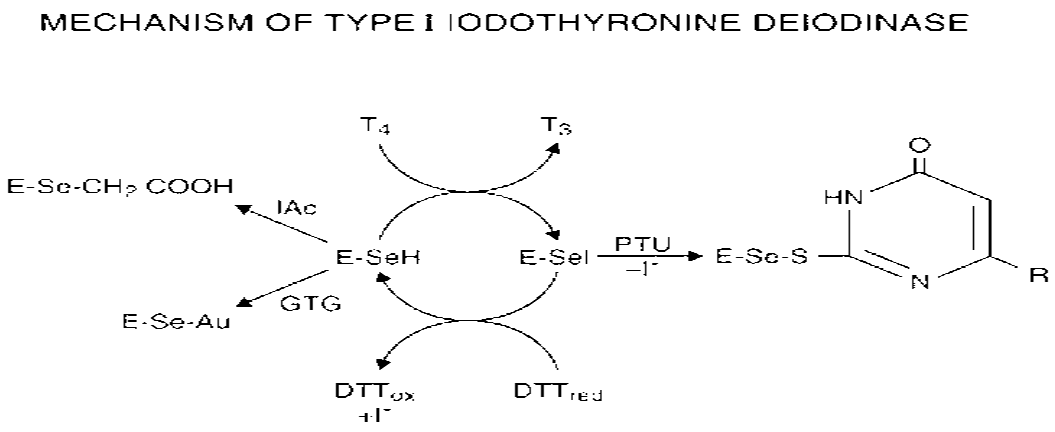


Fig. 2.2 Catalytic mechanism of Type 1 Iodothyronine deiodinase and inhibition by PTU, IAc and GTG) Selenocysteine (Sec)

2.6.2 Biochemical Functions of Selenium and Thyroid Hormone Metabolism

In addition, thyroidal selenium plays an important role as a component of glutathione peroxidase that detoxifies H_2O_2 as an active cofactor for iodination of thyroglobulin during synthesis of T_4. Thus, glutathione peroxidase being a synthesis regulator also protects organelles from H_2O_2 surplus (Aaseth, 2009). Selenium deficiency may be an active factor in the pathogenesis of endemic cretinism. The known metabolic role for selenium in mammals was as a component of the enzymes glutathione peroxidase which, together with vitamin E, catalase and superoxide dismutase, is a component of one of the antioxidant defence systems of the body (Combs and Combs, 1986). Several different selenium-containing enzymes have been described in microproteins other than glutathione peroxidase, but yet to be discovered in higher animals (Lyn, 2004). Selenium is retained and deiodinase expression is maintained at almost normal levels in the thyroid gland, the brain and several other endocrine tissues during selenium deficiency, thus guaranteeing adequate local and systemic levels of the active thyroid hormone T_3.

Selenium is also found in the selenoprotein I 5'-deiodinase (5'DI) which is essential for metabolism of the thyroid gland (Ma *et al.,* 1993; Reilly, 1998). The iodothyronine deiodinase enzyme, which helps to convert T_4 to T_3 is a selenoprotein (Reilly, 1998). Selenium supplementation of adult humans decreased plasma thyroxine (T_4) concentrations by increasing the deiodinase activity to convert T_4 to T_3 (Kohrle & Jakob, 2005). Results from a study by Beckett *et al.,* (1993) suggested that Se deficiency increases the hypothyrodism resulted from the deficiency of iodine. Endemic goitre is common in Se deficiency subjects (Keshan disease subjects) (Ma *et al.,* 1993) and Se deficiency in parallel with the deficiency of iodine is also reported to result in goitre in Sri Lanka and cretinism in Zaire (Vanderpas *et al.,* 1993; Fordyce *et al.,* 2000).

Three different selenium-dependent iodothyronine deiodinases (types I, II, and III) can both activate and inactivate thyroid hormones, making selenium an essential micronutrient for normal development, growth, and metabolism. Furthermore, selenium is found as selenocysteine in the catalytic center of enzymes protecting the thyroid from free radicals damage. In this way, selenium deficiency can exacerbate the effects of iodine deficiency and the same is true for vitamin A or iron deficiency (Triggiani *et al.,* 2009). Substances introduced with food, such as thiocyanate and isoflavones or certain herbal preparations, can interfere with micronutrients and influence thyroid function. Results from several studies have proposed that deficiency of Se may introduce a loss of

immunocompetence in humans via impairment of cell-mediated and humoral immunity (Rayman, 2000; Arthur *et al.,* 2003). Selenium deficiency impairs the functions of T and B cells and it reduces the number of antibodies in the body (Arthur *et al.,* 2003). Hypothyrodism, which could be resulted by the deficiency of Se, has adverse effect on the ability of neutrophils to fight with foreign organisms (Arthur *et al.,* 2003). Supplementation of Se in humans is observed to stimulate the immunity responses even to the Se replete individuals (Rayman, 2000). In addition to a decline in neutrophil microbial activity (Arthur and Beckett, 1989) selenium deficiency markedly reduces the activity of the 5'-deiodinase enzymes responsible for the production of triiodothyronine (T_3) from thyroxine (T_4); this fundamentally important selenium/iodine interaction is likely to influence the human response to iodine deficiency in areas (e.g Zaire) where subjects are concurrently deficient in both selenium and iodine (Forbes, 1987). It may well be involved in the etiology of cases of iodine-responsive disorders whose prevalence is difficult to explain solely on the basis of environmental deficiencies of iodine.This combined deficiencies of iodine and selenium may have adverse effects on neonatal growth, development and survival (Arthur and Beckett, 1994).

In humans, this essential trace element is a highly probable factor in myxoedematous cretinism (Thilly *et al.*, 1993). In cretins, selenium supplementation caused a decrease in the already low level of thyroid hormones (T_3 and T_4) accompanied by an increase of thyroid stimulating hormone (TSH), which was elevated even before the trial (Zagrodzki *et al* 2000). On the other hand, excessive selenium administration to subjects with subtly impaired thyroid hormone synthesis did not cause any symptoms of hypothyroidism (Roti *et al* 1993). Selenium investigations and supplementation trials were also carried out in patients suffering from phenylketonuria because of protein restrictions in their diet; these patients have an extremely low selenium intake, which can influence thyroid hormone metabolism (Terwolbeck *et al.,* 1993; Calomme *et al.,* 1995). In areas where an excess of one of the two elements is present, the antagonism between sulfur and selenium also must be taken into account (Zagrodzki *et al.,* 2000). It has been suggested that selenium deficiency is a co-factor to iodine deficiency in the pathogenesis of myxoedematous cretinism. The mechanism proposed is that the generation of hydrogen peroxide is greatly increased in iodine-deficient thyroid glands, and that selenium is involved in the control of hydrogen peroxide and its derived free radicals (Contempre *et al.,* 1995).

2.6.3 Selenium in the Immune System:

Selenium has many biological functions mediated through an array of selenoproteins. These proteins can influence a range of biochemical systems in the body including those involved in thyroid hormone metabolism, antioxidant mechanisms and redox control. All these processes can impinge on elements of the immune system and it is therefore not surprising that selenium can influence both the humoral and cell mediated immune responses (Arthur *et al.*, 2003). In addition, dietary selenium intake can influence eicosanoid metabolism and modulate the balance between polyunsaturated fatty acid metabolites that may promote inflammatory processes with differing potencies. Since many cell-mediated immune processes involved reactive oxygen-derived species, these have the potential to damage host cells as well as the foreign organisms that are targeted by the immune process. Thus there is evidence that immune cells can damage themselves when there is insufficient selenium to protect against free radical species generated in response to infective challenges. The glutathione peroxidases are likely to play a role in the protective systems since peroxides are generated by the cells during these responses. There is now evidence that selenium can affect aspects of responses that do not rely upon generation of oxygen-derived free radicals, consistent with function of non-peroxidase selenoproteins (Arthur *et al.*, 2003). Selenocysteine is synthesized and inserted into proteins co-translationally by a complex process (Burk & Hill, 2003). Families of selenoproteins include the glutathione peroxidases, the iodothyronine deiodinases, and the thioredoxin reductases. These are redox enzymes that take advantage of the chemical properties of selenium to catalyze, respectively, removal of hydroperoxides by glutathione, deiodination of thyroid hormones, and support of cellular processes requiring reduction of disulfides. Approximately ten additional selenoproteins have been identified. One of them, selenoprotein P, is an extracellular protein that contains most of the selenium in plasma. Immunohistochemistry demonstrates that it associates with endothelial cells, probably through its heparin-binding properties. Selenoprotein P has been postulated to protect against oxidative injury and to transport selenium from the liver to peripheral tissues (Burk & Hill, 2003).

2.7 COPPER

Thyroid and immune system health are crucially dependent upon copper. Copper is one of the most important factors in the development of hyperthyroidism. Proper metabolism interrelates with and depends upon many other nutrients. Studies show that

because copper slows down the thyroid gland activity, this is the probable reason that drinkers of beer put on weight and get a "beer-belly". Foods that contain copper, such as nuts and seeds, beans and other legumes, lobster and crab are not eaten, especially by women who need more copper, because of the belief that these foods are fattening or contain toxic substance (shellfish) other foods like beer and chocolate are good sources of copper. Beer contains reasonable high levels of two critical minerals, Copper and Selenium. Probably, men have a drive to drink beer for its selenium content which is necessary for testosterone production. Once zinc is depressed the metabolic rate decreases depending upon dietary intake of copper and zinc, cadmium can either push the woman to hypothyroidism (low zinc) or hyperthyroidism (low copper). In humans Cu is necessary for the development of connective tissue, nerve coverings and bone. Copper (together with Fe) is an active centre of cytochrome-c oxidase, which catalyses the mitochondrial respiratory activities (Cobin *et al.,* 2006). Hence its deficit can result in impaired energy production in the same way as Fe deficiency. There is always a close relationship between dietary Cu and the status of Fe in the body. Deficiency of Cu has been reported to result into Fe deficiency anaemia (Kanumakala *et al.,* 2002; Auclair *et al.,* 2006). Several cuproenzymes are used in the metabolism of Fe; for instance, enzyme ceruloplasmin, which acts as ferroxidase to oxidise Fe^{2+} during cellular export process, needs Cu for its functions. Therefore deficiency of Cu impairs the mobilization of Fe to transferrin and results to anaemia (Auclair *et al.,* 2006). Several studies have related Cu with human body immunity (Tong *et al.,* 1996). The deficiency of Cu in animals has been associated with decreased production of antibodies and impaired functions of T and B cells (Lukasewycz *et al.,* 1985; Tong *et al.,* 1996). Results demonstrated that animals fed with low-Cu diet had impaired neutrophil function, increased susceptibility to bacterial infections decreased resistance to tumour challenge and reduced humoral and cell-mediated immunity (Mulhern *et al.,* 1987). In humans, infants with Menken's disease (a genetically Cu deficiency disorder) have high morbidity rate due to pulmonary and urinary tract infections (Lukasewycz *et al.,* 1985). The deficiency of Cu was found to alter the structure of immune cell membranes and the activity of enzymes which mediate antioxidant defence, ATP production and mitosis (Tong *et al.,* 1996). The trace metal copper is a cofactor for a number of enzymes which have critical roles in biological processes. Consequently, Cu uptake at the cell surface and incorporation of Cu into Cu-requiring proteins are essential events in normal physiology. Its capacity to convert

between oxidation states accounts for its essential role in many proteins in oxidative metabolism, yet can lead to the generation of free radicals. These conflicting properties demand close regulation of copper levels (Armendariz *et al.,* 2003).

2.8. IRON

Iron is central to healthy functioning of the human body because it is the main constituent of hemoglobin - the oxygen-carrying protein inside red blood cells. Iron is also essential for many bio-chemical processes including the formation of Cytochromes & certain respiratory enzymes (Horton and Levin, 2001). Iron deficiency anemia is associated with lower plasma thyroid hormone concentrations in rodents and in some human studies (Beard *et al.,* 1998). In developing countries, many children are at high risk for both goitre and iron-deficiency anemia because iron deficiency may impair thyroid metabolism (Zimmermann *et al.,* 2000). Aluminum interferes with iron metabolism; studies have found that people who eat food cooked in aluminum pots get anemia. Getting these people to switch to iron cookware greatly reduces the rate of anemia (Adish *et al.,* 1999). Numerous enzymatic reactions involving oxidation and reduction use iron as the agent through which oxygen is added, hydrogen is removed, or electrons are transferred. The classes of enzymes dependent on iron for activity include the oxidoreductases, exemplified by xanthine oxidase/dehydrogenase; monooxygenases, exemplified by the amino acid oxidases and cytochrome P_{450}; dioxygenases, exemplified by amino acid dioxygenases, lipoxygenases, peroxidases, fatty acid desaturases, and nitric oxide synthases; and miscellaneous enzymes such as aconitase.

Among the trace elements, iron has the longest and best described history. By the seventeenth century, a recognized treatment for chlorosis, or iron deficiency anemia, was drinking wine containing iron filings. The physiological signs of iron deficiency include anemia, glossitis (smooth atrophy of tongue surface), angular stomatitis (fissures at the angles of the mouth), koilonychia (spoon nails), blue sclera, lethargy, apathy, listlessness, and fatigue. Pathological consequences of iron deficiency include impaired thermoregulation, immune function, mental function, and physical performance; complications in pregnancy, including increased risk of premature delivery, low birth weight, and infant mortality; and possibly increased risk of osteoporosis. Concerns have been expressed about high intakes of iron being a health issue. The toxic potential of iron arises from its biological importance as a redox element that accepts and donates electrons to oxygen that can result in the formation of reactive oxygen species or radicals

that can damage cellular components such as fatty acids, proteins and nucleic acids. Antioxidants are enzymes or molecules that prevent the formation of oxygen radicals or convert them to nonradical products. When not properly controlled by antioxidants, reactive oxygen damage can lead to premature cell aging and death. An iron overload disease known as hereditary hemochromatosis is caused by a defective regulation of iron transport with excessive iron absorption and high transferrin (transport form) iron in plasma. Iron and copper status have also been linked to decreased plasma T_3 concentrations in animals and man (Ohm *et al.,* 1994; Beard *et al.,* 1998). As with zinc, these changes have not yet been associated with specific changes in enzymes involved in thyroid hormone metabolism. It remains to be determined whether the changes in thyroid metabolism are a direct result of the iron and copper deficiencies or a non-specific response to poor health (Arthur & Beckett, 1999). Most iron in the body is incorporated into heme or hemoglobin and a smaller proportion as myoglobin, heme containing enzymes and iron sulfur proteins. A significant amount of Fe is stored as ferritin and haemosiderin, primarily in the bone marrow, spleen and liver.

2.8.1 Iron Homeostasis: In an adult male, the average loss of Fe per day must be replaced by dietary sources (Beard, 1996) Pregnant or premenopausal women and children have grater iron requirements, often obtained by dietary supplementation. Undesirable side effects associated with iron supplementation seem minimized by use of borine hemoglobin (Walter *et al.,* 1993) and of lactofelt (Cherki *et al.,* 1992). Iron is well conserved by the body therefore absorption of iron from the intestine is the primary means of regulating the amount of iron within the body. When plasma iron is decreased, more iron is absorbed from the small intestine and vice versa. Substances present in the intestinal lumen, such as anionic phosphate and phytate may bind and interefere with absorption (Conrad, 1987). It is believed that, before absorption, dietary Fe III must be reduced to the more soluble Fe II (Han *et al.,* 1995) by the action of HCl and reducing agents such as ascorbic acid (Beard, 1996). Entry of Fe II into the mucosa cell may be aided by the presence of a trans membrane enzyme (Pountney *et al.,* 1994; Goldenberg, 1997) with ferric reductase activity (Riedel *et al.,* 1995; Ekmekciogly, 1996)

Iron and vitamin A deficiencies are prevalent in areas of endemic goitre, although iodine intake is adequate. Watts, 2004) It is now recognized that the response to iodine therapy is ineffective in the presence of iron deficiency. Iron deficiency is associated with

alterations in many metabolic processes that may impact brain functioning; among them are mitochondria electron transport, neurotransmitter synthesis and degradation, protein synthesis, organogenesis, and others.

2.9 ZINC

Zinc is an essential trace nutrient in all biological systems (Prasad, 2003) and has close interrelationships with the endocrine system (Masayuki, 2001). It has a fundamental role in the structure and function of numerous proteins, metalloenzymes, transcription factors and hormone receptors. The importance of micronutrients in health and nutrition is undisputable, and among them, zinc is an essential element whose significance to health is increasingly appreciated and whose deficiency may play an important role in the appearance of diseases (Jayant et al., 2013). Nearly two billion people in the developing world are deficient in zinc (Prasad, 2003). It has a widespread role in metabolism because it is present in all tissues, organs and fluids of the human body (IPCS, 2001); a component of enzymes or a functional cofactor of a large number of enzymes. Zinc is second to iron as the most abundant trace element in the human body (Prasad, 2003) and the fourth most widely used metal after iron, aluminium and copper. It has an estimated total body content in a normal 70kg male of 1.4 - 2.3g. The zinc content of erythrocytes is about ten – fold that of plasma because of their rich content of carbonic anhydrase (catalytically involved in the transport of CO_2 in the blood) and other zinc metalloenzymes (Aggett, 1985). The role of zinc in several immune mechanisms has been extensively studied in humans, showing that even moderate deficiencies can rapidly diminish antibody and cell-mediated responses (Fraker *et al.*, 2000). Zinc is essential for growth and reproductive function. Zinc deficiency causes growth retardation, delayed sexual development and inhibits DNA synthesis, although which of the numerous zinc-dependent proteins underlie these effects is not clear (Cole and Lifshitz, 2008); its supplementation is effective to promote growth and sexual development in zinc-deficient children. Zn prevents Fe and Cu ions to initiate the lipid peroxidation on cells. This is because Zn in the state of Zn^{2+} has no unpaired electrons which could allow it to participate in redox reactions. On the other hand, Fe^{2+} and Cu^{+} due to their redox properties, play a big role in the initiation and propagation of lipid peroxidation. Lipid peroxidation is a process whereby a free radical such as HO**.** Removes electrons from the

lipids in cell membranes. The HO**.** radical is formed when a single electron is transferred to hydrogen peroxide (H_2O_2).

e^- + H_2O_2 ---------- HO**.** + OH, since Fe^{2+} and Cu^+ have multiple oxidation states, they can easily donate the required electron to oxidise H_2O_2.

Fe^{2+} + H_2O_2 ---------- HO**.** + OH- + Fe^{3+}

Cu+ + H_2O_2 ---------- HO**.** + OH- + Cu^{2+}

Thus, by competing for binding sites with Fe and Cu, Zn antagonises their abilities to promote formation of HO**.** from H_2O_2. Zn is also a cofactor of an enzyme Cu/Zn superoxide dismutase, (Cu/Zn SOD). This enzyme catalyses the disproportionation of reactive oxygen superoxide (O_2 -) into less active oxygen (O_2) and hydrogen peroxide (H_2O_2) (Nath, 1997; Ren *et al.,* 2006). Therefore, the deficiency of Zn reduces the activities of MTs and Cu/Zn - SOD hence, increasing the levels of lipid peroxidation in mitochondria and microsomal membranes which might damage the cells and result into the development of chronic diseases such as cancer.

2.9.1 Zinc and thyroid function:

There are indications that zinc is also important for normal thyroid homeostasis. Its roles are complex and may include effects on both the synthesis and mode of action of the hormones (Arthur & Beckett, 1999). Marked alterations in zinc homeostasis were observed in patients with thyroid diseases. In hyperthyroid patients, erythrocyte zinc content was shown to be significantly lower than normal, and inversely related to plasma thyroxine concentration. Plasma zinc concentration was, however, shown to be within normal range. Hyperthyroid patients excreted significantly greater amounts of zinc than controls, indicative of a catabolic process. This increased urinary loss might reflect a shift in the distribution of plasma zinc between ultrafilterable fraction and zinc-albumin complexes. On the other hand, in hypothyroid patients erythrocyte zinc content was shown to be significantly higher than normal, and plasma zinc concentration, leukocyte zinc content and urinary zinc excretion were lower than normal (Aihara *et al.,* 1984; Dolev *et al.,* 1988). Yoshida *et al.,* (1990) measured erythrocyte zinc concentrations in 28 healthy volunteers and 46 patients with hyperthyroidism, and observed a significant negative correlation between the concentrations of erythrocyte zinc and those of both plasma thyroxine (T_4) and triiodothyronine (T_3). They also revealed that after the

treatment of 17 hyperthyroid patients with antithyroid drugs, both mean plasma T_4 and T_3 concentrations became normal within four weeks, but the normalization of erythrocyte zinc concentrations lagged about two months behind them. Therefore, they suggested that erythrocyte zinc concentration in hyperthyroid patients reflected the patients' mean thyroid hormone levels over the preceding several months (Yoshida *et al.*, 1990).

Zinc deficiency was shown to impair the metabolism of thyroid hormones. Kralik *et al.*, (1996) reported that male Sprague-Dawley rats fed low-zinc diet for 40 days showed decreased serum concentrations of T_3 and free T_4 by approximately 30% compared with those fed adequate-zinc diet. Wada and King (1986) measured basal metabolic rate and plasma thyroid hormones in healthy young men fed low-zinc diet for 54 days and thereafter fed adequate-zinc diet for 9 days. They observed that basal metabolic rate and serum free T_4 levels decreased significantly during the low-zinc period, and increased during adequate-zinc period. Effects of zinc supplementation on thyroid function have been evaluated under some pathological states. Arreola *et al.*, (1993) reported that oral zinc supplementation increased plasma levels of TSH, T_3, T_4 and zinc in uremic patients under peritoneal dialysis, who had been zinc-deficient at the beginning of the study. They also revealed a close correlation between the changes in plasma zinc levels and the changes in TSH, T_3 and T_4 levels, suggesting that zinc deficiency might play a role in the biosynthesis or release of hormones of the hypothalamic-pituitary-thyroid axis in chronic renal failure (Arreola *et al.*, 1993). Nishiyama *et al.*, (1994) evaluated the effects of zinc supplementation on thyroid function in disabled patients with mild to moderate zinc deficiency that had low serum free T_3 and normal serum T_4 levels and showed enhanced reaction of serum TSH after TRH injection. They reported that oral zinc supplementation for 12 months resulted in normalization of the serum free T_3 levels and the TRH-induced TSH reactions. They suggested the contribution of zinc to conversion of T_4 to T_3 in humans (Nishiyama *et al.*, 1994).

It is well known that thyroid dysfunction is common in patients with Down syndrome. It was also shown that a marginal, but biologically relevant, zinc deficiency was usually present in children with Down syndrome (Fabris *et al.*, 1984). It is suggested that zinc has an inhibitory effect on calcitonin secretion from thyroid tissue. Nishiyama *et al.*, 1991 observed that zinc infusion resulted in a decrease in serum calcitonin concentration, but did not change the concentrations of ionized calcium and parathyroid

hormone in patients with short stature and insulin-dependent diabetes mellitus, and in age-matched controls. Since thyroid hormone receptors contain zinc-finger structures alike to steroid hormone receptors, it has been speculated that zinc deficiency might cause insufficient thyroid hormone action. However, there have been no clinical or experimental data to support the hypothesis. Changes in zinc nutriture, especially zinc deficiency affects the endocrine system in many ways, and a lot of hormones influence zinc status in the body. Various symptoms with zinc deficiency mainly derive from impaired activities of zinc enzymes and dysfunction of zinc-containing proteins. Abnormal zinc metabolism occurs commonly in thyroid disease. Zinc levels in red blood cells have become an important marker of peripheral tissue response to thyroid hormones, indicating duration of pre-existing thyroid disease (Tiran 1993, Vitoux *et al.*, 1999).

2.10 TOXIC METALS

As a class of agents, toxic metals are a concern of the highest priority for human exposure (Waalkes *et al.*, 2000). The metals have a vast array of remarkably adverse effects, including those of carcinogenicity, neurotoxicity and immunotoxicity. Metals are also non-biodegradable and persist in the *environment*. Anthropogenic use has led to global dispersion of metals in the environment; because of their wide distribution and extensive use in modern society, some human exposure to toxic metals is inevitable.

2.10.1 Lead (Pb)

Lead is one of the most useful elements in the industry; however unlike some other metals such as iron and copper, lead has no known biologic function in man and animals (Loghman-Adhman, 1997). It has therefore been suggested that in an individual that has not been exposed to lead, the ideal whole blood lead level is zero (Landrigan, 1987) however, this is not feasible since sources of exposure of the population abound in the environment. The permissible exposure level (PEL) for lead in the blood in between 10 – 20 ugldl and values below 10ug/dl is regarded as the most probable permissible level (Christian, 1978). It alters the hematological system by inhibiting the activities of several enzymes involved in heme biosynthesis. Once absorbed, it is distributed particularly to the liver, kidney, heart and male gonads as well as it affect the immune system (ATSDR, 2005). Calcium can affect the toxicity of lead by interacting with the Pb at its primary site of action (Peraza *et al.*, 1998). Therfore increasing the availability of the micronutrients may decrease the toxicity of the toxic metal. Zinc has been shown to counteract the toxic

effects of lead both invivo and invitro (Granick *et al.*, 1978). Zinc influences both tissue accumulation of lead and suscptibilty to lead toxicity (Mahaffay, 1981). Available evidence suggests that as dietary zinc increases, lead absorption and its subsequent toxicity decrease (Granick *et al.*, 1978; Mahaffay, 1981). Zinc exerts its effect on lead absorption in the gastrointestinal tract.

In lead poisoning, if lead gets into the blood, the body will try to get rid of it. Since the metal atoms are too heavy compared with the body's immune forces, the removal may be impossible. Lead can initiate a chronic inflammatory response and remain in the body permanently. Studies show that lead poisoning reduces T_4 in exposed workers. Statistical correlation of levels with environmental factors showed that lead (Pb) levels were most strongly correlated with the age of the house, with maternal exposure to heavy traffic for more than 5 years (Kurkjian *et al.*, 2002). The maximum values of lead are found in industrial areas and housing areas with buildings erected before 1920 (in those areas, average soil Pb concentration is higher compared to traffic areas). This might be the result of a higher Pb content in construction materials, emissions of coal-fired house heating, and of more intensive construction earth mixing and exchanging activities nearer to roads, which influences the Pb accumulation process in the soil. The direct neurotoxic actions of lead include apoptosis, excitotoxicity, infuences on neurotransmitter storage and release processes, mitochondria, second messengers, cerebrovascular endothelial cells, and both astroglia and oligodendroglia (Lidsky and Schneider, 2003). Zinc acts as a stimulator and copper as a suppressor of the thyroid and immune systems. Lead and mercury also have a direct effect on neuronal development leading to learning deficits. These are the same type of birth defects produced by maternal iodine deficiency and hypothyroidism.

2.10.2 CADMIUM (Cd)

Cadmium is one of the most potent and long-lasting toxic metals known. Cadmium has been demonstrated to damage thyroid cells and to decrease both T_4 and T_3 (Yoshizuka *et al.*, 1991). Cadmium accumulates in the thyroid, kidneys, liver and pancreas – all areas that seem to be involved in thyroid disease (Yoshida *et al*, 1987). It appears to be the largest single contributor to autoimmune thyroid disease, absolutely very toxic heavy& powerful metal (Chapman, 2008) which seems to be placed at the very centre of thyroid story. Not only does Cd appear to play a pivotal role in thyroid disease,

it is a very unique material, extremely toxic and has toxic biological effects at concentrations smaller than most commonly found mineral. It is distributed in the earth's crust (Selinus, 2005) and it is found in water, air, soil and plant and animal tissues (Zasadowski *et al.,* 1999). Problems with Cd has been studied by several authors in humans (Mueller *et al.,* 1989; Ikeda *et al.,* 1996), rabbits (Chan *et al.,* 2004), rats (De Wolf *et al.,* 2004) and sheep (Stoyebo *et al.,* 2003). It is gradually accumulated in animals especially in kidneys and liver (Berlin &Ullberg, 1963; Ware, 2007). There is some evidence that Cd can also affect the thyroid function (Gupta & Kar, 1999; Pilaz-Marcinkiewiez *et al.,* 2003). In adult sheep, poor wool growth, decreased milk yield, reduced weight gain, impaired reproductive performance with loss of libido in rams, and late abortions or birth of weak lambs with visibly enlarged thyroid glands has been associated with Cd (Khalil *et al.,* 2009). Cadmium or nickel toxicity affects hormone absorption by affecting the levels of calcium, sodium and other critical minerals. Despite this great toxicity, there is some evidence that cadmium is an essential nutrient with biological function. One of the greatest effects of cadmium is that it depletes selenium in the body because Se is essential for Cd removal via the bile system. Cadmium decreases T_3 by interfering with T_4 to T_3 conversion. Cadmiun plays a principal role in the etiology of thyroid disease. While being a female is the largest risk factor for developing thyroid disease, tobacco smoking is another major risk factor identified especially for hyperthyroidism due to cadmium toxicity (Anon, 2000). The heavy metal cadmium is one of the most potent and long - lasting toxic metals known. Cadmiun has been shown to kill animals at a concentration lower than any other commonly occurring toxic metal. Cadmiun has been demonstrated to damage thyroid cells and this damage can be viewed in invitro studies of thyroid cells in a culture. (Anon, 2000) To further demonstrate the toxicity of cadmium; an experimental study was carried out on castrated male and female rats showed that estrogen caused the cadmium to be incorporated and retained by the body, while testosterone caused the cadmium to be excreted from the body. This further explains why 90% of the people with thyroid disease are female. Cadmium occurs naturally and is also introduced into the soil by the use of manure and phosphate fertilizers as well as from industrial processes and sewage sludge. (WHO, 1992) In food, Cd concentrations vary as a function of the species grown. For example, leafy vegetables and potato tubers accumulate higher levels of Cd than fruits and cereals (WHO, 1992). Cadmium in soils is practically immobile, hence its concentrations in drinking water is

generally low (<1 ppb) (Rojas *et al.,* 1999). For that reason, 98% of the ingested Cd is reported to come from terrestrial foods, while only 1% comes from aquatic foods such as fish and shellfish, and 1% arises from drinking water. The retention of Cd in the body is reported to be low with only 2% to 6% of the ingested Cd absorbed into the body (WHO, 1992). However, its absorption could also be influenced by the iron status of the individual as mentioned earlier. Cadmium biological half life inside a body is very long (5 to 30 years) hence long term accumulation could pose a problem to health (Castelli *et al.,* 2005). Cadmium in the body replaces Zn in many enzymes causing impairments in some of the enzymatic functions of the body (Brzoska & Moniuszko-Jakoniuk, 2001). Cd is considered as category I carcinogen (Trzcinka-Ochocka *et al.,* 2004; Castelli *et al.,* 2005) by mechanisms involving overproduction of reactive oxygen species (Sarkar *et al.,* 1997).

2.10.3 ARSENIC (As)

Arsenic (As) is a class 1 human carcinogenic (IARC, 2004; Ahsan *et al.,* 2006) to which approximately 140 million people worldwide (Kinniburgh & Smedley, 2001) are chronically exposed to through contaminated drinking water. A study by Hall *et al.,* 2009 also showed that improved nutritional status could constitute a key strategy for reducing the risk of arsenic related disease, since nutritional factors are known to influence arsenic metabolism in adults, and poor nutriotional status – as reflected in part by a lack of various B vitamins and antioxidants- is thought to confer greater susceptibility to arsenc toxicity. Arsenic is widely distributed throughout the earth's crust, occurring in most soils and rocks in detectable quantities (WHO, 1981). Its compounds have also been used in agriculture as insecticides (Abernathy *et al.,* 2003). The mechanism of arsenic-induced carcinogenesis is unknown. It disrupts the mitotic spindle in normal human peripheral lymphocytes (Ramirez *et al.,* 1997) and exerts its toxic effects through an impairment of the cellular respiration by inhibition of various mitochondrial enzymes, and the uncoupli-ng of oxidative phosphorylation. Its toxicity results from its ability to interact with sulfahydryl groups of proteins and enzymes to substitute phosphorous in a variety of biochemical reactions (Patlolla *et al.,* 2005). Arsenic compounds are soluble in water hence they can easily dissolve and contaminate groundwater. In sea foods, it exists mostly in organic compounds which are much less harmful to health because they are readily eliminated by the body as waste (Abernathy *et al.,* 2003); for that reason, the threat of As to public health is more from drinking water than from foodstuffs. Inorganic

As is reported to be carcinogenic in humans (Ahsan *et al.*, 2006). Its carcinogenicity might be related to its ability to induce chromosome abnormality, to inhibit DNA repair and to induce oxidative stress in the body cells (Abernathy *et al.*, 2003).

2.10.4 NICKEL (Ni)

Nickel is a transition metal, a toxic trace element and exposure may occur in nickel refining, electroplating, Nickel/Cadmium battery manufacturing and glass-making. Nickel (as well as Hg) has been found to trigger autoimmune thyroid disorders and other autoimmune disorders such as systemic lupus (Moore, 2010). The main effects noted in humans are through inhalation and skin contact. Toxic effects are local rather than systemic such as asthma, and skin irritation among others Nickel activates the enzyme arginase and influences oxidative processes. Enzymes known as hydrogenases in bacteria contain nickel. Nickel is also important in plant ureases, required for the enzyme urease to break down urea to liberate the nitrogen into a usable (Joseph *et al.*, 2012). Nickel is required for iron absorption; Daily intake of nickel is about 250 µg/day most of which is excreted in the faeces. It is not known if it is essential although deficiency in some animals causes retarded growth, anaemia, and decreased enzyme activities. Nickel content of cigarettes is about 3 µg of which 10-20% is released in smoke and may be absorbed in lungs. This silvery-white metal found naturally in the earth's crust is the world's 24th most abundant element, a transition metal which has chemical properties that enable it to form multiple chemical compounds, some of which are toxic. It is also an excellent conductor of both heat and electricity. As the electrochemistry of nickel became better understood, it was adapted for use in batteries. Nickel and cadmium compounds are used to produce rechargeable nickel-cadmium (Ni-Cd) batteries.

Hazards and Risks associated with Nickel: Nickel metal powder is a fire hazard. Unless known otherwise, all nickel compounds should be regarded as toxic. Some are carcinogenic and teratogenic. The highly volatile nickel carbonyl [Ni $(CO)_4$] is extremely toxic and only to be handled by competent persons in very well-ventilated areas. Excess intake of nickel causes some morphological transformations in numerous cellular systems and chromosomal aberrations (Coen *et al.*, 2001).

Nickel Antagonists: Calcium, iron, magnesium, zinc and phytates reduce GIT absorption of nickel. Nickel toxicity can lead to magnesium deficiency or excess concentration of iron or zinc.

2.11 Interactions between the Trace elements - Iodine, Copper, Iron, Selenium, Zinc, Arsenic, Cadmium, Lead and Thyroid Metabolism

Normal thyroid status is dependent on the presence of many trace elements for both the synthesis and metabolism of thyroid hormones (Arthur & Beckett, 1999). The importance of the thyroid gland in maintaining human health is well recognised. Since iodine is an integral constituent of thyroid hormones, it is not surprising that thyroid dysfunction is very common in geographical areas of iodine deficiency. However, even when this trace element is present in adequate supply, thyroid disease is present in 3-5% of the population (Arthur & Beckett, 1999). Furthermore, the regulated supply of thyroid hormone to specific tissues is crucial during fetal development (Hetzel & Wellby, 1997).

2.12 Environmental metals, chemicals and thyroid function

There is growing evidence that environmental chemicals can disrupt endocrine systems. (Boas *et al*, 2006) Most evidence originates from studies on reproductive organs. However, there is also suspicion that thyroid homeostasis may be disrupted. Thyroid disruption may be caused by a variety of mechanisms, as different chemicals interfere with the hypothalamic–pituitary–thyroid axis at different levels. Mechanisms of action may involve the sodium–iodide symporter, thyroid peroxidase enzyme, receptors for THs or TSH, transport proteins or cellular uptake mechanisms. The peripheral metabolism of the THs can be affected through effects on iodothyronine deiodinases or hepatic enzymes. Even small changes in thyroid homeostasis may adversely affect human health, and especially fetal neurological development may be vulnerable. There is substantial evidence that polychlorinated biphenyls, dioxins and furans cause hypothyroidism in exposed animals and that environmentally occurring doses affect human thyroid homeostasis. Similarly, flame retardants reduce peripheral thyroid hormone (TH) levels in rodents, but human studies are scarce. Studies also indicate thyroid-disruptive properties of phthalates, but the effect of certain phthalates seems to be stimulative on TH production, contrary to most other groups of chemicals. It is therefore urgent to clarify whether the animal data showing effects of chemicals on thyroid function can be extended to humans.

Environmental metals or chemicals may interfere with thyroid homeostasis through many mechanisms of action, i.e. at the receptor level, in binding to transport proteins, in cellular uptake mechanisms or in modifying the metabolism of the thyroid

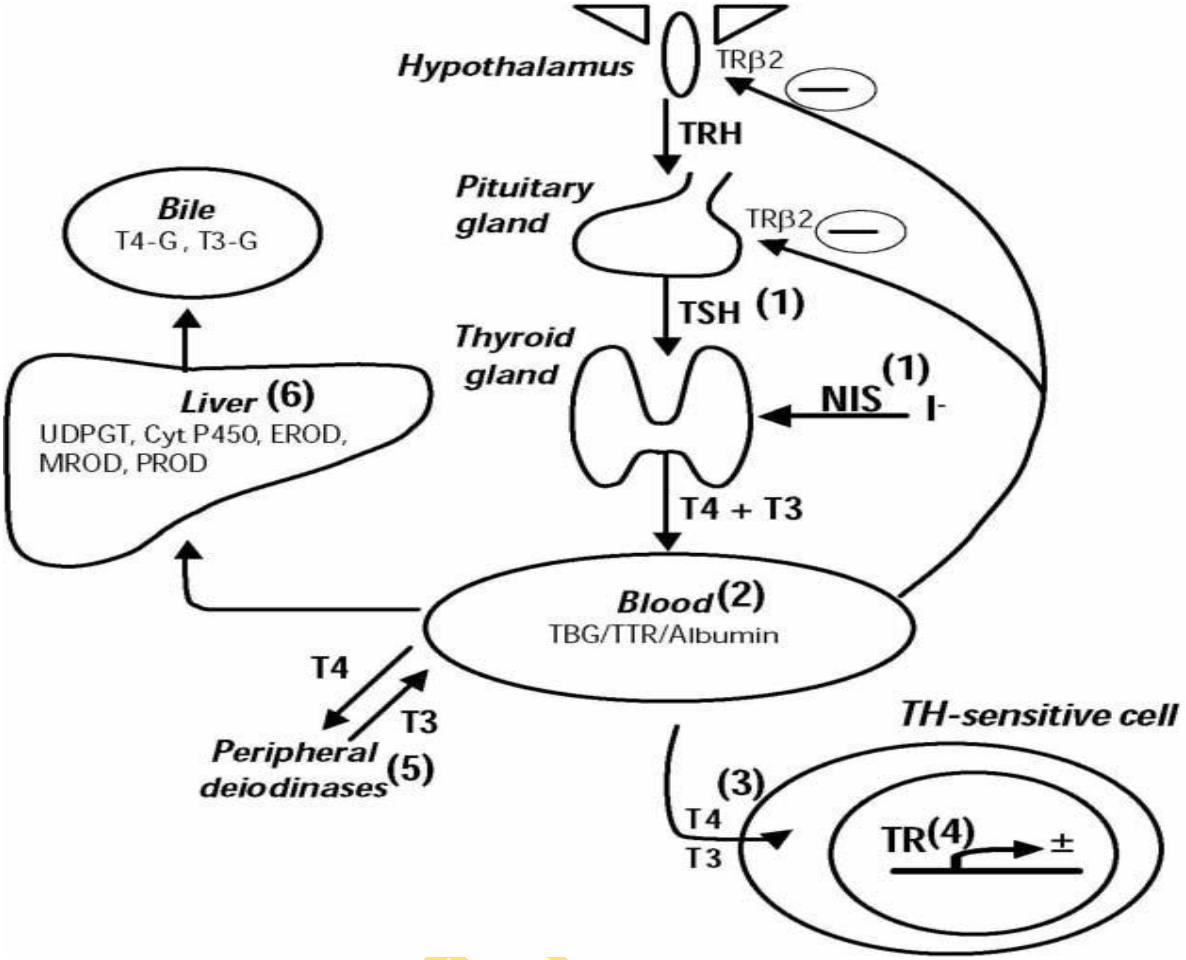


Fig. 2.3 **Possible mechanisms of action of environmental chemicals or elements on the Hypothalamic–Pituitary–Thyroid Axis (Boas *et al.*, 2006).**

(1) Synthesis of THs: interference with NIS, TPO or TSH receptor.

(2) Transport proteins.

(3) Cellular uptake mechanisms.

(4) The TH receptor

(5) Iodothyronine deiodinases.

(6) Metabolism of THs in the liver

hormones (THs). Several environmental chemicals have a high degree of structural resemblance to the THs thyroxine (T_4) and triiodothyronine (T_3) and therefore interfere with binding of THs to receptors or transport proteins. This, in turn, may lead to subclinical hypothyroidism, which in adults is often diagnosed only by chance because of subtle symptoms. However, growth and development in fetal life and childhood is highly dependent on normal levels of THs. Particularly during gestation, normal levels of THs are crucial for the development of the central nervous system. This critical phase may be vulnerable to even subtle effects of synthetic chemicals on fetal and maternal TH levels. Such developmental deficiencies may not be identifiable until later in life (Morreale de Escobar *et al.,* 2004). Perchlorate, has well known antithyroidal effects, which has been exploited in diagnosis and treatment of thyrotoxicosis (Wolff, 1998). It has therefore been of concern that perchlorate is found in drinking water (Strawson *et al.*, 2004). A study of workers in an ammonium perchlorate production plant found a significant decrease in thyroid gland iodine uptake related to presence at work (Bravermann *et al.,* 2005). However, human studies are contradictory concerning the effect of environmentally occurring levels of perchlorate on neonatal thyroid function (Brechner *et al.,* 2000; Li *et al.,* 2000; Kelsh *et al.,* 2003).

2.13 THYROID HORMONE DISORDERS

Thyroid disorders are a group of metabolic disease affecting mostly the female population; auto - immunity has been identified as a major cause. The alteration of the balance between certain essential and toxic trace elements has a strong role in the pathogenesis of autoimmune thyroid disease (AITD) as well as non auto immune thyroid disease (NAITD) and this has received little attention. This study examines the contribution and relationships of this imbalance to the patterns of various thyroid disorders in Nigerian patients.

Thyroid disorders were thought to be rare in Africans in the early 1960s (Olurin, 1972); however the 1970s witnessed an upsurge in reported cases of thyroid disorders in Africans. It is generally accepted that the incidence of thyroid disease is subject to geographic variation (Olurin, 1972). A review of the experience with the thyroid gland disease in Ibadan shows that Thomas in 1959 published the first report from Ibadan on "The large goitres". In another review of 300 thyroidectomies over a five year period, Adesola, (1962) found three cases (0.2%) of acute suppurative thyroiditis which required

urgent drainage. Davy and Ogunlesi's study (1963) inferred that a thyroid case (thyrotoxicosis) was rare in Nigeria. In a review of 404 hospital - based retrospective study, Oluwasanmi and Alli (1968) identified two main areas of endemic goitre in Southern Nigeria, viz (a) Ijesha-Ekiti, Ondo area and (b) Ijebu area. They found 3% of their cases had adenocarcinoma of the thyroid gland and 5% had toxic goitres. Taylor (1968) in same year sectioned 75 goitres and found 7% of them to be primary adenocarcinoma of the thyroid (follicular 54%, papillary 16%, mixed papillary and follicular 11% and undifferentiated 11%. He reported 2 cases of sub acute thyroiditis of De Quervain. Laditan and Johnson (1979) also described severe hypothyroidism and cretinism in Ibadan. Another review study by Famuyiwa and Bella, (1990) on thyrotoxicosis in Nigeria – An analysis of a five year experience stated that among the forty cases of thyrotoxicosis seen in the endocrinology unit of the teaching hospital in Ibadan over a five-year period, the clinical manifestation were not different in any way from the pattern described in other parts of the world; there was a female preponderance in a ratio of 4:1. The ratio of diffuse toxic goitre to toxic multi nodular goitre was only 3:2, underscoring the relative uncommonness of Graves' disease, an autoimmune disorders in Nigeria, Africa. They also demonstrated an increase in the hospital prevalence of thyrotoxicosis in Ibadan in the last 10-15 years. However, the incidence rate of 8 cases per year is still low when compared to experiences in other parts of the world.

Dietz, (2003) on analysis of the etiology and management of thyroid disorders in patients older than 55 years found out that hyperthyroidism was a common disease in the elderly. His findings showed that in such patients (older than 55 years) etiology of spontaneous hyperthyroidism, followed by Graves' disease and toxic adenoma. Investigations by Ojule and Osotimehin, (1998) to further appraise the public health importance of iodine deficiency disorders in Saki, Ibadan (a locality known to have environmental iodine deficiency and located within the goitre belt of Southwestern Nigeria) indicated that there was environmental iodine deficiency and related chemical hypothyroidism of mothers and neonates in Saki when compared with mothers and neonates from non-iodine defieicnt areas. Previous studies in Saki have shown that this area is iodine deficient with significant IDD (Iodine deficiency disorders) problems (Olurin, 1974 and Ojule, 1992); which results in deficient production of thyroid hormones associated with hypothyroidism, still births, miscarriages, impairment of the

nervous system development and function, cretinism as well as lesser degrees of loss of physical and mental function (Hetzel, 1983; 1989). These disorders affect the foetus, neonates, young children and adults. The sensitivity of different organs to thyroid hormone deficiency varies; the brain is particularly susceptible to damage during foetal and early post natal period. It has been estimated that nerve cells require thyroid hormone for their development (Bleichrodt *et al.,* 1987). The developing foetus begins its own thyroid hormone production only after the 10^{th} – 12^{th} week of gestation (since it is depends on the mother's) and as estimated from plasma values, the thyroid hormone production becomes appreciable only in the 20^{th} week. The formation of nerve cells precedes or coincides with the foetus' own thyroid hormone production. Experimental data shows that prior to foetal endogenous hormone production, development depends on the mother's thyroid (Morreale *et al.,* 1986). Thyroid hormone deficiency (hypothyroidism) occurring during this period gives rise to the syndrome of cretinism, which is characterized by retardation of physical, neuromotor, auditory and intellectual maturation. Although earlier reports gave some information about thyroid maladies and works done in Ibadan, it was difficult to get comprehensive information on the types, characters and problems of the diseases of the thyroid gland (Olurin *et al.,* 1986). From this study, thyroid gland diseases, in Ibadan can be classed as:

2.13.1 Simple Goitre

It constitutes about 84.5% of all cases, with a female to male ratio of 10:1 and occurring in all age groups though the peak age incidence was 31-40 years. It was often seen at puberty, in pregnancy and during lactation. It was the commonest form of goitres in endemic area of South-Western Nigeria. This is in contrast to the increase in frequency of and thyroid antibodies with age (Vanderpump, 2011)

2.13.2 Adenocarcinoma

It occurs in 12.5% of patients with peak age incidence of 31-40 years and a female to male ratio of 3:1. In about 36% of these patients, carcinoma was not suspected clinically but diagnosed histologically posthyroidectomy. This is a situation which fine needle aspiration biopsy could certainly have greatly improved. The incidence of cellular type of adenocarcinoma of the thyroid was; Papillary 18%, follicular 60%, anaplastic 10%, mixed papillary & follicular 12%. No case of medullary carcinoma was observed in the series.

2.13.2. Hyperthyroidism

It was found in 46 (5.3%) patients, primary in 59% and secondary in 41%. The peak age incidence was between 21 and 30years in the primary group and 31-40 years in the secondary group. The overall female to male ratio was 5:1. The youngest patient was nine years old. Subacute (granulomatous, giant cell) thyroiditis of De Quervain was confirmed in one case.

2.13.3. Autoimmune thyroiditis (Hashimoto's)

Riedel thyroiditis and chronic infective (tuberculous) thyroiditis were not found in this series. Perhaps some were missed in the retrospective study since quite a number of histologists were involved at different times in the interpretation of various sections.

2.13.4. Hypothyroidism

It was seen as a post thyroidectomy complication in three cases. These reports indicated that the thyroid maladies attracted the attention of the earlier workers in Ibadan. However, despite all these works, there still appear to be a paucity of data on the trace elements, autoimmunity and thyroid disorders in this environment; since thyroid disorders usually results from a disruption of the immuno-endocrine system.

Though thought to be rare in Africans; thyroid disorders are amongst the most prevalent of medical conditions, especially in women (UK guidelines, 2006). In endocrinology clinics in Nigeria, thyroid disorders are the second most common endocrine disorders seen (Ogbera *et al.*, 2007). The occurrence of thyroid diseases is determined by interplay between genetic and environmental factors (Knudsen *et al.*, 2004). The major environmental factor that determines goitre prevalence is iodine status, but other environmental factors influencing the entire populations have been identified such as goitrogens in food and drinking water (Geelhoed, 1999; Lezama *et al.*, 2001)

Thyroid disorders induce heavy metabolic and enzymatic damage to cell function including increased and decreased levels of blood lipid profiles in hypothyroidism and hyperthyroidism, respectively, accumulation of mucopolysaccharides in subcutaneous tissues in hypothyrodism, alterations in skin components and body fluid distributions and disturbances in serum enzymes concentrations. In addition associated immune disorders in some thyroid diseases increase thyroid stimulating or suppressing antibodies and anti-thyroid cell antibodies, which leads to other clinical manifestations (Bjoro *et al.*, 2000; Ardakanni *et al.*, 2005). Thyroid disorders result from a disruption of the immune and endocrine system (immuno-endocrine system). Nutritional deficiencies cause a disruption

of both the endocrine and the immune functions. The immune and endocrine disorders are closely related because their functions rely upon the same essential nutrients. If a nutrient is deficient which causes the endocrine system to be disrupted, then tne immune system will also likely be disrupted. While thyroid disorders sometimes occur without immune involvement, most cases of thyroid disease are thought to be the result of immune system dysfunction (Swain *et al.,* 2005). These are the autoimmune thyroid diseases, Graves' disease and Hashimoto's thyroiditis.

Studies by Ogbera *et al.,* 2007 which took place from June 2004 to August 2005 in the Department of Medicine of the Lagos State University Teaching Hospital, Ikeja, Nigeria showed a total number of patients with thyroid disorders seen in a 15-month period as 78. The female to male ratio was 5:1. The mean (standard deviation) age of all the subjects studied was 40 (12.4) years. The female to male ratio of those with thyrotoxicosis was 5.6:1. Cardiovascular complications of thyrotoxicosis, namely heart failure and atrial fibrillation, were the most common reasons for prolonged morbidity and hospitalizations. Hypothyroidism was present in five (7%), Graves' disease/hyperthyroidi sm in 63 (84%), and euthyroid in 10 (9%). Obstructive symptoms were documented in eight (13%) of the subjects with palpable goitres. The evidence from community studies is that the general testing of the population detects only a few cases of overt thyroid disease and is therefore unjustified except for certain high risk groups (Vander pump *et al.,* 1996). Among females the high risk group are adolescents, pregnant and lactating women. Puberty is a crucial period of hormonal interactions in a human life cycle (Hanna & Lafranchi, 2002). Marked changes in thyroid function occur during puberty as an adaptation to body and sexual development (Flueury *et al.,* 2001). That is why disorders affecting the thyroid gland are common in adolescents occurring, according to an estimate, in 3.7% of adolescents between the ages of 11 and 18 years (LaFranchi, 1994). Thyroid disorders in adolescents may present as goitre, a nodule or a general cluster of abnormal presentation of disorders substantially different from that in adults (Bettendorf, 2002).

There is a well known geographical dependency in thyroid diseases because of the different amounts of alimentary iodine intake that occur in different geographic locations (Knudsen *et al.,* 2000). Minimal diffuse enlargement of the thyroid gland is found in many teenage boys and girls and is almost a physiological response to the complex structural and hormonal changes occurring at that time (Flueury *et al.,* 2001; Hanna,

2002). It usually regressess (Rallision *et al.*, 1991) but occasionally it may persist, enlarge and become nodular depending on many factors like sex, family history, iodine intake and thyroid autoimmunity (Flueury *et al.*, 2001; Hanna & LaFranchi, 2002).

Low iodine intake enhances the Thyroid Stimulating hormone (TSH) sensitivity and positive influence of growth factors involved in the physiological regulation of thyroid growth. The outcome of such stimulation may be substantial in girls with mild iodine deficiency leading to the development of goitre during mid to late puberty (Flueury *et al.*, 2001). According to a study conducted in Lahore (Abbas *et al.*, 2003), it was found that the prevalence of mild iodine deficiency which seemed to result from limited amount of iodine available in food and so the resulting goitre was a physiological event by which the thyroid gland had adapted to an insufficient iodine supply. Studies by Elahi *et al.*, 2005 also showed a higher prevalence of goitre (60.0%) among female adolescents. Other studies conducted in two large cities of India also reported a 40% and 56% incidence of goitre among adolescents (Dodd & Samuel, 1993; Marwaha *et al.*, 1998). Similarly, in Northern Western part of Tunisia, 49.5% adolescents had goitre (elMay *et al.*, 1997).

Epidemiological studies have shown that the pattern of thyroid dysfunction in a community is largely determined by iodine intake level (Knudsen *et al.*, 2000). In iodine deficient communities, the incidence of hypothyroidism is low while nontoxic goitre and hyperthyroidism due to toxic nodular goitre is common and increases with age. On the other hand, in iodine sufficient areas, thyroid autoimmune diseases like Graves' disease and Hashimoto's thyroiditis are common reason of thyroid enlargement and etiologic factors for hyperthyroid and hypothyroid presentation respectively among children and adolescents (Hanna & LaFranchi, 2002). In areas where iodine deficiency is severe, they are of limited importance in the development of goitre (Fenzi *et al.*, 1986). Higher incidence of hypothyroidism particularly subclinical hypothyroidism (Milakovic, 2001) in recently developed goitre points towards a definite role of thyroid autoimmunity in goitrogenesis and nodularity among patients. This assumption is in accordance to a study in which 19.5% of goiterous adolescents are thyroid antibody positive in Lahore (Igbal, 2001). A potential reason for thyroid autoimmunity may be the consumption of excess iodized salt as a therapy to regress goitre.

2.14 INCIDENCE, PREVALENCE OF THYROID DISORDERS

Indeed, iodine deficiency is regarded as the most common cause of thyroid disorders worldwide (Delange, 1994; Vanderpump *et al.*, 1996). Epidemiological studies in iodine-deficient areas have mainly focused on the prevalence of goitre and cretinism (Gutekunst & Scriba, 1989; Gaitan & Dunn, 1992). The overall prevalence of goitre progressively increased with age (Aghini-Lombardi *et al.*, 1999).

The prevalence of thyroid disorders is higher in women than men and increases with age, reaching a peak of 21% in women and 16% in men over 74 yr of age (Canaris *et al.*, 2000). The American prevalence of subclinical hypothyroidism is estimated as 4 - 8.5% of the general population, and up to 20% of women older than 60 years, and about 2% general population are thought to be subclinically hyperthyroid (Wilson & Curry, 2005). Thyrotoxicosis is found in various age groups and in different clinical situations. For instance, a study by Luboshitzky *et al.*, 1995 and Willett, 2002 reported a prevalence of 0.8% for hyperthyroidism in children of various age groups, with lowest occurrence in age 1-2 years and peak occurrence in puberty. Muller *et al.*, 1997 and Kahaly & Dietlein, 2002 reported the occurrence of hyperthyroidism in the elderly.

In agreement with previous studies, the prevalence of both subclinical hyper- and hypothyroidism was found to be greater in women and increased with age (Boelaert & Frankyln, 2005). Studies by Edino *et al.*, 2004 in Kano, which is populated by people from various geographical areas showed that, of the 75 patients with goitres; 69(92%) were females. A 10 - year Study by Lawal *et al.*, 2003 in Ife showed that follicular cancer remains the predominant type, partly as a result of persisting dietary iodine deficiency; of the 279 (12.9%) patients, aged 13-85 years, thirty-six had malignant goitres, and most (80%) were young or middle-aged women. Of the well-differentiated cancers, follicular type was the most prevalent, being six- and 12-fold as frequent as papillary and medullary cancers (69% vs 11% and 5.6%), respectively. Lymphoma accounted for 5.6%, fibrosarcoma, 5.6% and anaplastic, 2.8%. No relationship was demonstrable between cancer type, duration of goitre and age at diagnosis.

2.16 AUTOANTIBODIES:

2.16.1 The Origin and role of Autoantibodies ((Thyroperoxidase and thyroglobulin))

Thyroglobulin antibodies (Tg Abs) are circulating immunoglobulins directed against different epitopes of the thyroglobulin molecule (Lambordia, 2005). Thyroid

microsomal antibodies (TPO Abs) are circulating immunoglobulins directed against a component of the smooth endoplasmic reticulum of thyroid cells. Several researchers have attempted to identify the mechanism triggering thyroid autoantibody production. All these studies have, until now, failed to provide a satisfactory answer. It is likely that more than one mechanism is involved and this would be consistent with antibody heterogeneity (Benveniste *et al.*, 1985*).* Various studies have suggested that there could be an antigenic stimulation at the initial step of antibody production; e.g, it has been proposed that a viral infection such as subacute thyroiditis could induce occult antigenic changes within the thyroid. This is rather unlikely in Graves' or Hashimoto's diseases where a vast majority of cases arise without any recent history of thyroid infection (Chiovato *et al.*, 1989).

Thyroid diseases are classified into two groups: autoimmune thyroid disease (AITD) and nonautoimmune thyroid disease (NAITD) on the basis of presence of antithyroid antibodies (Knobel *et al.*, 1994; Dayan & Daniels, 1996). Classic AITDs are: Graves' disease and Hashimoto's, atrophic, silent as well as postpartum thyroiditis; the rest of the thyroid disorders are considered NAITDs (Hasanat *et al.*, 2000) The autoimmune phenomenon has been observed to coexist in some cases of NAITD interfering with the disease process (Corrales *et al.*, 1994; Aust *et al.*, 1996). Although the nature of the disease may be suspected, diagnosis is often difficult, but it is essential for long term management. A few studies have observed the frequency of thyroid autoimmunity and cancer to be higher than it was before iodine prophylaxis in some countries (Harach *et al.*, 1985; Dayan & Daniels, 1996). Estimation of antithyroid antibodies has an important role for diagnosis (Ladenson, 1996). Absence of antibodies may not exclude autoimmunity, but presence of antibodies almost always confirms it except in a few situations (Dayan & Daniels, 1996). Antithyroglobulin antibody is a somewhat weaker marker for discriminating between AITD and NAITD (Toldy *et al.*, 1996) but antimicrosomal antibody is highly specific for AITD (Knobel *et al.*, 1994) and unless antibodies are checked, an appreciable number of patients with AITDs will remain undetected (Feldt-Rasmussen, 1996). Antithyroid antibodies may be positive in a limited percentage of healthy controls and in ~11% of NAITDs (Mariotti *et al.*, 1990). High iodine intake as well as widely varying concentration of dietary iodine day to day increases vulnerability to development of thyroid autoimmunity (Weetman and McGregor, 1994; Dayan and Daniels, 1996). In a Bangladesh study by Hasanat *et al.*, 2000, it was found that the prevalence of AITD among thyroid patients was 48.36%.

Specificity of antimicrosomal and antithyroglobulin antibodies were 93% and 87%, respectively**.** The prevalence of thyroid autoantibodies against thyroglobulin and microsomal antigen was investigated in a symptomless Asian population. Of 468 persons tested, an overall rate of 8.1% was detected for either antibody (i.e persons with antibodies to microsomal antigen and/or thyroglobulin) with a rate of 5.1% for autoantibodies to thyroglobulin and 6.4% for autoantibodies in women than in men especially in persons over 50 years of age.

In the study by Okosieme *et al.,* 2007 their prevalence of 4% for TgAb and 7% for TPOAb in healthy blood donors is comparable to that reported for African Americans, and greater than the reported prevalence from other African studies. The discrepancy between their findings and other African studies may in part be due to differences in methodology. Some of the studies in Africans measured thyroid antibodies using agglutination methods, which are less sensitive than more recent ELISA and radio-immunoassay techniques (Njemini *et al.,* 2002). In autoimmune diseases, autoantibodies may be the actual pathogenetic agents of the disease, the secondary consequences of tissue damage, or the harmless footprints of an etiologic agent **(**Plotz, 1993).

2.16.2 Thyroid Peroxidase (TPO) antibodies

Thyroid peroxidase a key memebrane-bound enzyme, a hemoprotein (Taurog *et al.,* 1970) active only in the presence of H_2O_2 (Magnusson *et al.,* 1987) in the formation of thyroid hormones and a major autoantigen in autoimmune thyroid diseases (Prummel & Wiersinga, 2009). The source of the H_2O_2-generating system within the thyroid is unclear. The iodination of tyrosine catalysed by peroxidase probably involves free radical mechanism (Vassart *et al.,* 1973). The TPO is the key thyroid enzyme catalyzing both the iodination and coupling reaction for the synthesis of thyroid hormone (Nakamura *et al.,* 1984): two steps of the biosynthesis of thyroxine: (a) the iodination of tyrosine residues of thyroglobulin and (b) the coupling of diiodotyrosine to produce thyroxine (Nunez, 1980). It is membrane bound and found in the cytoplasm and in high concentration on the apical microvillar surface of thyrocytes. It is of molecular weight between 100 to 105-kDa and previously known as thyroid microsomal antigen (McLachain & Rapport, 1992**)**. Multiple T & B cell epitopes exists within the molecule and the antibody response to TPO is restricted at the level of the germ line heavy and light chain variable (V) region (Mcintosh *et al.,* 1998**).** Anti-TPO autoantibodies are found in over 90% of patients with

autoimmune hypothyroidism and Graves' disease (Jaume *et al.,* 1999). Anti-TPO antibodies are mainly of the IgG class with IgG1 and IgG4 subclasses in excess (Silva *et al.,* 2003**).** The ability of an individual to develop TPO autoantibodies appears to be vertically transmitted (Phillips *et al.,* 1993) and is consistent with autosomal dominant transmission of a major gene on a polygenic background (Pauls *et al.,* 1993). The hallmark of the humoral response in Hashimoto's thyroiditis is autoantibodies to thyroid peroxidase (TPO); titres of TPO antibodies also correlate with the degree of lymphocytic infiltration in euthyroid subjects, and they are frequently present in euthyroid subjects (prevalence 12–26%) (Prummel & Wiersinga, 2009). Even within the normal range for thyrotropin (TSH), TPO antibody titres correlate with TSH levels, suggesting that their presence heralds impending thyroid failure. In assays for serum TPO antibodies, titres above an assay-dependent cut-off are a clear risk factor for hypothyroidism; in the Whickham survey the annual risk of developing hypothyroidism in TPO-positive women with normal thyrotropin levels was 2.1% (Prummel & Wiersinga, 2009). Measuring TPO antibodies in euthyroid subjects can be used to identify subjects with increased risk for hypothyroidism: e.g. as triage to measure thyrotropin.

2.16.3. Thyroglobulin (Tg) Antibodies

Thyroglobulin is a 660-kDa glycoprotein composed of two identical subunits of 330 kDa each. It is secreted by the thyroid follicular cells into the follicular lumen and stored as colloid. Each Tg molecule has around 100 tyrosine residues, a quarter of which are iodinated. These residues couple to form the thyroid hormones triiodothyronine (T_3) and thyroxin (T_4). When TSH stimulates the thyroid cell, Tg is endocytosed and hydrolyzed in lysosome releasing T_3 & T_4. The exact location of T and B cell epitopes within Tg is uncertain (Canayanniotis & Rao, 1997**)**. Thyroglobulin autoantibodies are found in less than 60% of patients with lymphocytic thyroiditis and 30% of Graves' disease patients. They are polyclonal and mainly of lgG class with all four subclasses represented. The TSH regulates the cell surface expression of TPO and Tg altering the mRNA transcription of these two proteins, possibly at the gene promoter level. These effects are mimicked by auto antibodies (both blocking and stimulating) in the sera of the patients with GD (Collison *et al.,* 1991**)**. In 1994 the World Health Organization (WHO/ICCIDD/UNICEF) proposed that a median thyroglobulin concentration of < 10 µg/L in both children and adults indicates iodine sufficiency (Vejbjerg *et al.,* 2009). Thyroglobulin (Tg), a thyroid-specific protein and precursor in the synthesis of thyroid

hormones, has been suggested as a marker of the iodine status not only in the population reflecting thyroid abnormalities, but also iodine deficiency in the population as a whole (Knudsen *et al.*, 2001; Rasmussen *et al.*, 2002). It is measurable in serum in most individuals and the serum concentration increases with increasing thyroid mass, with inflammation of the thyroid and if the TSH receptors are stimulated (Spencer, 2003).

2.16.4 Thyroid Stimulating Hormone receptor (TSH-R) Antibodies

The TSH-R is the prime autoantigen in Graves' disease and atrophic thyroiditis. It is located on the basal surface of thyroid follicular cells. In Graves' disease thyroid stimulating antibodies (TSAbs) bind to the receptor and stimulate the thyroid cell to produce excessive amount of thyroid hormones resulting in hyperthyroidism. In patients with atrophic thyroiditis the major antibody is the TSH-R blocking antibody. After binding to the receptor this antibody blocks the binding of TSH to its receptor, thus preventing stimulation of thyroid cell. This results in diminished thyroid hormone output, atrophy of thyroid gland and the clinical state of hypothyroidism. The TSH-R has 398 amino acid extra cellular domains, a 266 amino acid transmembrane domain (organized in seven loops) and an 83 amino acid intracellular domain. Antibodies binding to the amino terminal area are stimulatory whereas those binding to amino acids 261 to 370 or 388 to 403, near the cell surface, have blocking activity (Prabhakar *et al.*, 1997**).**

2.16.5 Other autoantibodies: The Na^{+}/I^{-} symporter (NIS) is the fourth major thyroid autoantigen. About a third of Graves' disease sera and 15% of Hashimoto's sera contain antibodies that inhibit NIS mediated iodide uptake *in vitro* (Ajjan *et al.*, 1998**)**. Antibodies to thyroid hormone can be found in 10% to 25% of patients with AITD and non-specific autoantibodies against DNA, tubulin and other cytoskeletal proteins can also be detected in a small proportion of patients.

2.17. THYROID DISORDERS AND AUTOANTIBODIES

Autoantibodies to thyroglobulin was first discovered by Doniach and Roitt (1956) in patients with Hashimoto's thyroiditis and subsequently led to the concept that thyroid diseases involved the progressive destruction of the thyroid gland by an autoimmune process (Bigazzi, 1979). Antithyroid antibodies are good markers for the assessment of thyroid autoimmunity (Pedersen *et al.*, 2006). The prevalence of thyroid antibodies increases with age in women as does the number of previous pregnancies; thus age would

confound the evaluation of association between thyroid antibodies and previous pregnancy.

Antithyroperoxidase antibody (TPOAb) and antithyroglobulin antibody (TgAb) are two important thyroid autoantibodies that are commonly found in patients with autoimmune thyroid diseases (Roberts & Ladenson, 2005). Several studies have suggested that TPOAb can induce antibody-dependent cell-mediated cytotoxicity (ADCC) and that TPOAb titers correlate with the severity of lymphocytic infiltration, regardless of the presence or absence of hypothyroidism (Kasagi *et al.*, 1996). However, the function of TgAb remains uncertain (Lindberg *et al.*, 2001). The two antibodies, either separately or combined, are also frequently present in the general population (Marcocci & Chiovato, 2000; Pedersen *et al.*, 2003). The National Health and Nutrition Examination Survey III study reported that more than 10% of disease-free populations are TPOAb or TgAb positive (Hollowell *et al.*, 2002). Although there have been several cross-sectional studies on the prevalence of thyroid autoantibodies, apart from the Whickham study (Vanderpump *et al.*, 1995), longitudinal studies have rarely explored the incidence and natural courses of these antibodies in the general population.

2.18. AUTOIMMUNE THYROID DISORDERS

Autoimmune thyroid disease (AITD), a multifactorial organ specific autoimmune disorder (Duntas, 2011); is seen mostly in women between 30-50 years of age (Swain *et al.*, 2005). Thyroid autoimmunity can cause several forms of thyroiditis ranging from hypothyroidism (Hashimoto's thyroiditis) to hyperthyroidism (Graves'disease). The prevalence rate of autoimmune mediated hypothyroidism is about 0.8 per 100 of which 95% of them are women. Graves' disease is about one tenth as common as hypothyroidism affecting mostly the younger individuals (Larry & Weetman, 2001). Both of these disorders share many immunologic features and the disease may progress from one state to another as the autoimmune process changes. Autoimmune hypothyroidism (AH) affects about 5 to 10% of middle aged and elderly women. If the greatest risk factor for autoimmune disease is being a female in the child-bearing years, then what could be the cause of this risk? The effect of estrogen on the immune system (Prummel *et al*., 2004). Another possibility is that foetal microchimerism could contribute to the pathogenesis of AITD. This hypothesis is also supported by the fact that chronic graft-vs-host disease shares similarities with some auto-immune diseases (Sarker & Miller, 2004). Since most

thyroid disorders are autoimmune disease; this means there is a problem with the body's normal immune system response. Although the pathogenesis of AITD has not been elucidated, several factors that have been associated with the disorder and include: genetic predisposition, nutrient deficiencies, use of certain medications affecting thyroid function and environmental factors including exposure to radiation, heavy metals, and chemical contaminants (Friedman, 2013).

Swain *et al.,* 2005 stated that genetic, environmental and endogenous factors are responsible for initiation of thyroid autoimmunity. At present, the only confirmed genetic factor lies in HLA complex (HLA DR-3) and the T cell regulatory gene (CTLA 4). A number of environmental factors such as viral infection, smoking, stress & iodine intake are associated with the disease progression. Circulating T - Lymphocytes are increased in AITD and thyroid gland is infiltrated with CD4+ and CD8+ T Cells. Wide varieties of cytokines are produced by infiltrated immune cells, which mediate cytotoxicity leading to thyroid cell destruction. About 2 to 4 percent of women and up to 1% of men are affected worldwide, and the prevalence rate increases with advancing age (Canaris *et al.,* 2000**)**. The AITD comprises a series of interrelated conditions including hyperthyroid Graves' disease (GD), Hashimoto's (goitrous) thyroiditis (HT), atrophic autoimmune hypothyroid ism, postpartum thyroiditis (PPT) and thyroid associated orbitopathy (TAO) among others. Among these, HT and GD are the commonest type and share many features immunologically. One form of the disease may change to other as the course of the immune process progresses. Many of these patients progress to hypothyroidism either spontaneously after treatment with antithyroid drugs or iatrogenically after radioiodine therapy or surgery. The development of antibodies to TPO, Tg and TSH-R is the main hallmark of AITD (Marcocci & Chiovato, 2000).

2.18.1 ETIOLOGY: The etiology of AITD is multifactorial (Swain *et al.,* 2005). Susceptibility to the disease is determined by a combination of genetic, environmental and constitutional factors among others.

2.18.2 Genetic Factors: Numerous studies show a higher frequency of AITD in family members of patients with autoimmune hypothyroidism and Graves' disease (Weetman & McGregor, 1994**)**. Both types of the disease cluster together in families, provides additional support that these conditions share common etiologic and pathogenic features. The autoimmune polyglandular syndrome type 2, involves the occurrence of autoimmune

thyroid dysfunction with other autoimmune diseases. (Type 1 diabetes mellitus, Addison's disease, pernicious anemia & vitiligo) Shared genetic factors are likely in this group of autoimmune disorders. Twin studies show increased concordance of GD and AH in monozygotic (MZ) twins, compared with dizygotic (DZ) twins (Brix *et al.*, 2001**)**.

Various techniques have been employed to identify the genes contributing to the etiology of AITD, including candidate gene analysis and whole genome screening. These studies have enabled the identification of several loci (genetic regions) that are linked with AITD, and in some of these loci putative AITD susceptibility genes have been identified. Some of these genes/loci are unique to Graves' disease (GD) and Hashimoto's thyroiditis (HT) and some are common to both diseases, indicating that there is a shared genetic susceptibility to GD and HT. The putative GD and HT susceptibility genes include both immune modifying genes (e.g. HLA, CTLA-4) and thyroid specific genes (e.g. TSHR, Tg). Most likely these loci interact and their interactions may influence disease phenotype and severity (Ban & Tomer, 2003). The most important susceptibility factor so far recognized is association of AITD with HLA-DR alleles. These MHC class-II genes play a critical role in the initiation of adaptive immune response. The HLA-DR3 is the best-documented genetic factor for GD and AH in Caucasians (Weetman, 2000**)**. In non-white populations, GD is associated with different HLA alleles.

2.18.3 Environmental Factors

2.18.3.1 Iodine: Fluoride added to water and dental products is another trigger for AITD. Furthermore, like chloride and lithium, fluoride is able to displace iodine, contributing to hypothyroidism (Moore, 2010).

2.18.3.2 Infection: No convincing evidence has indicated a role infection in AH except congenital rubella syndrome. An association has been proved between Yersinia infection and GD (Tomer & Davies, 1993**).** Yersinia contains proteins that mimic TSH-R immunologically (Jaspan *et al.*, 1996**)**.

2.18.2.3 Stress: Some studies suggest an association between antecedent major life events and Graves' disease, but a causal role of stress in autoimmune process remains to be clearly established. Smoking is a minor risk factor for the development of thyroid ophthalmopathy (Bartalena *et al.*, 1995**)**. The female preponderance of thyroid autoimmunity is most likely due to the influence of sex steroids. Estrogen use is

associated with a lower risk, and pregnancy with a higher risk for developing hyperthyroidism (Strieder & Prummel, 2003**)**.

2.19. AUTOIMMUNE FEATURES

All forms of thyroid autoimmunity are associated with a lymphocytic infiltrate in the thyroid, and these lymphocytes are largely responsible for generating both T and B cell-mediated autoreactivity. Other sites such as thyroid draining lymph nodes and bone marrow may also contain thyroid autoreactive lymphocytes in AITD. The initial autoimmune response by $CD4^+$ T cells appears to up regulate the secretion of IFN-ɤ resulting in the enhanced expression of MHC class II molecules on thyrocytes. This most likely triggers expansion of autoreactive T cells and gives rise to the characteristic inflammatory response and as the disease progresses; thyrocytes are targeted for apoptosis resulting in hypothyroidism. Another contributing factor to the observed hypothyroidism in Hashimoto's thyroiditis patients could be the circulating TSH inhibitory antibodies. Graves' disease on the other hand represents the other end of spectrum wherein the patients suffer from hyperthyroidism. The activation of thyroid specific $CD4^+$ T cells leads to the recruitment of autoreactive B cells and the mounting of thyroid stimulatory immune response via anti-thyroid antibodies (Prasad & Prabhakar, 2003).

2.19.1 AUTOIMMUNE THYROID DISEASE AND NEOPLASMS

Thyroiditis and thyroid antibodies are found in a quarter to a third of patients with thyroid cancer, and such patients have an improved prognosis (Baker & Fosso, 1993). Preexisting Hashimoto's thyroiditis is the major risk factor for the development of non-Hodgkin's lymphoma of thyroid (Matsuzuka *et al.*, 1993). Studies show an increased frequency of autoimmune thyroiditis in women with breast cancer (Giani *et al.*, 1996**).**

Autoimmune thyroid disease, the result of a complex interaction between genetic and environmental factors results when the autoreactive lymphocytes escape tolerance or ignorance. Both cell mediated and humoral immune responses contribute to tissue injury in autoimmune hypothyroidism. In Graves' disease, production of TSAbs leads to hyperthyroidism. The multistep development of disease suggests that it will be possible to restore normal tolerance and treat Graves' disease immunologically. Current approaches to medical therapeutic intervention in AITD include the use of monoclonal antibodies to selectively deplete specific T lymphocytes subsets and blocking of the T-Cell receptor MHC interaction, by vaccination with chemically altered auto antigens. The association

of thyroid antibody positivity, sometimes with transient thyroid dysfunction, has been reported in the course of immunotherapy with recombinant cytokines, interleukin-2 and interferon-γ for various cancers (Weijl *et al.*, 1993; Franzke *et al.*, 1999). Thyroid antibody related hypothyroidism has been suggested as being associated with a favourable tumour response to such therapies. Selenium deficiency is associated with AITD perhaps as a result of increased inflammatory activity arising from decreased activity of selenium containing antioxidative enzymes such as glutathione peroxidase (Zimmermann & Kohrle, 2002) whereas increasing dietary selenium or administration of selenomethionine have also been reported to diminish TPO antibody levels (Gartner *et al.*, 2002; Duntas *et al.*, 2003). Although it may seem unlikely, considerable evidence suggests there are significant gender differences in immune responses in both humans and mice. Immunization studies in both species suggest that females produce a higher titre of antibodies than males (Kuby, 2007). In fact, females in general tend to mount more vigorous immune responses. In humans, this is particularly apparent in young females. Women tend to have higher levels of CD4+ T cells and significantly higher levels of serum IgM.

2.19.2 DIAGNOSIS OF AITD

Diagnosis of AITD is based upon clinical features & supported laboratory investigations. The patient may be euthyroid, hypothyroid or hyperthyroid, according to disease type and stage. Investigations used to determine the existence and cause of hyperthyroidism (among others) are summarized (Swain *et al.*, 2005).

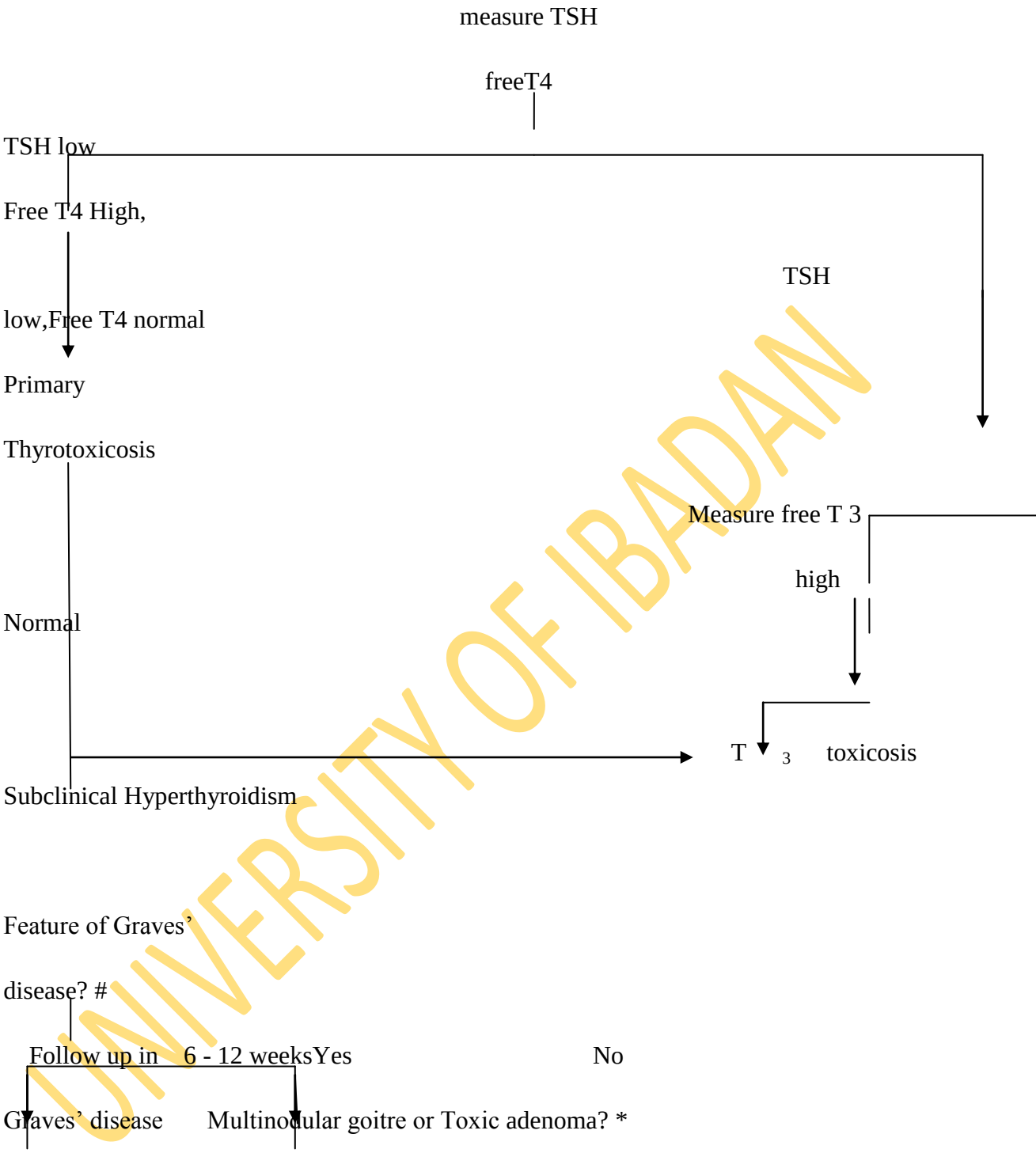
2.20 Investigations to determine the Etiology of Thyroid Dysfunction

Thyroid Antibody testing is not routinely available in developing countries; few studies have measured thyroid Abs in Africans. The significance of thyroid autoimmunity in an African setting is thus unclear (Okosieme *et al.*, 2007). The recognition that autoimmunity is a major cause of thyroid dysfunction has led to the development of tests for thyroid autoantibodies - TPOAb, TgAb and TSH receptor antibodies (TRAb). It is generally accepted that the indicence of thyroid disease is subject to geographic variation (Olurin, 1972; Okosieme *et al.*, 2007). Autoimmune thyroid disease is detected most easily by measuring circulating Abs against TPO and Tg (uncommon). Euthyroid women (5–15%) euthyroid men (2%) (Pedersen *et al.*, 2006; Fauci *et al.*, 2008) have thyroid antibodies; such individuals are at increased risk of developing thyroid dysfunction.

Almost all patients with autoimmune hypothyroidism, and Graves' disease (80%), have TPO antibodies, usually at high levels.

2.20.1 HYPOTHYOIDISM

It is a clinical syndrome resulting from a decreased or deficiency of thyroid hormones, which in turn results in a generalized slowing down of metabolic, processes (Baskin *et al.*, 2002). An insidious condition with significant morbidity and the subtle and nonspecific symptoms and signs may be attributed mistakenly to other illnesses, particularly in postpartum women and the elderly (Vanderpump & Tunbridge, 2004). Hypothyroidism is caused by any structural or functional derangement that interferes with the productions of adequate levels of thyroid hormone. It can result from a defect anywhere in the hypothalamic-pituitary-thyroid axis (Larsen *et al.*, 1998). Iodine deficiency is the most common cause of hypothyroidism worldwide. In persons living in iodine-replete areas, the causes are congenital, spontaneous because of chronic autoimmune disease (atrophic autoimmune thyroiditis or goitrous autoimmune thyroiditis (Hashimoto's thyroiditis), or iatrogenic because of goitrogens, drugs, or destructive treatment for thyrotoxicosis (Roberts & Ladenson, 2004; Vanderpump & Tunbridge, 2004). As in the case of hyperthyroidism, this disorder is divided into primary and secondary categories, depending on whether the hypothyroidism arises from an intrinsic abnormality in the thyroid or occurs as a result of pituitary disease; rarely, hypothalamic failure is a cause of tertiary hypothyroidism. Primary hypothyroidism accounts for the vast majority of thyroprivic (due to absence or loss of thyroid parenchyma or goitrous enlargement. The cause of primary hypothyrodism includes the following: Surgical or radiation-induced ablation of thyroid parenchyma. A large resection of the gland (total thyroidectomy) for the treatment of hyperthyroidism or a primary neoplasm can lead to hypothyrodism. The gland may also be ablated by radiation, whether in the form of radioiodine administration for the treatment of hyperthyroidism, or exogenous irradiation, such as external radiation therapy to the neck. In addition, primary hypothyroidism may be one manifestation of an autoimmune syndrome of poly glandular endocrine failure (Whitley, 1994). Autoimmune hypothyroidism is the most common cause of goitrous hypothyrodism in iodine-sufficient areas of the world (Whitley, 2001). The vast majority of cases of autoimmune are due to Hashimoto thyroiditis.



diffuse goitre, positive TPO antibodies, ophthalmopathy

*Confirmed by Radionuclide scan

Fig. 2.3 Algorithm for the diagnosis of AITD (Graves' disease)

Circulating autoantibodies, including anti-TSH receptor autoantibodies, are commonly found in Hashimoto thyroditis. Some patients usually do not have the goitrous enlargement lymphocytic infiltrate characteristic of Hashimoto thyroiditis. In the past, many of these patients were classified as having primary 'idiopathic" hypothyroidism, but the disease is now recognized as a type of autoimmune disorder of the thyroid, occurring either in isolation or in conjuction with intention to decrease-thyroid secretion e.g methimazole and propylthiouracil can cause hypothyroidism, as can agents used to treat non thyroid conditions (e.g. lithium, p-amino salicylic acid). Thyroid hormone resistance syndrome mutations in the thyroid hormone receptors (TR), which abolish the ability of the receptor to bind thyroid hormones (Refetoff, 2003). Patients demonstrate a generalized resistance to thyroid hormone, despite high circulating levels of T_3 and T_4, since the pituitary is also resistant to feedback from thyroid hormones, TSH levels tends to be high as well. In rare instances, there may be complete absence of thyroid parenchyma (thyroid agenesis), or the gland may be greatly reduced in size (thyroid hyperplasia). Mutations in the TSH receptor are a newly recognized cause of congenital hypothyrodism associated with a hypoplastic thyroid gland (Yen, 2000). Secondary hypothyroidism is caused by TSH deficiency, and tertiary (central) hypothyroidism is caused by TRH deficiency. Secondary hypothyroidism can result from any of the causes of hypopituitarism. Frequently, the cause is a pituitary tumor; other causes include post partum pituitary necrosis, trauma and non pituitary tumors. Tertiary (central) hypothyroidisms can be caused by any disorder that damages the hypothalamus or interferes with hypothalamic pituitary portal blood flow, thereby preventing delivery of TRH to the pituitary. This can result from hypothalamic damage from tumors, trauma, radiation therapy, or infiltrative disease factors or because of an intrinsic, inherited defect in thyroid hormone biosynthesis compensatory thyroid. Primary non goitrous hypothyroidism is characterized by loss or atrophy of thyroid tissue, resulting in maximum stimulating by TSH. Hashimoto's thyroiditis is the most frequently cause of primary hypothyroidism in developing countries where iodine intake is sufficient. Congenital hypothyroidism may be due to absence of the thyroid gland (athyreosis) or may occur secondarily to defects of thyroid hormones synthesis (Tietz, 2001). The disorder occurs once in every 3500 to 4000 births; early treatment with thyroid hormone replacement is critical if its irreversible neurological damage is to be prevented (Delange, 1997; Dussualt, 1997). Hypothyroidism can lead to metabolic

problems in adults and abnormal development during gestation and infancy (Braverman & Utiger, 2000). Severe hypothyroidism due to iodine deficiency during pregnancy is a preventable cause of cretinism, a permanent cognitive impairment of the developing foetus (Glinoer, 2000). Mild hypothyroidism during pregnancy has been associated with subtle cognitive deficits in children (Haddow *et al.*, 1999; Klein *et al.*, 2001), leading to the NRC to recommend that consideration be given to adding iodide to all prenatal vitamins (NRC, 2005). Subclinical hypothyroidism (SCH) also known as mild hypothyroidism is defined as a high serum thyroid stimulating hormone (TSH) and normal serum total/free thyroxine (T_4), triiodothyronine (T_3) concentrations associated with few or no symptoms/signs of hypothyroidism (Singh and Singh, 2011). Azad, 2010 defined SCH as TSH that is elevated in the presence of normal blood levels of triiodothyronine (T_3) and thyroxine (T_4) with or without thyroid antibodies. The prevalence of this condition is higher in women than men and increases with age, reaching a peak of 21% in women and 16% in men over 74 yr of age (Duntas & Wartofsky, 2007).

2.21 SIMPLE GOITRE (SG)

A simple (or non-toxic) goitre is defined as a diffuse or nodular enlargement of the thyroid gland that is not the result of an inflammatory or neoplastic process in an individual who is euthyroid (Studer & Ramelli, 1982) whereas a toxic goitre is one involving excessive production of thyroid hormone. It (SG) results from multinodular goitre together with diffuse goitre and in the absence of thyroid dysfunction, AITD, thyroiditis and thyroid malignancy (Laszlo *et al.*, 2006). Simple goitre occurs both endemically and sporadically (Brix *et al*, 1999). It is described as simple (endemic) goitre when the enlargement of the thyroid gland as a result of decreased production of the thyroid hormones but in sufficient quantities to meet the metabolic needs of the peripheral tissues. Although, this has been presumed to lead to increased secretion of TSH, most patients with SG have normal TSH. Therefore, the goitre maybe due to increased sensitivity to normal thyroid levels of TSH or the presence of other thyroid growth stimulating immunoglobulins. The enlargement of the thyroid gland and cellular activity help the patient to overcome the mild impairment of hormone synthesis and thus remain metabolically normal though goitrous. However, in severe cases, the compensatory responses which in such cases include high secretion and TSH are sufficient to compensate for the impairment, and the patient has both hypothyroidism and raised TSH.

2.21.1 ETIOLOGY: The etiology of goitre is interplay between environmental, individual and genetic factors (Krohn *et al.*, 2005; Brauer *et al.*, 2006) which can differ between geographic regions. In areas with endemic goitre, more than 10% of the population has a generalized localized thyroid enlargement, whereas the prevalence in nonendemic areas ranges from 0.4-5% (Wang & Crapo, 1997). The etiology of simple goitre, affecting up to 5% of a population in nonendemic areas, is incompletely understood. It is generally believed to be multifactorial in origin, but the relative contributions of genetic and environmental factors remain to be clarified (Brix *et al.*, 1999). It is generally accepted that iodine deficiency contributes to both endemic and sporadic simple goitre (Hennemann & Krenning, 1985). However, a number of environmental factors such as naturally occurring goitrogens, (Willett, 2002; Kahaly & Dietlein, 2002) goitrogenic drugs, certain infections, smoking are also of importance (Ber t-elsen & Hegedus, 1994) as well as intrinsic thyroid hormone production defects. The fact, however, that even in endemic areas, environment cannot alone account for the development of simple goitre suggests that individual factors that predispose a part of the population to goitre development must play a role in the etiology (Hennemann & Krenning, 1985). Another important factor involved in the pathogenesis of endemic goitre in Africa is thiocyanate. Thiocyanate overload results from cassava consumption. Cassava roots contain the cyanogenic glucoside linamarin (Gaitan & Cooksey, 1989). Linamarin metabolism releases cyanide, which is detoxified to thiocyanate, a known goitrogen (Delange, 1989). It competes with iodide for trapping by the sodium iodide symporter and for oxidation by the TPO (Wollman, 1962). Thiocyanate (SCN_2) promotes cell growth, has protective properties in case of toxic and mutagenic cell exposure and stimulates the immune response and the phagocytosis (Weuffen *et al.*, 1990). In addition SCN_2 affects thyroid function depending on the SCN_2 concentration. At low concentrations, stimulation of thyroid function was found (Virion *et al.*, 1980) whereas at pathologically elevated concentrations, it acts as a competitive inhibitor of the I_2 transport into the thyrocyte (Laurberg *et al.*, 2002); ie thiocyanate induces both a release of iodide from the thyroid cell and a decrease of thyroid hormone synthesis. Raw cabbage contains high doses of SCN_2 as well as other goitrogenes e.g. oxazolidone which have been supposed to harbor an even higher goitrogenous potential than SCN_2 itself. Therefore, unbalanced nutrition with cabbage has been associated with increased thyroid volume and goitre (Delange *et al.*, 1978). Smoking has been proposed as a risk factor for goitre

(Christensen *et al.,* 1984), and nodules were also found with higher prevalence in goitres of smokers compared with nonsmokers. The impact of smoking on thyroid disease could be due to increased thiocyanate levels in smokers exerting a competitive inhibitory effect on iodide uptake and organification (Knudsen *et al.,* 2002 a, b). The association is more pronounced in iodine deficiency (Knudsen *et al.,* 2002b). Thiocyanate aggravates the severity of iodine deficiency and worsens its outcome (Contempre *et al.,* 2004). However, the common association of these two factors in Central Africa is not sufficient to explain the more restricted prevalence of myxedematous cretinism (Ngo *et al.,* 1997). Among the individual factors, the influences of genes should be considered (Brix *et al.,* 1999).

A role of genetic factors is suggested by the aggregation of goitre within families. It has been hypothesized that the high familial prevalence of goitre is due to the inheritance of a predisposition gene or susceptibility to goitre development (Molla et al., 2014). This hypothesis is supported by the existence of families with vertical transmission of goitre (Bougacha-Elleuch et al., 2014), suggesting an autosomal dominant pattern of inheritance of disease susceptibility. Furthermore, two studies indicate that a number of genetic markers with association and or linkage with simple sporadic goitre may exist (Bignel *et al.,* 1997).

Radiation is another environmental risk factor not only for thyroid malignancy but also for benign nodular thyroid disease. An increased prevalence of nodular disease has been associated with exposure to radionuclear fallouts and therapeutic external radiation; this theory is being discussed by some authors to explain occupational exposure to low level radiation (Nagataki & Nystrom, 2002). A study by Sari *et al.,* 2003 has shown that that thyroid volume is also significantly correlated with body weight and body mass index. Nodular disease is more frequent (5- to 15-fold) (Reinwein *et al.,* 1988; Hegedus *et al.,* 2003) in women, but the reasons for this are poorly understood. Thus, at present, one can only speculate as to a genetic susceptibility for thyroid disease and/or a direct impact of steroid hormones. In fact, a growthpromoting effect of estrogen has been described *in vitro* in rat FRTL-5 cells and thyroid cancer cell lines and has been proposed as a possible contributing, constitutional effect of gender (Furlanetto *et al.,* 1999; Manhole *et al.,* 2001). In addition, 17-estradiol has been suggested to amplify growth factor-induced signaling in normal thyroid and thyroid tumors (Manhole *et al.,* 2001). On the other hand, pregnancy- related thyroid enlargement was clearly related to iodine

deficiency (Knudsen *et al.*, 2002), and in a study (Struve & Ohlen, 1990), increased MNG prevalence with parity was only observed in those women who had not taken iodine supplementation during an earlier pregnancy. In summary, the development of nodular disease is influenced by multiple environmental components interacting with constitutional parameters of gender and age.

2.22 MULTINODULAR GOITRE (MNG)

They are clinically recognizable enlargements of the thyroid gland characterized by excessive growth and structural or functional transformation of one or several areas within the normal thyroid tissue (Laszlo *et al.*, 2006). Thyroid glands from patients with multiple follicular nodules are referred to as multinodular goitre (Beckers, 1979). Glands from patients with a single follicular nodule are referred to by several terms, including follicular adenoma, adenomatoid nodule, or adenomatous follicular nodule, reflecting uncertainty about whether these lesions are true neoplasms or local hyperplasias (Gerber *et al.*, 1987 Hicks *et al.*, 1990). Multinodular goitre is prevalent, especially in iodine – deficient areas. Most goitre is relatively small and produces few or no clinical symptoms (Laszlo *et al.*, 2006). When present, the clinical manifestations of multinodular goitre are related to growth and function.

2.22.1 TOXIC MULTINODULAR GOITRE AND EPIDEMIOLOGY

Toxic multinodular goitre occurs when multinodular goitre is associated with clinical and laboratory evidence of thyroid hyperfunction (Laszlo *et al.*, 2006). Iodine deficiency is by far the best studied epidemiological risk factor for nodular thyroid disease (Krohn *et al.*, 2005). The prevalence of nodular thyroid disease (as well as goitre) is inversely correlated with the population's iodine intake (Delange, 1994; Delange *et al.*, 2001). This has formerly been assessed clinically by palpation, nowadays considered highly inaccurate (Jarlov *et al.*, 1998; Hegedus, 2001), but it's also clearly documented by thyroid ultrasonography. Based on ultrasound investigation, a frequency of thyroid nodular disease as high as 30–40% (in women) and 20–30% (in men) of the adult population has been reported in iodine-deficient areas. Furthermore, even minor differences in the ambient iodine supply may be reflected in the different prevalence of thyroid abnormalities; Knudsen *et al.*, (2000) found a difference in goitre prevalence (15% in mild and 22.6% in moderate deficiency) and nodule size (increased in the moderate iodine deficiency group). The prevalence of thyroid nodules seems to increase

with age (Hampel *et al.*, 1995; Aghini-Lombardi *et al.*, 1999). In a borderline iodine deficiency area, MNG was present in 23% of the studied population of 2656 Danish people aged 41 to 71 yr and increased with age in women (from 20 to 46%) as well as men (from 7 to 23%) (Knudsen *et al.*, 2000). In contrast, the relation between age and thyroid volume is less coherent, whereby in iodine-deficient areas (except for severe deficiency), thyroid enlargement peaks around 40 yr with no tendency for further increase (Knudsen *et al.*, 2002a). Interestingly, similar observations have been made in an iodine-sufficient area. Thyroid nodules are found with higher frequency in enlarged thyroid glands, although all clinicians will agree that they may also be present in an otherwise normal thyroid gland (Hampel *et al.*, 1995; Aghini-Lombardi *et al.*, 1999). The correlation between iodine supply and prevalence of nodular thyroid disease can similarly apply to TMNG. The high frequency of thyroid autonomy, which accounts for up to 60% of cases of thyrotoxicosis in iodine deficient areas, is largely due to TMNG (Laurberg *et al.*, 1991; Delange *et al.*, 2001). Although goitre and euthyroid and toxic nodular thyroid disease share the common and important epidemiology of iodine deficiency, it needs to be stressed that most epidemiological conclusions are derived from cross-sectional studies (Delange *et al.*, 2001). Thyroid nodules (and goitre) also occur in individuals without exposure to iodine deficiency, and not all individuals in an iodine-deficient region develop goitre. Moreover, there is a strong clustering of goitre in families. Screening has been performed for other "environmental factors" (Knudsen *et al.*, 2002a). Smoking has been proposed as a risk factor for goitre (Christensen *et al.*, 1984). The impact of smoking on thyroid disease could be due to increased thiocyanate levels in smokers exerting a competitive inhibitory effect on iodide uptake and organification (Knudsen *et al.*, 2002b). The association is more pronounced in iodine deficiency (Knudsen *et al.*, 2002b). Radiation is another environmental risk factor not only for thyroid malignancy but also for benign nodular thyroid disease. An increased prevalence of nodular disease has been associated with exposure to radionuclear fallouts and therapeutic external radiation, and this theory is being discussed by some authors to explain occupational exposure to low level radiation (Shibata *et al.*, 2001; Nagataki & Nystrom, 2002). Nodular disease is more frequent (5- to 15-fold) (Reinwein *et al.*, 1988; Hegedus *et al.*, 2003) in women, but the reasons for this are poorly understood. Thus, at present, one can only speculate as to a genetic susceptibility for thyroid disease and/or a direct impact of steroid hormones. In fact, a growthpromoting effect of estrogen has been

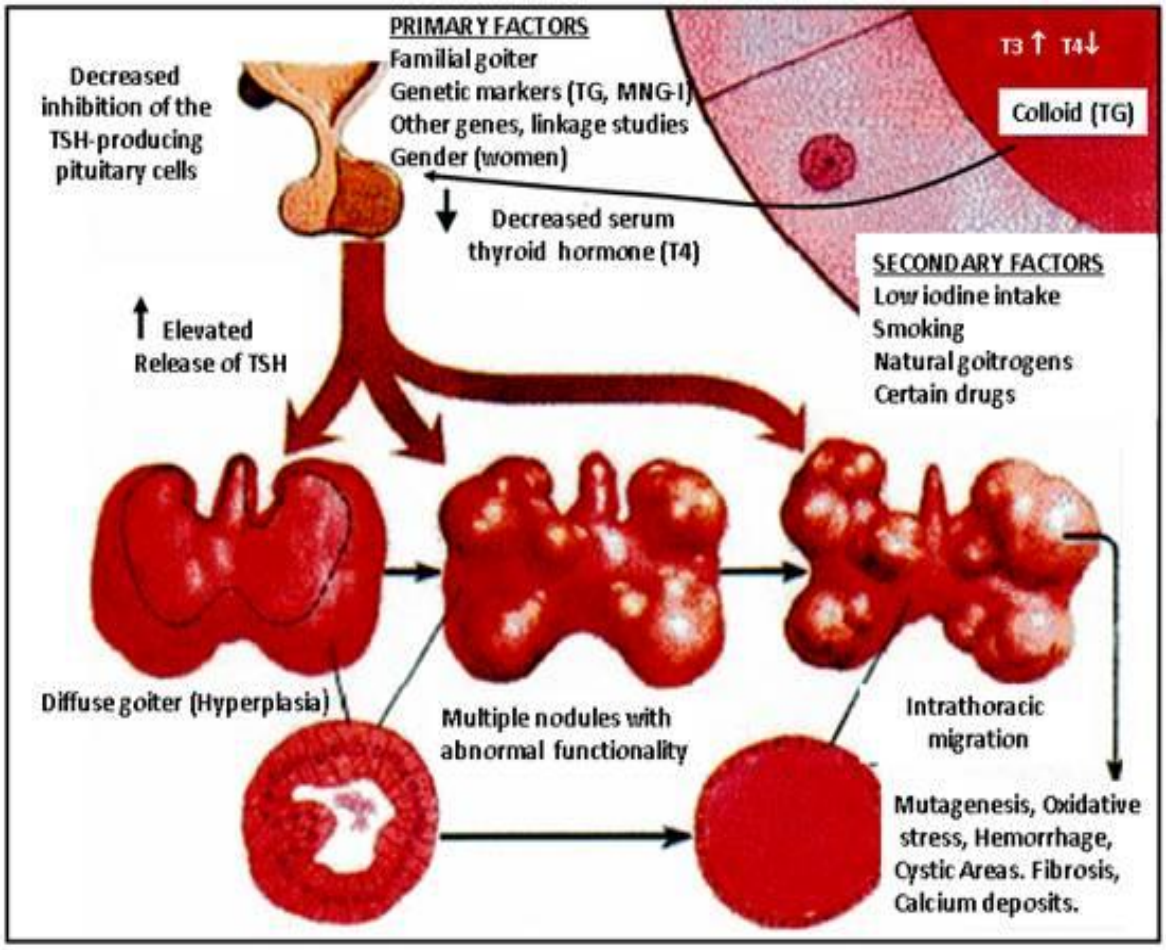


Fig. 2.5: Multinodular goitre formation. Mild iodine deficiency associated or not with smoking, presence of natural goitrogenic, drugs, familial goitre, genetic markers and gender (women) will decrease the inhibition of serum T_4 on the pituitary thyrotrophs. Increased TSH production will cause diffuse goitre followed by nodule formation. Finally, after decades of life, a large multinodular goitre is present with cystic areas, hemorrhage, fibrosis and calcium deposits (Geraldo Medeiros-Neto, 2010)

described *in vitro* in rat FRTL-5 cells and thyroid cancer cell lines and has been proposed as a possible contributing, constitutional effect of gender (Furlanetto *et al.*, 1999; Manole *et al.*, 2001).

In addition, 17β-estradiol has been suggested to amplify growth factor-induced signaling in normal thyroid and thyroid tumors (Manhole *et al.*, 2001).

2.23 THYROID CANCERS

INCIDENCE AND PREVALENCE:

Most thyroid cancers are sporadic and no single cause can be identified; (radiation and genetics are two important causal factors) A proportion of thyroid cancers are associated with radiation as an etiologic factor and a proportion have a genetic link (McDougall, 2007). The radiation is usually external (McDougall, 2007). Radiation causes mutations that can be carcinogenic. There are also familial thyroid cancers that are associated with genetic abnormalities. This is best understood for familial medullary cancer and multiple endocrine neoplasia (MEN 2) syndromes. There is increasing evidence that some cancers of follicular cells are also familial. Genetic defects in the *RET* protooncogene are the cause of 25%–30% of medullary cancers and 100% of the MEN 2A and 2B syndromes. Approximately 1.1% of all cancers arise from the thyroid and 1.7% of cancers in women compared with 0.5% in men are primary thyroid cancers (McDougall, 2007). Thus, thyroid cancer is about three times more common in women. This One exception to the gender difference occurs in prepubertal children, in whom the incidence in boys and girls is about equivalent (McDougall, 2007). The average age of the patient with differentiated thyroid cancer is 35–40 years. The peak incidence at about 40 years is different from most malignancies that are more prevalent with advancing age. From 2005-2009, the median age at diagnosis for cancer of the thyroid was 50 years of age (Howlader *et al.*, 2009). Approximately 1.8% was diagnosed under age 20; 15.5% between 20 and 34; 20.4% between 35 and 44; 24.3% between 45 and 54; 19.0% between 55 and 64; 11.7% between 65 and 74; 5.9% between 75 and 84; and 1.4% 85+ years of age. The age-adjusted incidence rate was 11.6 per 100,000 men and women per year. These rates are based on cases diagnosed in 2005-2009 from 18 SEER (Surveillance epidemiology and end results) geographic areas (National Cancer Institute, 2011).

Although some thyroid cancers can spread and cause death, for many people thyroid cancer has also long been recognized to exist in a subclinical form. More than 50

years ago, pathologists reported that thyroid cancer (particularly papillary histology) was a common autopsy finding, despite its never having caused symptoms during a person's life (VanderLaan, 1947). This finding has been replicated in several autopsy studies, (Solares *et al.*, 2005) the most methodical of which was from Harach *et al.*, (1985) who systematically sectioned 101 thyroid glands in 2- to 3-mm slices. They found that 36% of people not known to have thyroid cancer during their lifetime nonetheless had 1 or more foci of thyroid cancer. However, because many of the cancers identified by Harach *et al* were small (far smaller than the 2-3 mm between the slices), they reasoned that many were missed by their technique. They went on to calculate that, if sectioned finely enough, virtually every person would be found to harbor a thyroid cancer. As diagnostic techniques for thyroid cancer have become more sensitive, particularly with the advent of ultrasound and fine-needle aspiration, it has become possible to detect this subclinical reservoir. Thus, while increasing incidence of thyroid cancer might reflect an increase in the true occurrence of disease, it might also reflect increased diagnostic scrutiny or changes in diagnostic criteria. Examination of the reasons underlying an increase in the incidence of thyroid cancer is important, because if there is an increase in the true occurrence of disease, efforts should be made to address its cause and aid those at greatest risk of developing the disease. If the observed increase is based simply on increased diagnostic scrutiny, then the challenge to the health care community is how to identify which patients truly warrant treatment, with its attendant risks. Previous studies have indicated that ionizing radiation, particularly during childhood, is the main established risk factor for thyroid cancer. History of benign nodules/adenoma, goiter, iodine deficiency or high-iodine intake might be other associated factors (Seyed *et al.*, 2011). The wide variety of dietary, lifestyle and environmental exposures, as well as the genetic variation among people in developing countries can provide valuable new information on factors that contribute to cancer or that protect against it (Tanuja *et al.*, 2004).

The most common presentation for thyroid cancer is as a painless nodule in the thyroid gland or an enlarged lymph node in the neck, although the cancer is sometimes first detected as a distant metastasis. There is no role for serum thyroglobulin assessment in distinguishing benign from malignant thyroid diagnoses. However, when the presenting tumor is distant from the neck, immunohistochemical assessment of thyroglobulin can often elicit the primary source of the tumor. Both normal and

malignant thyroid follicular cells usually produce thyroglobulin that can be measured in the peripheral circulation. Since no other tissues have been found to produce thyroglobulin, this protein serves as a specific marker for papillary or follicular thyroid cancers after complete surgical resection of the thyroid and radioiodine ablation of remnant thyroid tissue, removing all benign sources of thyroglobulin. Although nuclear scanning with radioactive iodine provides an effective means of detecting thyroid cancer after resection of the primary tumor and thyroid gland, thyroglobulin measurements often prove more sensitive for this purpose (Whitley & Ain, 2004). Appreciation of the clinical utility of serum thyroglobulin in the follow-up of differentiated thyroid carcinomas has prompted consensus panels to report tumor assessment strategies based upon serum thyroglobulin measurements (Mazzaferri *et al.*, 2003; Schlumberger *et al.*, 2004).

Thyroid cancer is statistically a minor health problem that accounts for about 0.4% of all cancer deaths (Pacini and Degroot, 2006). In general, lifestyle factors have only a small effect on the risk of thyroid cancer; a possible protective effect of tobacco smoking has been reported. Because of the (small) increase in risk of thyroid cancer associated with iodination programs, these should be supervised, so that the population does not receive excess iodine (Nagataki & Nystrom, 2002). The thyroid gland is highly sensitive to radiation-induced oncogenesis. Numerous investigations have shown that thyroid cancers are 2 - 4 times more frequent in females than males (Nagataki & Nystrom , 2002). The incidence of thyroid cancer is approximately the same for males and females before puberty and after the female menopause. This suggests an effect of estrogen and/or other factors associated with pregnancy (Nagataki & Nystrom, 2002) several investigations have thus shown a positive correlation between parity and the incidence of thyroid cancer, the relative effect increasing with the number of pregnancies. The increase in risk is pronounced in childbearing in the latter half of the reproductive life and in artificial menopause (Nagataki & Nystrom, 2002). Its clinical importance, however, is much greater because up to 4% of the population harbour clinically detectable thyroid nodules that must raise the possible diagnosis of thyroid cancer. The Overall incidence of thyroid cancer is between 1 and 10 per 100,000 populations in most countries (Grebe and Hay, 1995). The two main types of differentiated cancers (papillary and follicular) comprise approximately 60% to 90% of this.

2.23.1 DNA METHYLATION AND THYROID NEOPLASIA

Benign and malignant thyroid neoplasms have a monoclonal origin suggesting

that selected, early genetic events play a key role in determining the clonal expansion of a single follicular cell (Namba *et al.*, 1990; Apel *et al.*, 1995). One of the earliest events observed in the progression of thyroid neoplasms is a widespread modification of methylation pattern of genomic DNA (Matsuo *et al.*, 1993). Abberant DNA methylation is a general rather than a site - specific process and represents a predisposing event for silencing or mutating critical genes. The Cpg islands, infact are sensitive targets of abberant methylation, methylated cytosines residues being susceptible to subsequent deamination to thiamine. Indeed, forced chemical demethylation of thyroid cancer cells by 5'azacytidine treatment is able to restore thyroid differentiation properties, including iodine uptake (Venkataraman, 1999)

2.23.2 ANTIGENICITY OF THYROID EPITHELIAL CANCERS.

Expression of Expression of thyroid antigens: Two different types of molecules may serve as antigens in thyroid cancer. One class of these molecules cell specific differentiati on proteins, such as thyroglobulin, thyroid peroxidase, and the thyroid stimulating hormone (TSH) receptor. A second class include antigens associated with cellular transformation and the progression to neoplasm.

2.23.3 Differentiated thyroid antigens: The study of autoimmune thyroid disease has identified thyroid specific proteins that are antigenic in humans and that are also present on cell membrane of thyroid cancers (Haapala *et al.*, 1994).These include thyroglobulin, thyroid hormones, thyroid peroxidase (TPO) and the TSH receptor. Monitoring some of these proteins frequently is useful in the diagnosis and treatment of thyroid cancer and may have important implications for future immunotherapy.

2.23.4 Receptor for Thyroid Stimulating hormone: The thyroid stimulating hormone is the main autoantigen in Graves' disease (Barnett & McGregor, 1994). Several investigators have identified a higher than expected incidence of thyroid cancer in patients with Graves' disease and have suggested a possible link between TSH – stimulating antibodies and the progression of thyroid cancer (Belfiore *et al.*, 1990; Mazzaferri, 1990). A strategy that selectively blocks the TSH receptor on thyroid cancer cells could possibly inhibit some aspect of tumorigenesis. This is currently being investigated in several thyroid cancer cell lines. Although no evidence has definitively

implicated this receptor in tumorigenesis, it has been suggested that TSH can act as a growth factor for differentiated carcinoma (Mazzaferri, 1990; Brabant *et al.*, 1991).

CHAPTER THREE
MATERIALS AND METHODS

3.1 The Study Design

The study was a case control study conducted in the Surgery Outpatient (SOP) and Medical Outpatient (MOP) clinics, University College Hospital - (UCH) Ibadan and the Endocrine Unit of the Lagos University Teaching Hospital (LUTH), Nigeria. The UCH and LUTH are referral centres that receive samples from all parts of the country. Study protocol was approved by the UI/UCH Institutional Review Committee (UI/IRC/04/0027, see Appendix). Informed consent was obtained from the subjects before recruitment into the study. An average of five to seven patients was seen at each clinic day and all new cases of thyroid disorders that met the inclusion criteria and gave consent to participate in the study were recruited.

Exclusion Criteria: Thyroid disorder patients already on treatment, Conditions related to endocrine or immune system infections, pregnancy, contraceptives, blood donors who had a personal or family history of thyroid disease and those receiving supplements, radioiodine treatment, immunomodulatory medications e.g. corticosteroids, amiodarone or lithium were excluded from the study.

3.1.1 The study population: The study population consisted of one hundred and thirty-seven (137) subjects drawn from the potential target groups for evaluation of study**.**

3.1.2 Control subjects: Subjects who as far as possible are known to be apparently healthy without any nutritional deficiency and are not on any known nutritional supplement or contrapceptives were selected. They were made up of one hundred and thirty-six (136) apparently healthy individuals. Participation in the study was purely voluntary.

3.1.3 Sample size determination: The prevalence of thyroid disorders is yet to be established in this environment, therefore the sample size was determined as follows:

From the Surgery Outpatient records department of the University College Hospital (UCH).

Incidence was calculated based on the 2006 Medical records of the UCH Ibadan; one of the years which had the most comprehensive record of patients during the period this research was carried out. Other years within the research period had records varying from 3-6 months. During this one - year period of approximately 12 months in 2006; 42 patients with all varieties of thyroid disease were seen and treated in this hospital, ranging from congenital hypothyroidism (2 cases), simple and diffuse non-toxic goitre (16 cases), hyperthyroidism (2 cases), thyrotoxicosis with diffuse goitre (12 cases), thyrotoxicosis with toxic multinodular goitre (5 cases), nontoxic goitre, unspecified (3), non-toxic multinodular goitre (1case), iodine-deficiency related (endemic) goitre, unspecified (1 case). There was a noticeable yearly increase in the number of new cases from about sixteen in 2004 to about thirty - six in 2009 (pls see appendix). The age of the patients ranged from 12 to 70 years.

3.2. Anthropometric measurement

3.2.1. Weight: This was taken with a beam balance scale on a flat surface with the subject on light clothing, without shoes. The subject stood on the pre-zeroed scale and the readings were recorded in kilograms (Kg).

3.2.2. Height: This was measured against a flat, vertical surface with the subjects standing upright on a firm level ground without raising their heel from the ground. The feet were kept together without the shoes on; while the back and heel were aligned with a ruled bar against a vertical surface. A sliding headpiece was placed on the vertex of the subject's head and the reading was recorded to the nearest meter.

3.2.3. Body Mass Index (BMI): The BMI was calculated from the weight in Kg (kilograms) and height in (meter) of an individual; unit in (kg/ m^2).

$$\textbf{BMI} = \frac{\text{Weight (kg)}}{\text{Height}^2\ (m^2).}$$

3.2.4 Systolic and Diastolic Blood Pressure Measurement

This was measured using a Sphygmomanometer after the subjects were allowed to be relaxed in a sitting position for at least 5 minutes. The blood pressure of the control group was also measured.

3.3 RECRUITMENT OF SUBJECTS

The recruited subjects made up of two hundred and seventy - three (52 males, 221 females) consenting age-matched subjects, 18 – 65 years were consecutively enrolled for this study based on clinical symptoms and thyroid function tests. They were divided into four groups as shown below:

Group 1: 136 apparently healthy subjects (controls) (110 females and 26 males)

Group 2: 79 Hyperthyroid (62 females and 17 males)

Group 3: 15 Hypothyroid (12 females and 3 males)

Group 4: 43 Simple non toxic goitres (37 females and 6 males)

Socio-demographic characteristics and anthropometric indices were obtained. Spot urine samples were collected for assessment of iodine status (IOD) using colorimetric method (Sandell Kolthoff, 1937).

3.3.1 COLLECTION OF SAMPLES:

About 10ml of venous blood sample was obtained from the antecubital fossa using disposable pyrogen-free needles and syringes (Bectron-Dickinson, Dublin, Ireland), this was put into heparinized tubes and centrifuged at 3000g rpm for 5 minutes. The plasma samples were decanted and frozen at -20^{o}C until time for analysis for the following: Trace elements - Copper, Iron, Zinc, Selenium, Arsenic, Cadmium, Lead and Nickel were determined in plasma using Atomic Absorption Spectrophotometer (AAS); Buck Scientific, 205 Atomic Spectrophotometer, Connecticut USA.

Thyroid function tests (TFTs) - Total Triiodothyronine (T_3), Total Thyroxine (T_4), Free Triiodothyronine (FT_3), Free Thyroxine (FT_4), Thyroid Stimulating Hormone (TSH), Thyroglobulin (Tg), autoantibodies (Antithyroid Peroxidase (TPOAb) and Antithyroglob u-lin (TgAb) were determined in plasma using Enzyme – Linked Immunosorbent Assay (ELISA). Random urine samples (20ml) were also collected in acid-washed containers

for the determination of urinary iodine (UI). Urine samples were also stored at -20^{o}C until analyzed.

3.4 DETERMINATION OF TRACE ELEMENTS BY ATOMIC ABSORPTION SPECTROPHOTOMETRY

The technique of atomic absorption spectrophotometer (AAS) technique was employed for the determination of Zinc, Copper, Iron, Selenium, Arsenic, Cadmium and Lead. Serum zinc was by the method of Smith *et al.*, (1979); copper by method of Osheim (1983); selenium by method of Phleban *et al.*, (1982). Alpha atomic absorption spectrophotometer BUCK 205 model (USA) at observed wavelength (pls see table 3.0) for each trace metal at IITA Ibadan, Institute of international and Tropical Agriculture, Ibadan was used in the analysis. Results were displayed digitally on the instrument in ppm (parts per million). They were converted to µg/100ml by multiplying by 100 as for zinc, e.g reading of 0.88ppm, is equivalent to 88µg/100ml; copper, 1.0ppm = 100µg/100ml copper.

Another commercial standard with a target value of 200µg/100ml copper (2ppm) obtainable from Boerhinger Manheim (Boerhinger house, Sussex, England) served as quality control samples.

3.4.1 PRINCIPLE OF ATOMIC ABSORPTION SPECTROPHOTOMETRY

In AAS, the element of interest is not appreciably excited in the flame, but is merely dissociated from its chemical bonds and placed in an unexcited or ground state (neutral atom). This neutral atom at a low energy level it is capable of absorbing radiation at a very narrow bandwidth corresponding to its own line spectrum. A hollow cathode lamp with the cathode made of the material to be analyzed is used to produce a wavelength of light specific for the analyte. When the light from the hollow cathode lamp enters the flame, some of it is absorbed by the ground-state atoms in the flame, resulting in a net decrease in the intensity of the beam from the lamp. The process is referred to as atomic absorption. The amount of light absorbed is proportional to the concentration of the element in solution.

Quality Control: This was done using known quality control standards for each element of interest; these standards were first validated and run alongside with all the samples. The standardization was repeated periodically to assay for any intra-assay drift.

3.5 PLASMA ZINC DETERMINATION: (The method of Smith *et al.,* 1979)

3.5.1 Principle

A fivefold dilution of serum was aspirated and the zinc level was determined by comparing the signal from the diluted serum with signal of aqueous standards prepared in a matrix of 5% glycerol to stimulate the viscosity of the diluted serum. The wavelength employed is 214mm. In the AAS vaporized ground state (unexited) zinc atoms absorb light of the same wavelength as that emitted by the element in the excited state. The amount of light absorbed is proportional to the concentration of Zinc in the solution. Zinc is not appreciably excited in the flame but merely dissociated from its chemical bonds and placed in an unexcited or ground state where it absorbs radiation at a very narrow bandwidth corresponding to its line of spectrum; a hollow cathode lamp with the cathode made up of zinc is used to produce a wavelength of light 213.9 nm specific for Zn.

Results and Calculations: Values were displayed digitally in ppm; these were converted to µg/100ml as for Zinc.

3.6 DETERMINATION OF PLASMA COPPER

The method of Osheim, 1983 was used for copper determination

3.6.1 Principle

Copper is not appreciably excited in the flame but merely dissociated from its chemical bonds and placed in an unexcited or ground state where it absorbs radiation at a very narrow bandwidth corresponding to its line of spectrum; a hollow cathode lamp with the cathode made up of Cu is used to produce a wavelength of light 324.8nm specific for Copper.

3.7 DETERMINATION OF PLASMA ARSENIC

The method of Prasad *et al.*, 1996 modified by Kaneko, 1999 was used for Arsenic assay

3.7.1 Principle:

Arsenic is not appreciably excited in the flame but merely dissociated from its chemical bonds and placed in an unexcited or ground state where it absorbs radiation at a very narrow bandwidth corresponding to its line spectrum; a hollow cathode lamp with the cathode made up of As is used to produce a wavelength of 195.7 nm specific for As.

3.8 DETERMINATION OF PLASMA IRON

Samples for plasma iron, as well as their standards were diluted 1:1 with ultra pure water (1 part of plasma plus 1 part of ultra pure water. Blanks and standards were prepared with 10% glycerin) and read with a wavelength of 213.9nm (Prasad, 1966; Sunderman, 1973).

Table 3.0: The operating characteristics of AAS for Fe, Cu and Zn assays

Element	Fe	Cu	Zn
Wavelength	248.3	324.7	213.9
Slit	1	2	2
Burner height	6	7	7
Gas mixture	Air acetylene	Air acetylene	Air acetylene
Acetylene flow	3,3	1.8	13.5
Lamp current (mA)	8	4	4
Detection limit (µg/dl)	0.003	0.001	0.009
Sensitivity (µg/dl)	0.05	0.03	0.009

KEY:

mA = milliampere

µg/dl = microgram per decilitre

mg/l = ppm = RXD

Where,

R = AAS reading

D = Dilution factor

3.8.1 DETERMINATION OF PLASMA CADMIUM.

Samples for plasma cadmium as well as their standards were diluted 1:1 with ultra pure water (1 part of plasma plus 1 part of ultra pure water. Blanks and standards were prepared with 10% glycerin) (Prasad, 1966; Sunderman, 1973; Analytical methods for AAS, Norwalk, 1976 and Tietz 1976).

3.8.2 DETERMINATION OF SELENIUM

Samples for plasma selenium as well as their standards were diluted 1:1 with ultra pure water (1 part of plasma plus 1 part of ultra pure water. Blanks and standards were prepared with 10% glycerin) (Prasad, 1966; Sunderman, 1973; Analytical methods for AAS, Norwalk, 1976 and Tietz 1976).

3.8.3 DETERMINATION OF BLOOD LEAD.

Samples for blood lead analysis as well as their standards were diluted 1:1 with ultra pure water (1 part of plasma plus 1 part of ultra pure water. Blanks and standards were prepared with 10% glycerin) (Prasad, 1966; Sunderman, 1973; Analytical methods for AAS, Norwalk, 1976 and Tietz 1976) and analysed.

Principle

3.9. URINARY IODINE ASSAY (SANDELL-KOLTHOFF METHOD, 1937)

3.9.1 Principle of Urinary Iodine

Urine is digested in strong ammonium persulphate. Following this step, iodide is measured by its catalytic action on the reduction of the ceric ion (Ce^{4+}) to the cerous ion (Ce^{3+}) coupled to the oxidation of arsenite As^{3+}, to As^{5+}. This reaction called the Sandell-Kolthoff reaction is represented thus:

$$2Ce^{4+} + 2I \longrightarrow 2Ce^{3+} + I_2$$

The ceric ion (Ce^{4+}) has a yellow color, while the cerous (Ce^{3+}) is colourless. Thus, the course of reaction can be followed by the disappearance of yellow color. Sulfuric acid and chloride are both important and component of the reaction mixture: Sulfuric acid increases catalysis and chloride stabilizes it by inhibiting oxidation of iodide to iodate.

Quality Control: Known iodine standards were included and run alongside with each batch of the sample. All samples were run in duplicates.

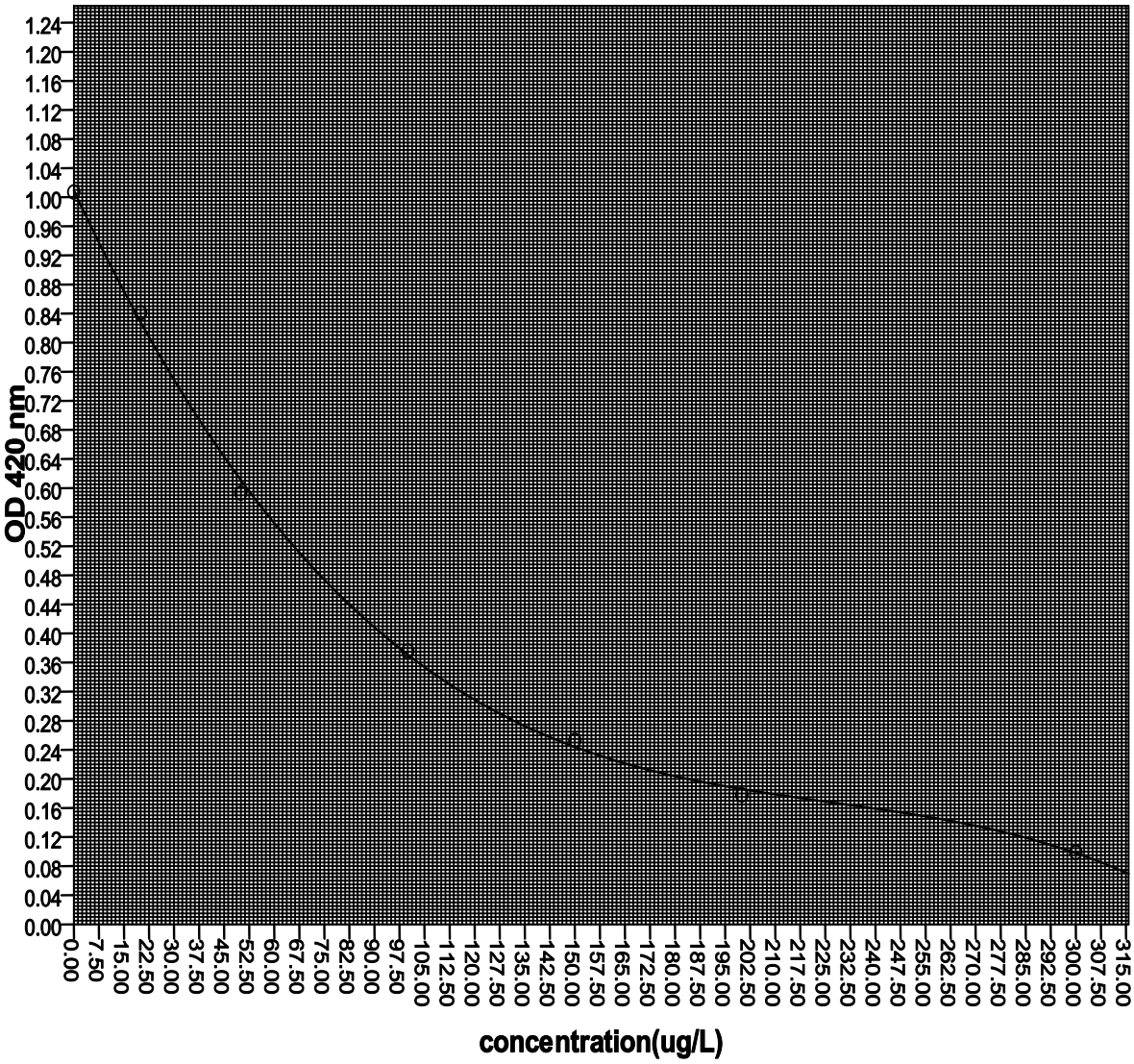


Fig. 3.0: Standard curve of urinary iodine concentration

3.9.2 CALCULATION OF URINARY IODINE IN SUBJECTS

A standard curve was constructed by; plotting the concentration of iodine of each standard on the abscissa against its optical density at 420nm on the ordinate. The results were calculated as micrograms of iodine per litre of urine.

3.5. Quantification of Thyroid Peroxidase IgG antibodies by Enzyme linked immunosorbent assay (ELISA) method

The method is essentially based on the adaptation of Rapoport and McLachlan, 1996 by the manufacturer of the kit (Genesis diagnostics, Cambridgeshire CB6 1SE, UK)

3.5.1. Principle

Diluted serum samples are incubated with TPO immobilised on microtitre wells. After washing away unbound serum components, rabbit anti-human IgG conjugated to horseradish peroxidase is added to the wells, and this binds to surface-bound antibodies in the second incubation. Unbound conjugate was removed by washing, and a solution containing 3, 3', 5, 5'- tetramethylbenzidine (TMB) and enzyme substrate added to trace specific antibody binding. Stop Solution terminated the reaction and provides the appropriate pH for colour development. The optical densities (directly proportional to antibody activity in the sample) of the standards, controls and samples were measured using a microplate reader at 450nm.

Quality Control: All assays were carried out as instructed by the manufacturers of the kit, provided by the kit suppliers.

Values above 200U/ml were regarded as positive;

values below 200U/ml were regarded as negative;

values above 3000U/ml were repeated at a higher dilution.

Intra-assay precision was determined by testing 3 samples including a low, moderate and high specimen 6 times within a single assay run to obtain their mean, standard deviation (SD) and coefficient of variation were calculated **CV**= $\frac{\textbf{S.D}}{\textbf{Mean (й)}}$ **x 100**.

The kit was calibrated against the NIBSC standard 66/387.

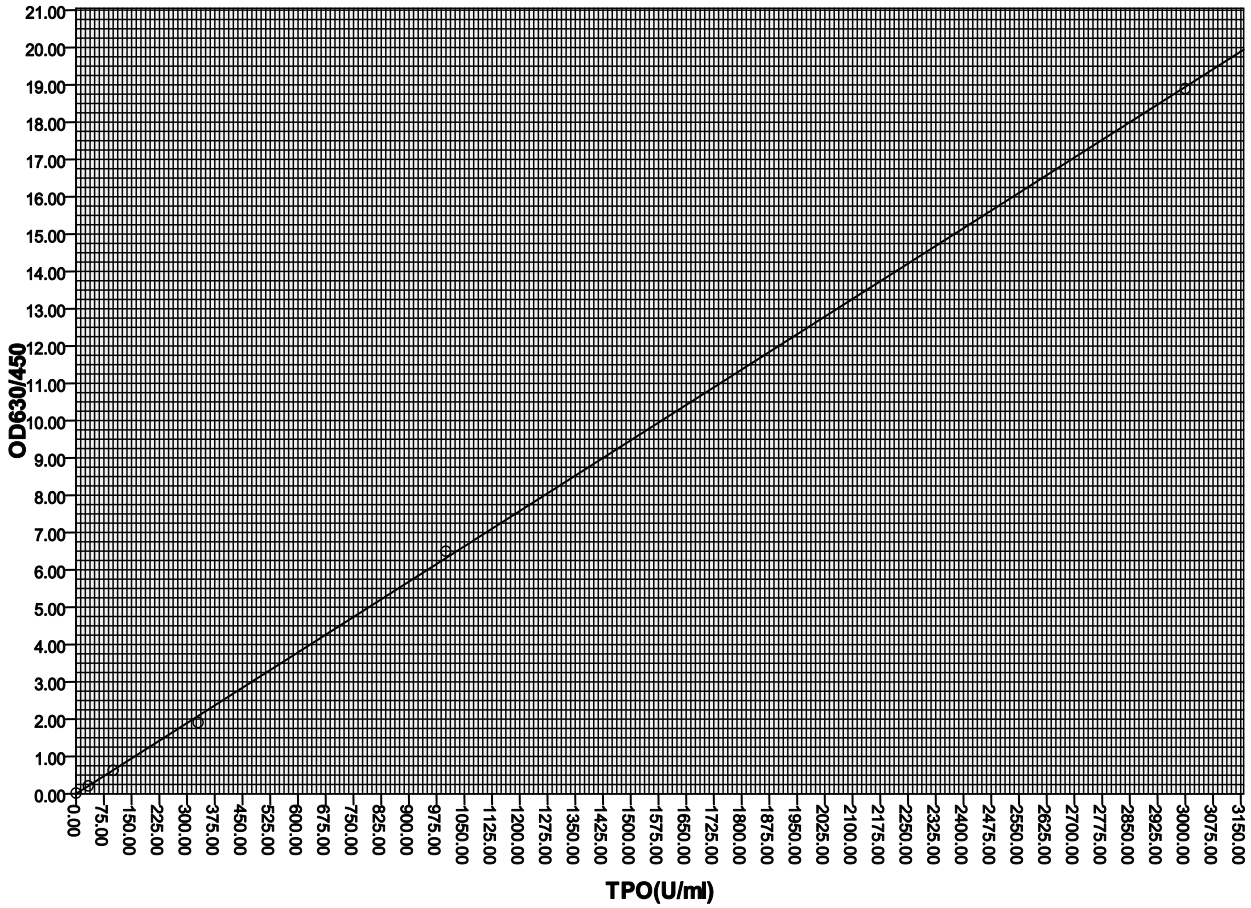


Fig. 3.1: Standard curve for thyroid peroxidise (TPO) assay

Sample	1U/ml	2U/ml	3U/ml
Mean	93.1	287.3	942.5
S.D	1.75	8.6	88.1
C.V%	2.01	3.1	9.6

3.6. Quantification of Thyroglobulin IgG (TgAb) antibodies by Enzyme linked immunosorbent assay (ELISA) method.

The method is essentially based on the adaptation of Kuppers *et al*., 1993 by the manufacturer of the kit (Genesis diagnostics, Cambridgeshire CB6 1SE, UK (Genesis Diagnostics, UK)

3.6.1. Principles

Diluted serum samples are incubated with thyroglobulin immobilized on microtitre wells. After washing away unbound serum components, rabbit anti-human IgG conjugated to horseradish peroxidase is added to the
wells, and this binds to surface-bound antibodies in the second incubation. Unbound conjugate is removed by washing, and a solution containing 3, 3', 5, 5'-tetramethylbenzidine (TMB) and enzyme substrate is added to trace specific antibody binding. Addition of stop solution terminates the reaction and provides the appropriate pH for colour development. The optical densities of the standards, controls and samples are measured using a microplate reader at 450nm. Optical density is directly proportional to antibody activity in the sample.

Quality Control

Positive and Negative controls were obtained.
Values above 120 IU/ml were considered positive
values below 120 IU/ml were considered negative and
values above 9000 IU/ml were repeated at a higher dilution.
The antithyroglobulin antibodies ELISA was calibrated against the WHO 1st International Reference Preparation 65/93.

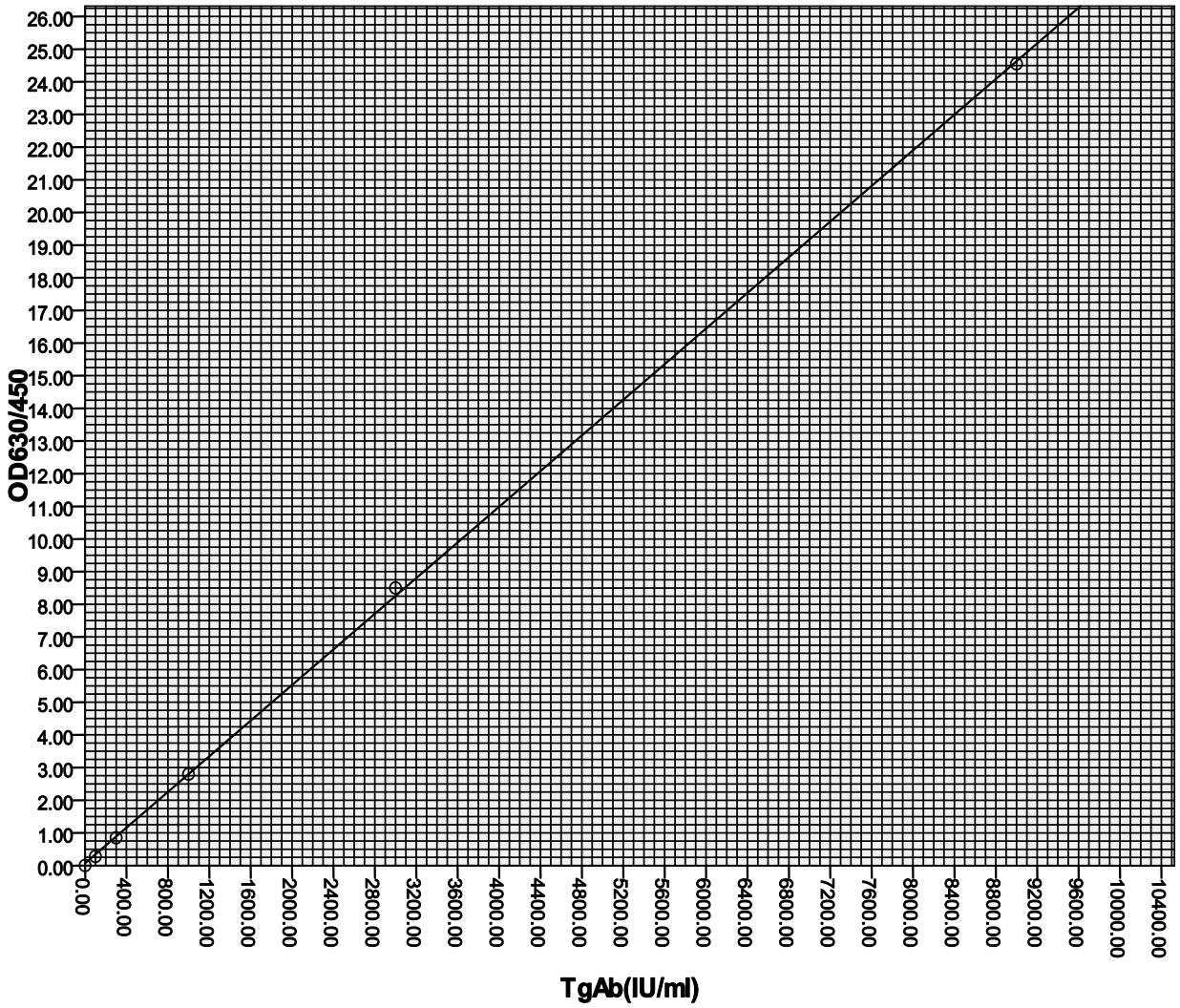


Fig. 3.2: Standard curve for thyroglobulin antibodies assay

3.7. Quantification of Thyroglobulin antigen (TgAg) IgG antibodies by Enzyme linked immunosorbent assay (ELISA) method.

The method is essentially based on the adaptation of Mariotti *et al.*, 1995 by the manufacturer of the kit (Genesis diagnostics, Cambridgeshire CB6 1SE, UK) (Genesis Diagnostics, UK)

3.7.1. Principles

Serum specimens were incubated for 60 minutes to allow Tg to bind to anti-Tg antibody-coated wells. After washing away unbound serum constituents, bound Tg was detected using rabbit anti-human Tg conjugated to horseradish peroxidase. After 30 minutes incubation, unbound conjugate was removed by washing, and TMB Substrate was added. A blue colour develops in Tg-positive wells. Addition of Stop Solution gave a yellow colour and the optical densities of standards, controls and samples were measured using a microplate reader.

Quality Control

Values >400ng/ml were considered positive;

values <400ng/ml were considered negative;

values >400ng/ml were repeated at a 1:5 dilution.

Normal and Elevated controls were obtained from the standard curve.

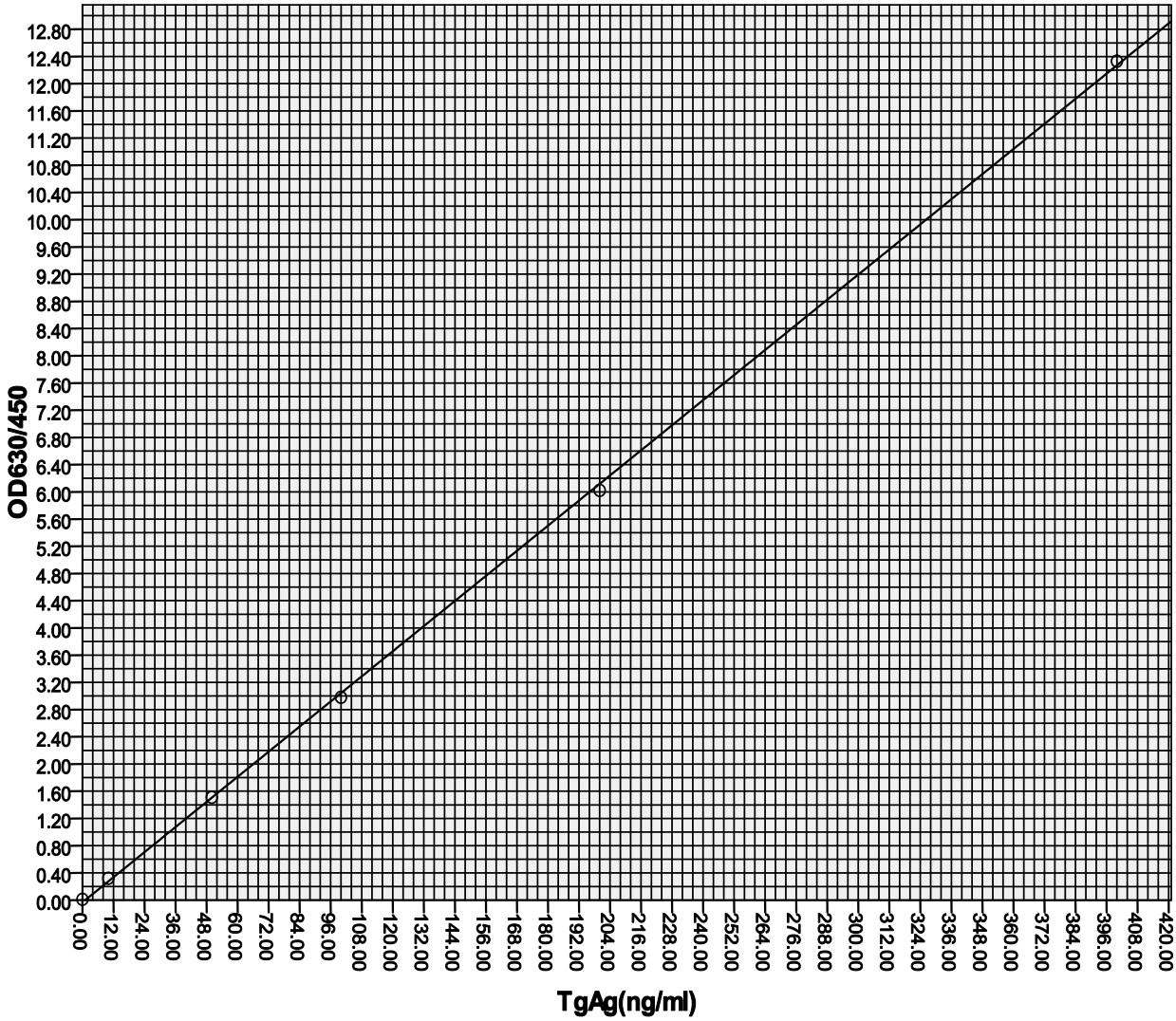


Fig. 3.3: Standard curve for thyroglobulin assay

3.8. Quantification of total Thyroxine (T_4) in human serum by Enzyme-Immunoassay (EIA).

The method is essentially based on the adaptation of Schuurs and Van Weeman, 1977 by the manufacturer of the kit (Genesis diagnostics, Cambridgeshire CB6 1SE, UK) (Pathozyme Omega Diagnostics, UK)

Serum levels of TT_4 were measured by ELISA. The minimum detectable level of TT_4 was 0.1 ng/mL.

3.8.1. Principle

The principle is based on the specific anti-T_4 antibodies being coated onto microtitration wells. Test sera were applied. T_4 with Horseradish Peroxide enzyme (Conjugate) was added which competes with the released serum T_4 for available binding sites on the solid phase. After incubation, the wells were washed with water to remove any unbound T_4 or T_4 enzyme conjugate. On addition of the substrate (TMB), a colour develops only in those wells in which enzyme were present, indicating a lack of serum T_4. The reaction was stopped by the addition of dilute hydrochloric acid and the absorbance was then measured at 450nm.

This assay provides a rapid and sensitive method for measuring T_4 in human serum using highly specific T_4 monoclonal antibody and a T_4 enzyme labelled conjugate solution.

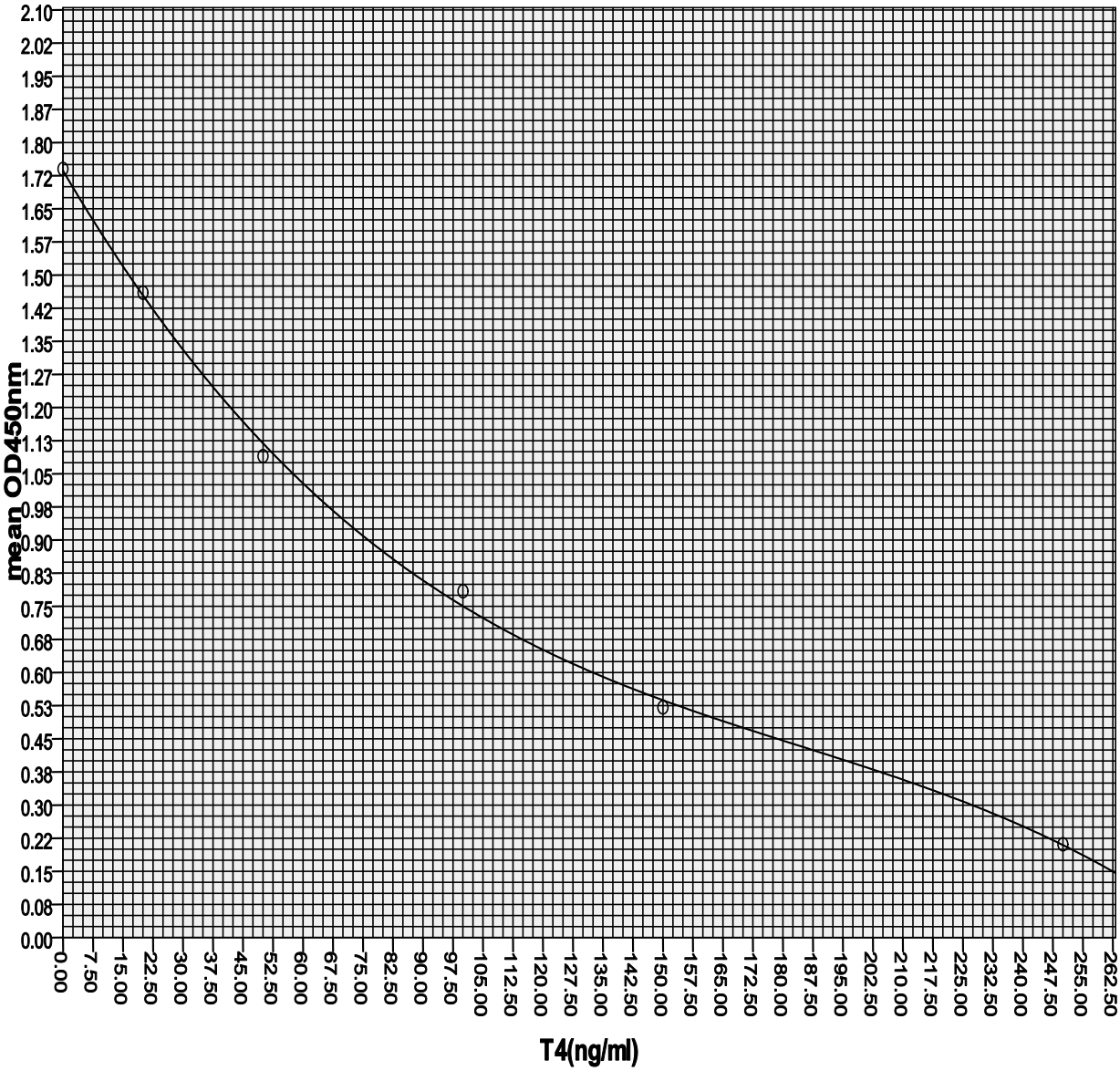


Fig. 3.4: Standard curve for Total Thyroxine (T_4) assay

3.9. Quantification of free Thyroxine (fT_4) in human serum by Enzyme-Immunoassay (EIA).

The method is essentially based on the adaptation of Dussault *et al*., 1976 by the manufacturer of the kit (Genesis diagnostics, Cambridgeshire CB6 1SE, UK) (Pathozyme Omega Diagnostics, UK)

Serum levels of FT_4 were measured by ELISA. The minimum detectable level of FT_4 was 0.1 pg/mL.

3.9.1. Principle

The FT_4 test is a solid phase competitive enzyme immunoassay. The principle was based on the addition of the patient serum samples, standards, and Thyroxine-Enzyme Conjugate to wells coated with monoclonal T_4 antibody. After incubation at room temperature, the wells were washed to remove unbound T_4 conjugate. On the addition of Substrate (TMB), a colour developed only in those wells in which enzyme were present, indicating a lack of fT_4. The reaction was stopped by the addition of dilute Hydrochloric Acid and the absorbance was then measured at 450 nm. The intensity of the colour formed is proportional to the amount of enzyme present and is inversely related to the amount of unlabelled fT_4 in the sample.

FT_4 tests are designed to directly reflect the equilibrium existing in serum between T_4 and TGB-bound T_4. These methods, including the fT_4 tests, can generally reflect thyroid status in a single assay.

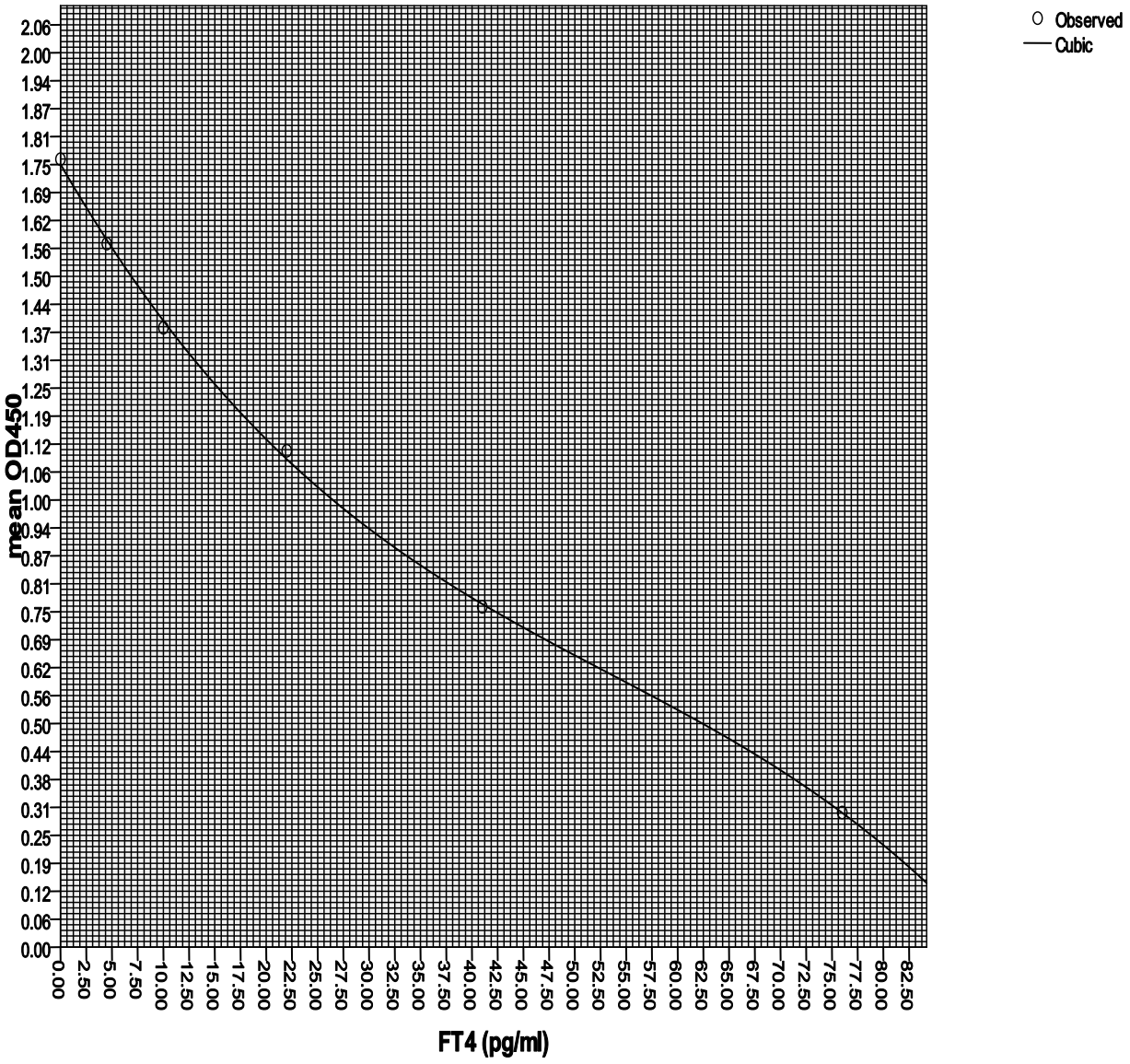


Fig. 3.5: Standard curve for free Thyroxine (FT_4) assay

3.10. Quantification of free Triiodothyronine (fT_3) in human serum by Enzyme-Immunoassay (EIA).

The method is essentially based on the adaptation of Horworth and Ward, 1972 by the manufacturer of the kit (Genesis diagnostics, Cambridgeshire CB6 1SE, UK) (Pathozyme Omega Diagnostics, UK)

Serum levels of FT_3 were measured by ELISA. The minimum detectable level of FT_3 was 0.1 pg/mL.

3.10.0. Principle

The FT_3 test is a solid phase competitive enzyme immunoassay. Patient serum samples, standards, and T_3-Enzyme conjugate are added to wells coated with monoclonal T_3 antibody. FT_3 in the specimen and the T_3 labelled conjugate compete for available binding sites on the antibody. After incubation at room temperature, the wells are washed with distilled water to remove unbound T_3 conjugate. On addition of the substrate (TMB), a colour develops only in those wells in which enzyme are present, indicating a lack of serum FT_3. The reaction is stopped by the addition of dilute Hydrochloric Acid and the absorbance measured at 450 nm.

The concentrations of the carrier proteins are altered in many clinical conditions, such as pregnancy. In individuals with normal thyroid function, as the concentrations of the carrier proteins change, the total T_3 levels also change so that the free triiodothyronine (FT_3) concentration remains constant.

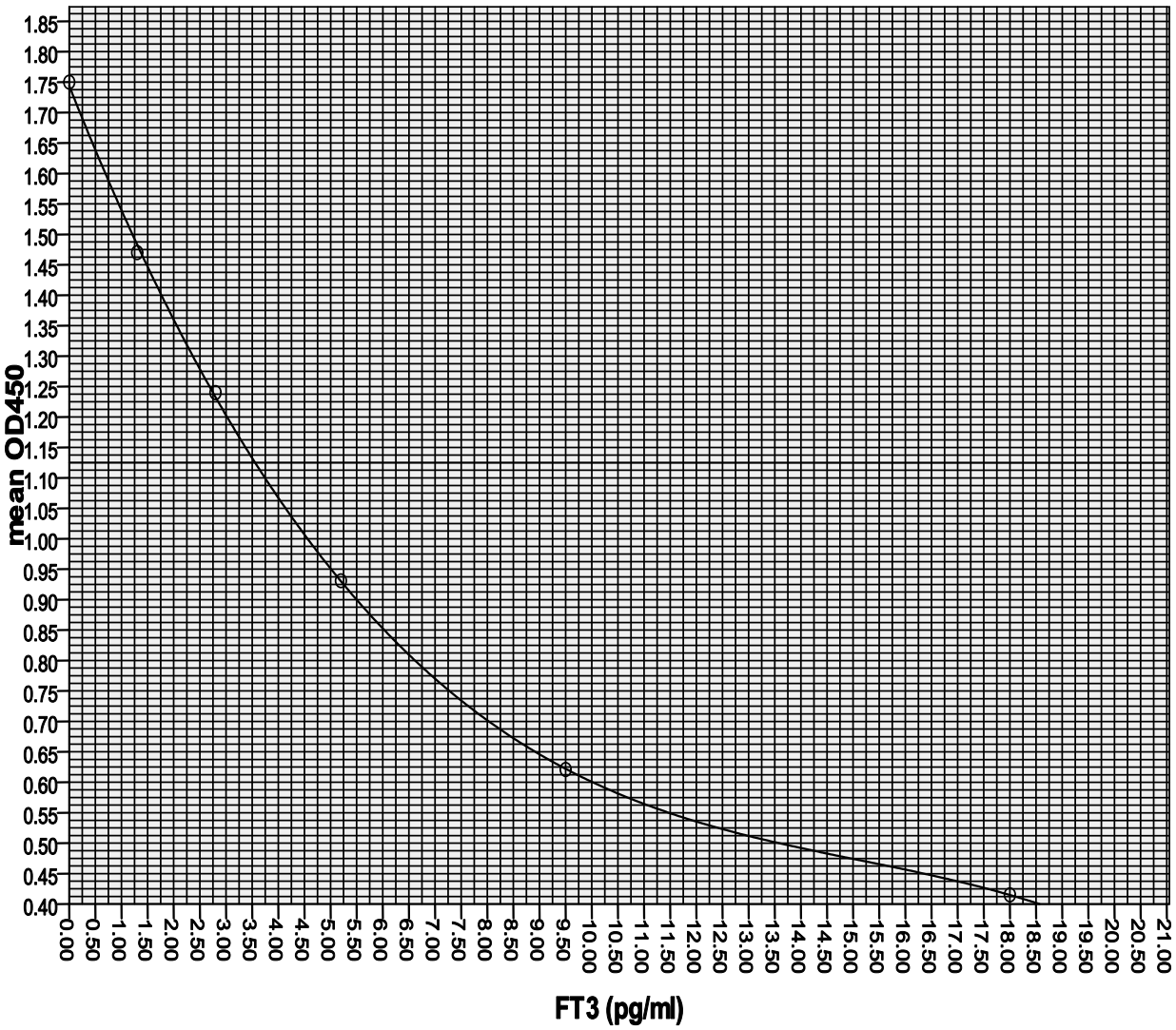


Fig. 3.6: Standard curve for free Triiodothyronine (FT_3) assay

3.11. Quantification of total Triiodothyronine (T_3) in human serum by Enzyme-Immunoassay (EIA).

The method is essentially based on the adaptation of wisdom, 1976 by the manufacturer of the kit (Genesis diagnostics, Cambridgeshire CB6 1SE, UK) (Pathozyme Omega Diagnostics, UK)

Serum levels of TT_3 were measured by ELISA. The minimum detectable level of TT_3 was 0.1 ng/mL.

3.11.1. Principle

The principle is based on goat anti – mouse IgG antibodies which are coated onto microtitration wells. Test sera are applied along with antibody reagent. T_3 enzyme Conjugate is added which competes with the serum T_3 for available binding sites on the solid phase. After incubation, the wells are washed to remove any unbound T_3 or T_4 enzyme conjugate. On addition of the Substrate (TMB), a colour develops only in those wells in which enzyme are present, indicating a lack of serum T_3. The reaction is stopped by the addition of dilute Hydrochloric Acid and the absorbance in then measured at 450nm.

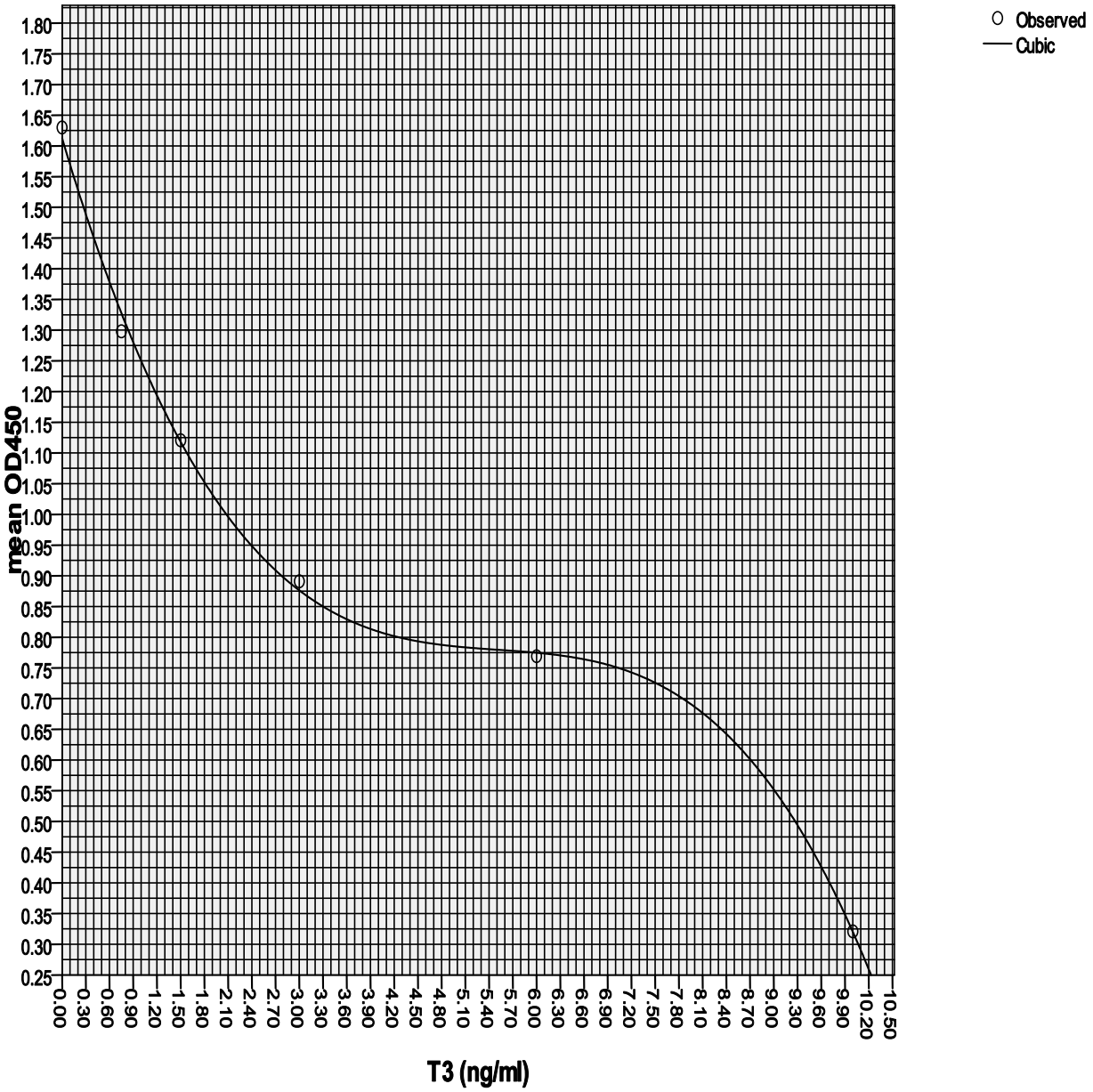


Fig. 3.7: Standard curve for total Triiodothyronine (TT_3)

3.12. ULTRASENSITIVE TSH (µTSH)

Quantification of Low levels of TSH in human serum by Enzyme-Immunoassay (EIA).

The method is essentially based on the adaptation of Uotila *et al*., 1981 by the manufacturer of the kit (Genesis diagnostics, Cambridgeshire CB6 1SE, UK) (Pathozyme Omega Diagnostics, UK)

Serum levels of TSH were measured by ELISA. The minimum detectable level of TSH was 0.1 µIU/mL.

3.12.1. Principle

Specific mouse monoclonal anti-TSH antibodies are prepared, purified and coated onto microtitration wells. Test sera are applied and goat anti-TSH antibody labelled with Horseradish Peroxidase enzyme (Conjugate) is added. If human TSH is present in the sample it will combine with the antibody on the well and the enzyme conjugate, resulting in the TSH molecule being sandwiched between the solid phase and the enzyme linked antibodies. After incubation the wells are washed to remove unbound labelled antibodies. On addition of the Substrate (TMB), a colour will develop only in those wells in which the enzyme Conjugate is present, indicating the presence of TSH. The enzyme reaction is stopped by the addition of dilute Hydrochloric Acid and the absorbance is then measured at 450nm. The concentration of TSH is directly proportional to the colour intensity of the test sample.

Quality Control

The optical density of the calibrator A (0µIU/ml) was <0.75

Calibrator F (10µIU/ml) was >1.5 and were regarded as valid.

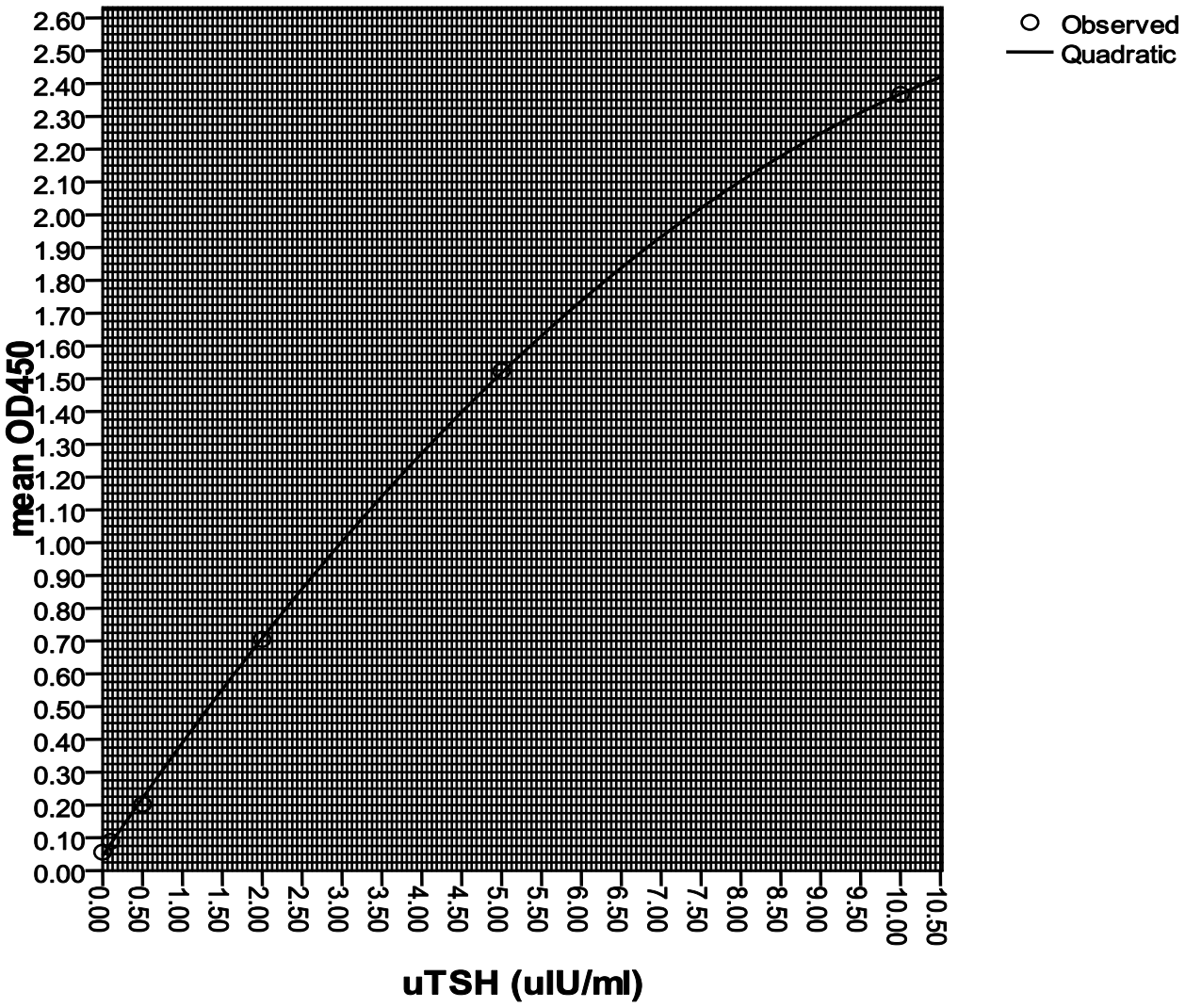


Fig. 3.8: Standard curve of ultra sensitive Throid Stimulating Hormone (µTSH) assay

3.13. STATISTICAL ANALYSIS

All data from this study were analysed and presented as mean ± standard error of mean (SEM). The Statistical software package of the Social Science (SPSS) version 17.0 was used for analysis of data.

For quantitative variables, the Student's t-test was used to test the significance of difference between the mean values while Analysis of variance (ANOVA) was used to test significance of variations within and among the group; post-Hoc was used for comparism of multiple variable

Pearson's correlation technique was used for the comparison of the strength of association among variables.

Chi square test was used for the comparison of means for non quantitative variables. Significant values were considered at $p < 0.05$.

CHAPTER FOUR

RESULTS

The results obtained from the study are summarized in tables 4.1 to 4.28 and figures 4.0 to 4.1. The sex distribution in this study exhibited a preponderance of female patients in the entire group. A description of anthropometric and clinical indices among all the groups comprising 79 hyperthyroidism (Hyper), 15 hypothyroidism (Hypo) 43 simple non – toxic goitre (SNTG) patients and 130 apparently healthy controls (Ctrls) are represented in table 4.1. There was no significant difference in the mean age of patients: 42.86 ± 1.92, 44.13 ± 1.92, 44.37 ± 1.80 years when compared with control group (42.24 ± 0.88 years); ($p>0.05$) respectively. There was a significant difference in the body mass index of the cases compared with the controls.

Indices of thyroid function, T_3, T_4, FT_3, and FT_4 displayed highly significant increase in hyper-thyroidism and SNTG patients, but a highly significant decrease in the levels of T_3, T_4, FT_3,and FT_4 in hypothyroid patients compared with control. In contrast, there was a highly significant decrease in TSH value in SNTG and hyperthyroid patients but a significant increase in hypothyroid patients compared with control group; ($p < 0.05$) respectively (Table 4.2).

Studies on trace element demonstrated a highly significant decrease in the levels of Cu, Fe, Zn and Se in hyperthyroidism, hypothyroidism and SNTG patients compared with those of the controls ($p < 0.05$) respectively (Table 4.3). Levels of the toxic trace elements As, Pb, Cd and Ni revealed highly significant increases in the different groups compared with the controls ($p < 0.05$) respectively (Table 4.4).

The levels of anthropometric and blood pressure measurements revealed a significant increase in the systolic blood pressure but not in diastolic blood pressure in SNTG patients (table 4.5) while, a significant increase was observed in systolic as well as diastolic blood pressure in hyperthyroidism compared with controls ($p<0.05$) (Table 4.10).

The T_3, T_4, FT_3, and FT_4 levels displayed highly significant increase in SNTG patients and hyperthyroidism compared with control group, the result also show a highly significant decrease, in the levels of indices of thyroid function, T_3, T_4, FT_3, and FT_4 in hypothyroid patient compared wi with the control group ($p<0.05$) respectively. Similarly, there was a highly significant decrease in TSH value of SNTG and hyperthytoid patients and a highly significant increase in TSH value of hy-pothyroid patients compared with controls ($p<0.05$) respectively (Tables 4.2, 4.11 and 4.16)

Table 4.1. Age, anthropometric and clinical indices of patients and controls

Indices	Group1 SNTG (n=43)	Group 2 Hyper (n=79)	Group3 Hypo (n=15)	Group 4 Ctrls (n=130)	F	p-value
Age (yrs)	44.37± 1.80	42.86± 1.92	44.13 ± 1.92	42.24 ± 0.88	0.435	0.728
Weight(kg)	71.78± 1.89	67.24± 1.42	77.97± 3.79	67.26± 0.88	4.159	0.001*
Height (m)	1.64± 0.01	1.63± 0.01	1.63± 0.02	1.61± 0.01	5.337	0.007*
SBP (mmHg)	122.32±2.15	128.18±2.14	118.67± 3.63	116.76± 1.15	9.366	0.000*
DBP (mmHg)	76.25± 1.70	77.47± 1.25	75.33± 2.74	75.66± 0.82	1.175	0.320
BMI(kg/m^2)	26.65± 0.65	25.31± 0.55	31.50± 0.98	26.23± 0.40	5.750	0.000*

BMI = Body mass index

SBP = Systolic blood pressure

DBP = Diastolic blood pressure

SNTG= simple non-toxic goitre

Hyper = hyperthyroidism

Hypo=hypothyroidism

CTRLs= controls

F = F-ratio

* = significant at p<0.05

p-value = significant level

Values are reported as mean± SEM (standard error of mean)

Table 4.2: Levels of T_3,T_4, FT_3, FT_4 &TSH in subjects with thyroid disorders and Controls

Indices	Group1 (SNTG) (n=43)	Group 2 (Hyper) (n=79)	Group3 (Hypo) (n=15)	Group 4 (Ctrls) (n=130)	F	p-value
T_3(nmol/l)	1.31± 0.04	2.03± 0.02	0.63± 0.03	1.01 ± 0.01	698.97	0.000*
T_4(nmol/l)	106.47± 1.83	134.80± 1.10	54.82± 2.82	104.72± 2.63	60.944	0.000*
FT_3(pmol/L	3.71± 0.02	4.41± 0.02	1.12± 0.03	3.61± 0.05	239.343	0.000*
FT_4(pmol/L	14.88± 0.20	27.18± 0.44	7.43± 0.08	13.13± 0.13	659.665	0.000*
TSH (µIU/L	1.38± 0.22	0.31±0.04	8.71± 0.15	1.60± 0.05	1607.941	0.000*

T_3, FT_3 = Total and free triiodothyronine

T_4, FT_4 = Total and free thyroxine

TSH = thyroid stimulating hormone

SNTG= simple non-toxic goitre

CTRLs= controls

Hyper = hyperthyroidism

Hypo=hypothyroidism

F = F-ratio

* = significant at $p<0.05$

p-value = significant level

Values are reported as mean± SEM (standard error of mean)

Table 4.3: Levels of Cu, Fe, Zn, Se of subjects with thyroid disorders and controls

Indices	Group1 SNTG (n=43)	Group 2 Hyper (n=79)	Group 3 Hypo (n=15)	Group 4 Ctrls (n=130)	F	p-value
Cu(µmol/l)	11.89± 0.27	11.76± 0.16	16.06± 0.52	16.13± 0.22	92.18	0.000*
Fe(µmol/l)	20.05± 0.49	19.76± 0.42	15.04± 0.27	28.66± 0.19	294.81	0.000*
Zn(µmol/l)	18.67± 0.28	19.74± 0.29	16.16± 0.41	21.49± 0.17	42.70	0.000*
Se(µmol/l)	0.54± 0.01	0.95± 0.12	0.46± 0.03	3.82± 0.03	556.96	0.000*

Cu = copper

Fe = Iron

Zn = Zinc

Se = Selenium

SNTG= simple non-toxic goitre

CTRLs= controls

Hyper = hyperthyroidism

Hypo=hypothyroidism

F = F-ratio

* = significant at $p<0.05$

p-value = significant level

Values are reported as mean± SEM (standard error of mean)

Table 4.4: Levels of As, Pb, Cd, Ni of subjects with thyroid disorders and controls

Indices	Group1 SNTG (n=43)	Group 2 Hyper (n=79)	Group 3 HYPO (n=15)	Group 4 Control (n=130)	F	p-value
As(µmol/L)	0.18± 0.01	0.16± 0.01	0.16± 0.02	0.12± 0.01	33.777	0.000*
Pb(µmol/L)	1.19± 0.03	1.16± 0.03	1.20± 0.04	0.59± 0.01	206.601	0.000*
Cd(µmol/L)	0.39± 0.01	0.35± 0.01	0.31± 0.03	0.11± 0.01	196.143	0.000*
Ni(µmol/L)	3.33± 0.04	3.16± 0.05	3.27± 0.09	0.73± 0.01	1461.150	0.000*

As = Arsenic

Pb = Lead

Cd = Cadmium

Ni = Nickel

SNTG= simple non-toxic goitre

CTRLs= controls

Hyper = hyperthyroidism

Hypo=hypothyroidism

F = F-ratio

* = significant at $p<0.05$

p-value = significant level

Values are reported as mean± SEM (standard error of mean)

Table 4.5. Age, anthropometric and clinical indices in Simple non toxic goitre Females and Controls

Indices	Group (SNTG) (n=37)	Group (Ctrls) (n=37)	t	p-value
Age (yrs)	44.51± 1.93	43.65± 1.69	0.337	0.737
Weight (kg)	72.06± 2.12	67.40±1.30	1.867	0.066
Height (m)	1.64± 0.01	1.61± 0.01	2.763	0.007*
SBP (mmHg)	122.91±2.05	109.64± 1.24	5.527	0.000*
DBP (mmHg)	73.93± 1.77	69.91± 1.16	1.901	0.134
BMI (kg/m^2)	26.84± 0.73	26.48± 0.70	0.357	0.722

SBP = systolic blood pressure
DBP = Diastolic blood pressure
BMI = body mass indes
SNTG= simple non-toxic goitre
CTRLs= controls
t = student's t-test
* = significant at $p<0.05$
p-value = significant level
Values are reported as mean± SEM (standard error of mean)

Table 4.6. Levels of T_3,T_4, FT_3, FT_4 &TSH among Simple non toxic goitre Females and Controls

Indices	Group (SNTG) (n=37)	Group (Ctrls) (n=37)	t	p-value
T_3(nmol/L)	2.01± 0.01	0.95± 0.02	46.104	0.000*
T_4(nmol/L)	105.42± 1.99	89.91± 3.12	4.183	0.000*
FT_3(pmol/L)	3.72± 0.02	3.02± 0.09	7.641	0.000*
FT_4(pmol/L)	14.90± 0.23	12.31± 0.20	8.425	0.000*
TSH (µIU/L)	1.35± 0.04	1.07 ± 0.04	5.092	0.000*

T_3, FT_3 = Total and free triiodothyronine

T_4, FT_4 = Total and free thyroxine

TSH = thyroid stimulating hormone

SNTG= simple non-toxic goitre

CTRLs= controls

t = Student's t-test

* = significant at $p<0.05$

p-value = significant level

Values are reported as mean± SEM (standard error of mean)

The levels of autoantibodies, TPO Tg; Thyroglobulin and urinary iodine demonstrated that there was a highly significant increase in the levels in SNTG, hyperthyroidism and hypo-thyroidism patients compared with controls; ($p<0.05$) respectively (Tables 4.7, 4.12 and 4.17)

The essential trace elements Cu, Fe, Zn and Se exhibited highly significant decreases in SNTG, hyperthyroidism and hypothyroidism compared with that of the control (tables 4.8, 4.13 and 4.18). In contrast, a significant increase in toxic trace elements As, Pb, Cd and Ni was seen in SNTG, hyperthyroidism and hypothyroidism group as compared with the control group ($p<0.05$) respectively (4.9, 4.14 and 4.19).

The results of correlation between indices of thyroid function (T_3, FT_3, FT_4 and T_4) and concentration of essential and toxic trace elements in thyroid disorder patients is as shown in Tables 4.20 to 4.22. Highly significant positive correlations was observed between T_3 and autoantibodies (TPO, Tg), Thyroglobulin, Pb, Cd, Ni while, highly significant negative correlation was observed be tween T_3 and IOD, Cu, Fe, Zn, Se, TSH ($p<0.05$) (Table 4.20). Similarly, positive correlation was noticed between T_4 and Thyroglobulin, Cd, Ni, Pb, Se in thyroid disorder patients ($p<0.05$) (Table 4.22).

Highly significant positive correlation was observed between FT_3 and Thyroglobulin, Lead ($p<0.05$). Also, highly significant negative correlation was observed between FT_3 and TSH, IOD, TPOAb, Cu, Fe, As in patients; ($p<0.05$) (Table 4.23, 4.24). Significant negative correlations between FT_4 and TSH but significant positive correlations were observed between FT_4 and systolic, diastolic blood pressures in patients ($p < 0.05$) in each case (Table 4.25). Negative correlations were observed to be significant between Iodine and Tg, T_3, FT_3, FT_4 while a significantly positive correlation was seen between Iodine and TSH, TgAb, TPO in baseline patients ($p<0.05$) (Table 4.26 Similarly, positive correlation were observed between As and T_3, TSH, autoantibodies (TPO, Tg), systolic and diastolic blood pressures while a negative correlation was observed between As and the essential trace elements (Cu, Fe, Se, Zn), T_4, FT_3, FT_4, Tg ($p < 0.05$) in each case (Tables 4.27 and 4.28).

The Figure 4.0 displays the autoantibodies prevalence of subjects with positive TPOAb and TgAb in simple non toxic goitre, toxic nodular goitre, hyperthyroidism, hypothyroidism, thyroid cancer and control groups. Figure 4.1 shows the urinary iodine concentrations in SNTG, TNG, HYPERTH, HYPOTH, THY Cancer and the control groups respectively.

Table 4.7. Levels of Autoantibodies &Urinary Iodine levels among Simple non toxic goitre Female Subjects & Controls

Indices	Group (SNTG) (n=37)	Group (Ctrls) (n=37)	t	p-value
TPO (kIU/L)	1.16± 0.06	1.00± 0.02	2.640	0.010*
TgAb(kIU/L	1.13± 0.06	1.00± 0.01	2.372	0.020*
TgAg (µg/L)	1.67± 0.08	1.11± 0.01	6.602	0.000*
IOD (µg/L)	1.80± 0.11	2.05± 0.04	-2.290	0.025*

TPO = Thyroid peroxidase antibody

TgAb = Thyroglobulin antibody

TgAg = Thyroglobulin

IOD = urinary iodine

SNTG= simple non-toxic goitre

CTRLs= controls

t = student's t- test

* = significant at $p<0.05$

p-value = significant level

Values are reported as mean± SEM (standard error of mean)

Table 4.8. Levels of Cu, Fe, Zn, Se among Simple non toxic goitre Female Subjects & Controls

Indices	Group (SNTG) (n=37)	Group (Ctrls) (n=37)	t	p-value
Cu(µmol/L)	11.76± 0.27	15.27 ± 0.48	-6.322	0.000*
Fe(µmol/L)	19.64± 0.54	28.76 ± 0.33	-14.363	0.020*
Zn(µmol/L)	18.74 ± 0.32	20.82 ± 0.33	-4.507	0.000*
Se(µmol/L)	0.55 ± 0.01	3.76± 0.07	-44.909	0.000*

Cu = copper

Fe = Iron

Zn = Zinc

Se = Selenium

SNTG= simple non-toxic goitre

CTRLs= controls

t = student's t-test

* = significant at $p<0.05$

p-value = significant level

Values are reported as mean± SEM (standard error of mean)

Table 4.9. Levels of As, Pd, Cd, Ni among Simple non toxic goitre Female Subjects & Controls

Indices	Group (SNTG) (n=37)	Group (Ctrls) (n=37)	t	p-value
As(µmol/)	1.16± 0.06	1.01± 0.03	2.640	0.010*
Pb(µmol/l)	1.13± 0.06	1.02 ± 0.01	2.372	0.020*
Cd(µmol/)	1.67± 0.08	1.11± 0.01	6.602	0.000*
Ni(µmol/l)	1.80± 0.11	2.05± 0.04	2.290	0.025*

As = Arsenic

Pb = Lead

Cd = Cadmium

Ni = Nickel

SNTG= simple non-toxic goitre

CTRLs= controls

t = Student's t-test

* = significant at $p<0.05$

p-value = significant level

Values are reported as mean± SEM (standard error of mean)

Table 4.10. Age, anthropometric and clinical indices among Hyperthyroid Females and Controls

Indices	Group (HYPER) (n=62)	Group (Ctrls) (n=62)	t	p-value
Age (yrs)	44.82± 1.65	43.69± 1.30	0.537	0.592
Weight(kg)	67.72 ± 1.60	68.19±1.19	0.335	0.738
Height(m)	1.62± 0.01	1.61± 0.01	1.920	0.057
SBP (mmHg)	127.42 ± 2.06	112.58± 0.89	6.625	0.000*
DBP (mmHg)	78.79± 1.11	72.26± 1.04	4.290	0.000*
BMI (kg/m^2)	25.86± 0.63	26.85± 0.57	1.157	0.250

SBP = systolic blood pressure

DBP = Diastolic blood pressure

BMI = body mass indes

CTRLs= controls

Hyperth = hyperthyroidism

t = student's t-test

* = significant at $p<0.05$

p-value = significant level

Values are reported as mean± SEM (standard error of mean)

Table 4.11. Levels of T_3,T_4, FT_3, FT_4 &TSH among Hyperthyroid Female subjects and Controls

Indices	Group (HYPER) (n =62)	Group 4 (Ctrls) (n =62)	t	p-value
T_3(nmol/l)	2.65± 0.06	1.01 ± 0.02	25.500	0.000*
T_4(nmol/l)	135.04± 1.32	94.45 ± 2.37	14.783	0.000*
FT_3(pmol/L)	4.40 ± 0.02	3.07± 0.07	9.639	0.000*
FT_4(pmol/L)	27.26 ± 0.49	12.99 ± 0.19	27.251	0.000*
TSH (µIU/L)	0.32± 0.01	1.64 ± 0.08	17.163	0.000*

T_3, FT_3 = Total and free triiodothyronine

T_4, FT_4 = Total and free thyroxine

TSH = thyroid stimulating hormone

Hyperth = hyperthyroidism

CTRLs= controls

t = Student's t-test

* = significant at $p<0.05$

p-value = significant level

Values are reported as mean± SEM (standard error of mean)

Table 4.12. Levels of autoantibodies &Urinary Iodine among Hyperthyroid Female Subjects & Controls

Indices	Group (HYPER) (n=62)	Group (Ctrls) (n=62)	t	p-value
TPO (kIU/L)	1.50± 0.06	1.00± 0.01	7.074	0.000*
TgAb(kIU/L)	1.22 ± 0.05	1.00± 0.00	3.570	0.020*
TgAg (µg/L)	1.76± 0.11	0.11± 0.04	11.369	0.000*
IOD (µg/L)	1.73± 0.08	2.10± 0.05	-3.884	0.000*

TPO = Thyroid peroxidase antibody

TgAb = Thyroglobulin antibody

TgAg = Thyroglobulin

IOD = urinary iodine

Hyperth = Hyperthyroidism

CTRLs= controls

t = student's t- test

* = significant at $p<0.05$

p-value = significant level

Values are reported as mean± SEM (standard error of mean)

Table 4.13. Levels of Cu, Fe, Zn, Se between Hyperthyroid Female Subjects & Controls

Indices	Group (HYPER) (n=62)	Group (Ctrls) (n=62)	t	p-value
Cu(µmol/L)	8.99 ± 0.25	15.66 ± 0.36	-15.222	0.000*
Fe(µmol/L)	12.68 ± 0.49	28.63 ± 0.30	-27.508	0.020*
Zn(µmol/L)	19.07± 0.45	20.64 ± 0.27	-2.993	0.000*
Se(µmol/L)	0.85 ± 0.12	3.75± 0.05	-22.076	0.000*

Cu = copper
Fe = Iron
Zn = Zinc
Se = Selenium
Hyperth = Hyperthyroidism
CTRLs= controls
t = student's t-test
* = significant at $p<0.05$
p-value = significant level
Values are reported as mean± SEM (standard error of mean)

Table 4.14. Levels of As, Pb, Cd, Ni levels between Hyperthyroid Female Subjects & Controls

Indices	Group (HYPER) (n=62)	Group (Ctrls) (n=62)	t	p-value
As(µmol/l)	0.19 ± 0.01	0.12 ± 0.001	5.923	0.000*
Pb(µmol/l)	1.19± 0.02	0.63 ± 0.02	17.123	0.000*
Cd(µmol/)	0.36 ± 0.01	0.11± 0.01	16.580	0.000*
Ni(µmol/l)	3.10 ± 0.06	0.76 ± 0.02	36.962	0.000*

As = Arsenic

Pb = Lead

Cd = Cadmium

Ni = Nickel

Hyperth = hyperthyroidism

CTRLs= controls

t = Student's t-test

* = significant at $p<0.05$

p-value = significant level

Values are reported as mean± SEM (standard error of mean)

Table 4.15. Age, anthropometric and blood pressure indices between Hypothyroid Females and Controls

Indices	Group (HYPO) n=12	Group (Ctrls) n=12	t	p-value
Age (yrs)	43.50± 3.11	43.33± 3.09	0.038	0.970
Weight(kg)	84.92 ± 2.88	63.92±2.29	5.700	0.000*
Height(m)	1.63± 0.01	1.61± 0.01	1.297	0.208
SBP (mmHg)	138.33±3.66	110.00± 2.46	6.425	0.000*
DBP (mmHg)	86.25± 2.23	70.83 ± 2.29	4.824	0.000*
BMI (kg/m^2)	32.11± 1.15	25.00 ± 1.18	4.308	0.000*

SBP = systolic blood pressure

DBP = Diastolic blood pressure

BMI = body mass indes

CTRLs= controls

Hypoth = hypothyroidism

t = student's t-test

* = significant at $p<0.05$

p-value = significant level

Values are reported as mean± SEM (standard error of mean)

Table 4.16. T_3,T_4, FT_3, FT_4 &TSH levels among Hypothyroid Females and Controls

Indices	Group (HYPO) (n=12)	Group 4 (Ctrls) (n=12)	t	p-value
T_3(nmol/l)	0.93 ± 0.03	1.43 0.09	-5.370	0.000*
T_4(nmol/l)	57.69± 2.96	95.17 ± 3.87	-7.685	0.000*
FT_3(pmol/L)	1.13± 0.04	3.84± 0.09	-26.846	0.000*
FT_4(pmol/L)	7.44 ± 0.08	13.07 ± 0.36	-15.335	0.000*
TSH (µIU/l	8.72 ± 0.12	1.24 ± 0.05	55.427	0.000*

T_3, FT_3 = Total and free triiodothyronine

T_4, FT_4 = Total and free thyroxine

TSH = thyroid stimulating hormone

Hypoth = hypothyroidism

CTRLs= controls

t = Student's t-test

* = significant at $p<0.05$

p-value = significant level

Values are reported as mean± SEM (standard error of mean)

Table 4.17. Autoantibodies & Urinary Iodine levels between Hypothyroid Female Subjects & Controls

Indices	Group (HYPO) (n=12)	Group(Ctrls) (n=12)	t	p-value
TPO (kIU/L)	1.67± 0.14	1.00± 0.01	7.091	0.000*
TgAb(kIU/L)	0.72± 0.25	1.00± 0.001	3.00	0.007*
TgAg (µg/L)	1.33 ± 0.28	1.01± 0.02	4.690	0.000*
IOD (µg/L)	2.00 ± 0.25	2.17± 0.11	0.616	0.544

TPO = Thyroid peroxidase antibody

TgAb = Thyroglobulin antibody

TgAg = Thyroglobulin

IOD = urinary iodine

Hypoth = hypothyroidism

CTRLs= controls

t = student's t- test

* = significant at $p<0.05$

p-value = significant level

Values are reported as mean± SEM (standard error of mean)

Table 4.18. Copper, Fe, Zn & Se among Hypothyroid Female Subjects & Controls

Indices	Group (HYPO) (n=12)	Group (Ctrls) (n=12)	t	p-value
Cu(µmol/L)	16.99± 0.18	15.92 ± 0.53	1.911	0.069
Fe(µmol/L)	15.40± 0.23	27.64± 0.25	-35.451	0.000*
Zn(µmol/L)	16.64 ± 0.39	21.75 ± 0.53	-7.761	0.000*
Se(µmol/L)	0.47 ± 0.03	3.56± 0.15	-20.483	0.000*

Cu = copper

Fe = Iron

Zn = Zinc

Se = Selenium

Hypoth = hypothyroidism

CTRLs= controls

t = student's t-test

* = significant at $p<0.05$

p-value = significant level

Values are reported as mean± SEM (standard error of mean)

Table 4.19. Levels of As, Pd, Cd, Ni levels between Hypothyroid Female Subjects & Controls

Indices	Group (HYPO) (n=12)	Group (Ctrls) (n=12)	t	p-value
As(µmol/l)	0.47 ± 0.02	0.12± 0.003	13.749	0.000*
Pb(µmol/l)	1.25± 0.05	0.63± 0.05	9.213	0.000*
Cd(µmol/l)	0.35± 0.03	0.10± 0.01	7.960	0.000*
Ni(µmol/l)	3.35± 0.08	0.88 ± 0.05	25.760	0.000*

As = Arsenic

Pb = Lead

Cd = Cadmium

Ni = Nickel

Hypoth = hypothyroidism

CTRLs= controls

t = Student's t-test

* = significant at $p<0.05$

p-value = significant level

Values are reported as mean± SEM (standard error of mean)

Table 4.20. Correlations of T_3 and T_4, FT_3, FT_4, toxic trace elements, autoantibodies in all patients

Triiodothyronine T_3 (nmol/l) vs.	(n = 137)	Correlation coefficient(r)	p-value
T_4 (nmol/L)		0.718	0.000*
FT_3 (pmol/L)		0.774	0.000*
FT_4 (pmol/L)		0.727	0.000*
Pb (µmol/l)		0.654	0.000*
Cd (µmol/l)		0.656	0.000*
Ni (µmol/l)		0.761	0.000*
TPO (kIU/L)		0.271	0.000*
TgAb (kIU/L)		0.135	0.025*
Tg (µg/L)		0.446	0.000*

T_3, FT_3 = Total and free triiodothyronine

T_4, FT_4 = Total and free thyroxine

TSH = thyroid stimulating hormone

Pb = Lead

Cd = Cadmium

Ni = Nickel

TPO antibody= Thyroid peroxidase

TgAb antibody = Thyroglobulin

Tg = thyroglobulin As = Arsenic

* = significant at $p<0.05$

p-value = significant level

r = correlation coefficient

Table 4.21. Correlations of T_3 and TSH, IOD, Cu, Zn, Fe, Se, As, Sys, Dia, BMI in all patients

Triiodothyronine T_3(nmol/l) vs.	(n = 137) Correlation coefficient (r)	p-value
Dia (mmHg)	0.241	0.000*
Sys (mmHg)	0.004	0.000*
TSH (µIU/L)	-0.738	0.000*
IOD (µg/L)	-0.308	0.000*
Cu (µmol/l)	- 0.392	0.000*
Fe (µmol/l)	-0.795	0.000*
Zn (µmol/l)	-0.282	0.000*
Se (µmol/l)	-0.711	0.025*
As (µmol/l)	-0.602	0.000*
BMI (kg/m^2)	-0.120	0.048*

Cu = copper
Fe = Iron
Zn = Zinc
Se = Selenium
As = Arsenic
TSH = thyroid stimulating hormone
IOD = urinary iodine
BMI = body mass index
SBP = systolic blood pressure
DBP = diastolic blood pressure
* = significant at $p<0.05$

Table 4.22. Correlations of thyroxine and T_3, FT_3, FT_4, Se, Cd, Ni, Pb, Tg, in all patients

Thyroxine (T_4) (nmol/l) vs.	(n = 137)	Correlation coefficient(r)	p-value
T_3 (nmol/L)		0.563	0.000*
FT_3 (pmol/L)		0.886	0.000*
FT_4 (pmol/L)		0.830	0.000*
Se (µmol/L)		0.185	0.002*
Cd (µmol/L)		0.267	0.000*
Ni (µmol/L)		0.276	0.000*
Pb (µmol/L)		0.225	0.000*
TgAg (µg/L)		0.217	0.000*
Weight (kg)		- 0.185	0.002*

T_3, FT_3 = Total and free triiodothyronine

T_4, FT_4 = Total and free thyroxine

Ss = Selenium

Pb = Lead

Cd = Cadmium

Ni = Nickel

Tg = thyroglobulin

* = significant at $p<0.05$

p-value = significant level

r = correlation coefficient

A display of the anthropometric and clinical indices among the female subgroups comprising 37 simple non-toxic goitre (SNTG), 9 toxic nodular goitre (TNG), 47 hyperthyroidism (Hyper), 12 hypothyroidism (Hypo), 6 thyroid cancer (Thy Ca) patients and 100 apparently healthy controls is shown in Table 4.23. The mean age of these patients were 44.52±1.92, 41.11±4.24, 46.40±1.90, 43.50±3.11, 38.00±1.90 and 43.33±0.99 years, respectively. Significant differences were observed in the BMI and the systolic blood pressure ($p<0.05$), however such significant differences were not found in the mean age of the cases compared with the controls ($p>0.05$) (Table 4.23).

Among the female subgroups, T_3, T_4, FT_3, FT_4 levels displayed highly significant decrease in hypothyroidism patients compared with control group ($p>0.05$) (Table 4.24), the results also showed a highly significant increase in the levels of T_3, T_4, FT_3, FT_4, FT_3, FT_4 in SNTG, toxic nodular goitre (TNG), hyperthyroidism and thyroid cancer compared with control grou p ($p<0.05$). Similarly, a significant difference was observed in the level of urinary iodine in hypothyroidism and SNTG, TNG, Hyper, Thyroid cancer respectively. On the other hand, there was a highly significant increase in TSH value of hypothyroidism patients and a significant decrease in patients with SNTG, TNG, hyperthyroidism and thyroid cancer patients, respectively when compared with control group ($p<0.05$) (Table 4.24 and 4.25).

Copper showed highly significant decreases in SNTG, Toxic goitre, hyper and thyroid cancer but a highly significant increase in hypothyroidism patients compared with control ($p<0.05$). However, similar trend of significant decreases were also observed in the levels of Fe, Zn and Se in SNTG, TNG, hyperthyroidism, hypothyroidism and thyroid cancer patients compared with control; ($p<0.05$) (Table 4.26). Highly significant increases were observed in toxic trace elements, As, Pb, Cd and Ni in different groups compared with controls ($p<0.05$) (Table 4.27).

Table 4.23: Age, anthropometric & clinical indices among female subgroup

INDICES	Group1 SNTG (n=37)	Group 2 TNG (n=9)	Group 3 Hyper (n=47)	Group 4 Hypo (n=12)	Group 5 Thy Ca (n=6)	Group 6 Control (n=100)	F	p-value
Age (Years)	44.52± 1.92	41.11± 4.24	46.40± 1.90	43.50± 3.11	38.00± 1.90	43.33± 0.99	0.969	0.437
Weight (Kg)	72.05± 2.12	72.32± 5.18	66.16± 1.84	77.08± 4.29	71.03± 2.31	67.01± 0.89	3.187	0.008*
Height (m)	1.64± 0.01	1.61± 0.01	1.62± 0.01	1.62± 0.01	1.60± 0.01	1.59± 0.00	6.479	0.000*
SBP (mmHg)	122.43± 2.49	127.78± 4.94	126.17± 2.42	120.00± 4.26	138.67± 5.58	117.82± 1.35	3.863	0.002*

SBP = systolic blood pressure
SNTG = simple nontoxic goitre
Hyper = hyperthyroidism
Hypo = hypothyroidism
Thy Ca thyroid cancer
TNG = Toxic nodular goitre
CTRLs= controls
F = F - ratio
* = significant at $p<0.05$
p-value = significant level
Values are reported as mean± SEM (standard error of mean)

Table 4.24: Indices of thyroid function between female subgroups

Indices	Group1 SNTG (n=37)	Group 2 TNG (n=9)	Group 3 Hyper (n=47)	Group 4 Hypo (n=12)	Group 5 Thy.Ca (n=6)	Group 6 control (n=100)	F	p-value
T_3 (nmol/L)	1.41± 0.01	1.33± 0.10	3.01± 0.37	1.35± 0.14	1.29± 0.21	1.20± 0.21	16.316	0.000*
T_4 (nmol/L)	133.15± 5.96	136.64± 6.13	161.96± 9.25	57.69± 2.96	140.08± 9.07	108.79± 3.23	19.553	0.000*
TSH (µiu/L)	1.02± 0.32	0.52± 0.18	0.46± 0.15	8.47± 0.21	2.21± 0.98	1.46± 0.08	94.300	0.000*
IOD (ug/L)	124.83± 10.31	106.94± 9.06	140.76± 10.36	108.79± 6.67	114.58± 11.22	182.79± 6.67	8.553	0.000*

T_3, FT_3 = Total and free triiodothyronine

T_4, FT_4 = Total and free thyroxine

TSH = thyroid stimulating hormone

IOD = urinary iodine

SNTG = simple nontoxic goitre

Hyper = hyperthyroidism

Hypo = hypothyroidism

Thy Ca = thyroid cancer

TNG = Toxic nodular goitre

CTRLs= controls

F = F - ratio

* = significant at $p<0.05$

p-value = significant level

Values are reported as mean± SEM (standard error of mean)

Table 4.25 : Thyroid function and autoantibodies levels among female subgroups

Indices	Group1 SNTG (n=37)	Group2 TNG (n=9)	Group3 Hyper (n=47)	Group4 Hypo (n=12)	Group 5 Thy.Ca (n=6)	Group6 control (n=100)	F	p-value
FT_3(pmol/l)	6.19± 0.51	6.23± 0.72	8.10± 0.75	2.97± 0.25	4.12± 0.14	2.93± 0.15	16.046	0.000*
FT4(pmol/l	37.21± 2.30	39.71± 3.87	44.45± 0.48	15.29± 0.97	42.00± 0.86	14.50± 0.36	97.278	0.000*
TPO(iu/L)	278.21 ± 27.76	85.85± 39.55	524.39± 34.74	438.09 ± 22.38	49.75± 22.97	102.68 ± 11.13	2.898	0.016*
Tg (iu/L)	282.18 ± 28.74	343.06 ± 45.79	323.21± 22.66	297.81 ± 29.62	410.00± 3.00	22.41± 4.41	36.665	0.000*

SNTG = simple nontoxic goitre

Hyper = hyperthyroidism

Hypo = hypothyroidism

Thy Ca thyroid cancer

TNG = Toxic nodular goitre

CTRLs= controls

F = F - ratio

* = significant at p<0.05

p-value = significant level

FT_3 = free triiodothyronine

FT_4 = free thyroxine

TPO = thyroid peroxidase antibody

Tg = Thyroglobulin

Values are reported as mean± SEM (standard error of mean)

Table 4.26: Essential trace elements indices among female subgroups

Indices	Group1 (SNTG) n=37	Group 2 (TNG) n=9	Group3 (Hyper) n=47	Group4 (Hypo) n=12	Group 5 (Thy Ca) n=6	Group 6 (control) n=100	F	p-value
Cu (µmol/L)	11.76± 0.27	11.83± 0.51	11.62± 0.15	16.99± 0.17	13.28± 1.36	15.92± 0.23	48.704	0.000*
Fe (µmol/L)	19.64± 0.54	18.04± 1.19	20.46± 0.44	22.91± 0.75	19.82± 1.59	28.53± 0.23	101.516	0.000*
Zn (µmol/L)	18.74± 0.32	19.18± 0.56	20.18± 0.31	18.55± 0.51	17.22± 0.29	21.36± 0.19	10.243	0.000*
Se (µmol/L)	0.55± 0.01	0.62± 0.08	0.53± 0.00	0.47± 0.02	3.05± 0.13	3.81± 0.03	1402.587	0.000*

Cu = copper

Fe = Iron

Zn = Zinc

Se = Selenium

SNTG = simple nontoxic goitre

Hyper = hyperthyroidism

Hypo = hypothyroidism

Thy Ca thyroid cancer

TNG = Toxic nodular goitre

CTRLs= controls

F = F - ratio

* = significant at $p<0.05$

p-value = significant level

Values are reported as mean± SEM (standard error of mean)

Table 4.27: Toxic trace elements status of female subgroups

INDICES	Group1 (SNTG n=37	Group2 (TNG) n=9	Group 3 (Hyper) n=47	Group 4 (Hypo) n=12	Group 5 (ThyCa) n=6	Group 6 (Control) n=100	F	p-value
As (µmol/L)	0.18± 0.01	0.16± 0.02	0.18± 0.01	0.16± 0.02	0.15± 0.01	0.12± 0.01	18.352	0.000*
Pb (µmol/L)	1.21± 0.03	1.66± 0.03	1.19± 0.03	1.25± 0.04	1.26± 0.03	1.13± 0.03	275.814	0.000*
Cd (µmol/L)	0.40± 0.02	0.36± 0.05	0.37± 0.01	0.34± 0.03	0.36± 0.05	0.11± 0.01	100.706	0.000*
Ni (µmol/L)	3.40± 0.04	3.23± 0.09	3.11± 0.07	3.37± 0.08	3.31± 0.02	2.81± 0.04	11.393	0.000*

SNTG = simple nontoxic goitre

Hyper = hyperthyroidism

Hypo = hypothyroidism

Thy Ca thyroid cancer

TNG = Toxic nodular goitre

CTRLs= controls

As = Arsenic

Pb = Lead

Cd = Cadmium

Ni = Nickel

* = significant at $p<0.05$

p-value = significant level

F = F - ratio

Values are reported as mean± SEM (standard error of mean)

The overall prevalence of thyroid autoantibodies (TPOAb and TgAb) among the various groups is shown on table 4.28. There were significant increases in the autoantibody distribution observed among the different subgroups, SNTG, TNG, HYPERTH, HYPOTH, Thy Cancer and Controls respectively.

Among the different subgroups, some patients showed significant positivity for both TPO Ab and Tg Ab (but not for either of the two) (Table 4.28).

Significant differences was observed in the smoking habit ($p<0.05$) while alcohol use was not significantly different ($p>0.05$) among groups with SNTG, HYPERTH, HYPOTH and thyroid cancers.

Table 4.28: Comparison of Autoantibodies distribution in the subgroups

INDICES	SNTG	TNG	HYPER	HYPO	Thy Ca	Controls
TPO Ab (%)	16.27 (All F)	13.33 (All F)	32.69 (All F)	60.00 T 13.33 M 46.67 F	25.00T 8.33 M 16.67 F	4.61 (All F)
Tg Ab (%)	13.95 T 2.32 M 11.63 F	-	19.23 T 1.92 M 17.30 F	20.00 (All F)	41.66 T 16.66 M 25.00 F	3.07 (All F)
TPOAb Tg Ab (%)	9.30 2.32M 6.98 F	-	17.30 1.92 M 15.38 F	20.00 (All F)	25.00 16.66M 8.33 F	2.31 (All F)

SNTG = simple nontoxic goitre
Hyper = hyperthyroidism
Hypo = hypothyroidism
Thy Ca thyroid cancer
TNG = Toxic nodular goitre
CTRLs= controls
F = F – ratio
* = significant at $p<0.05$
p-value = significant level
Values are reported as mean± SEM (standard error of mean)

TPO Ab = thyroid peroxidase
TgAb = Thyroglobulin
% = percentage of subjects
$\chi 2$ = Chi - square
M = males
F = females
T = total

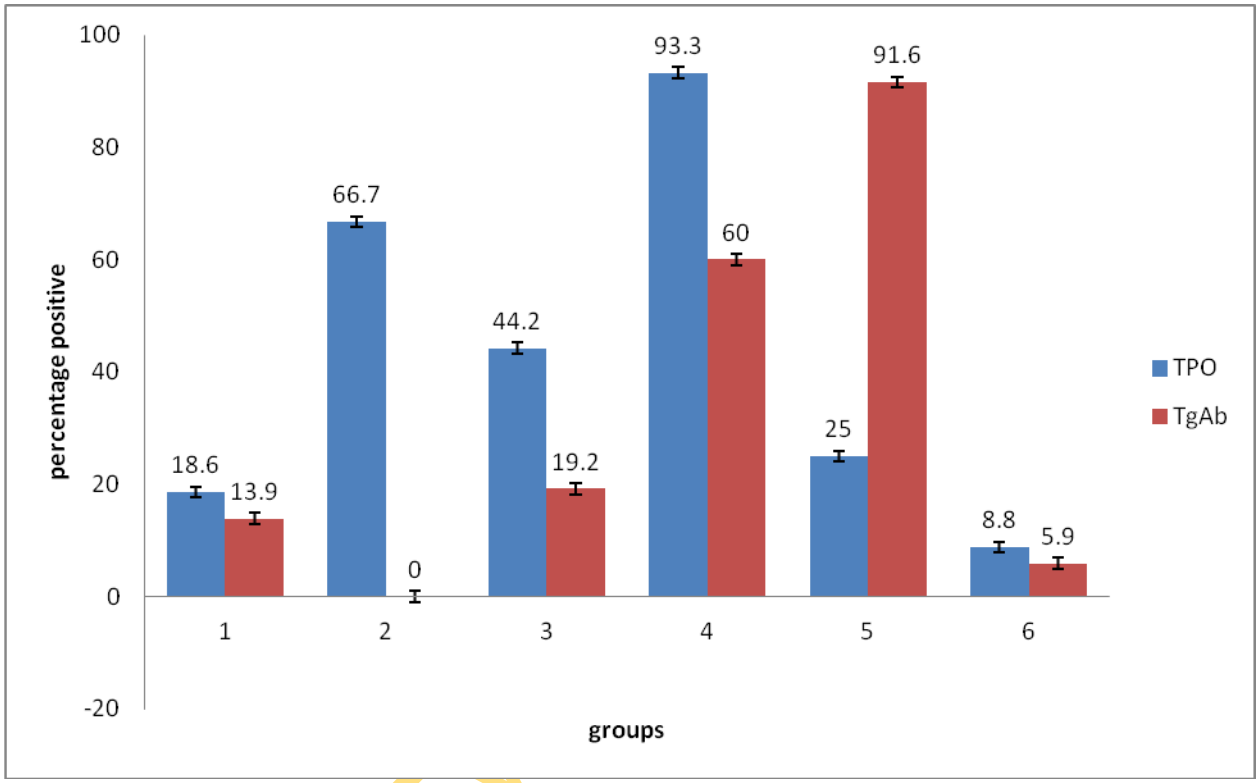


Figure 4.0: Autoantibody Prevalence of Subjects with positive TPOAb and TgAb in the various groups

Group 1 = Simple nontoxic goitre; Group 2 = toxic nodular goitre

Group 3 = hyperthyroidism; Group 4 = hypothyroidism

Group 5 = thyroid cancer; Group 6 = control

TPO = thyroid peroxidase antibody; TgAb = Thyroglobulin antibody

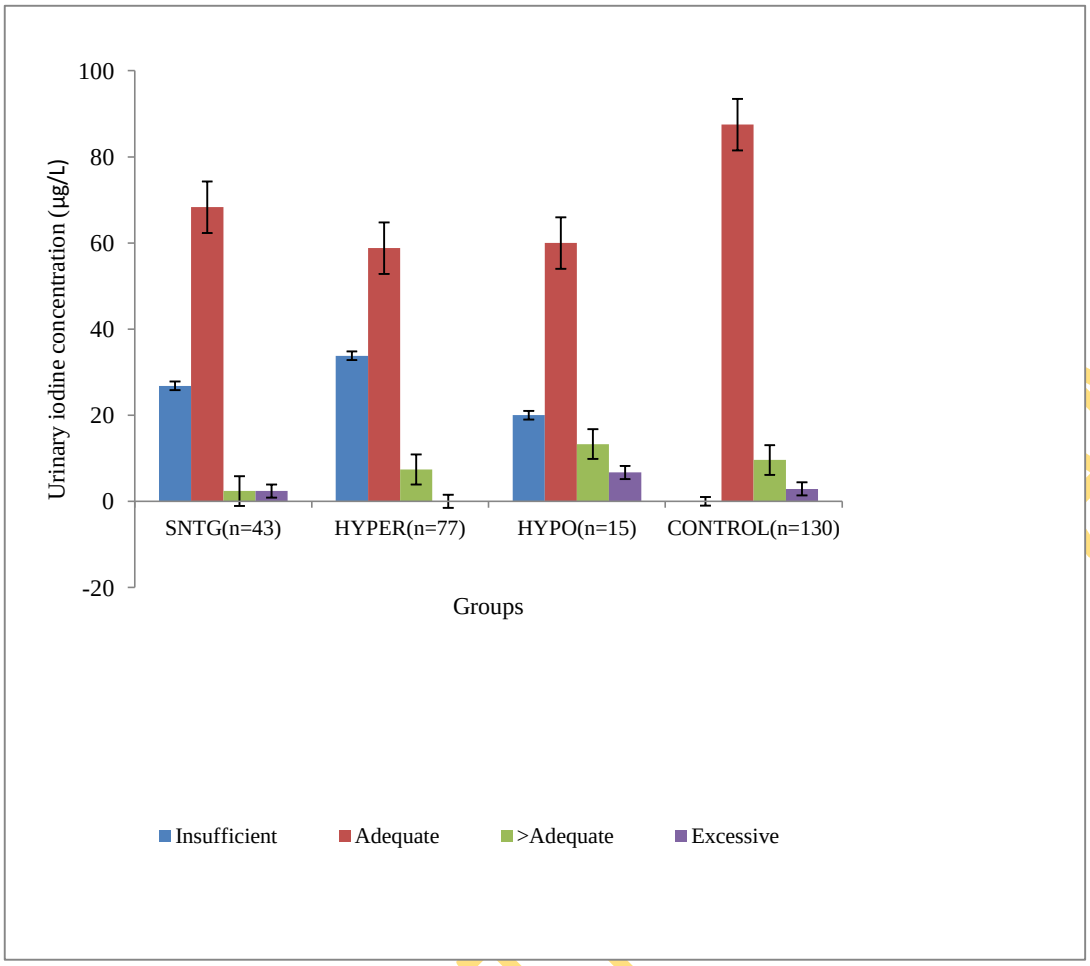


Fig. 4.1: Urinary iodine concentration (µg/L) in the various clinical groups and controls

SNTG = Simple nontoxic goitre

Hyper = hyperthyroidism;

Hypo = hypothyroidism

20 – 49(µg)= insufficient (moderate iodine deficiency)

50 – 99(µg) = insufficient (mild iodine deficiency)

100 -199(µg) = Adequate (optimal iodine nutrition)

200 – 299(µg) = More than adequate

300(µg) = Excessive

CHAPTER FIVE

DISCUSSION

Thyroid autoantibodies and imbalance between essential and toxic trace elements has been identified as the major cause of thyroid disorders; since thyroid disease are predominantly autoimmune in nature (Swain *et al*., 2005). Predisposing genetic factors seem to be evident, but environmental factors such as smoking, nutrition; especially an increased iodine supply as well as an increased age have also been shown to be conclusively linked to AID. Thyroid autoantibodies are an invaluable tool in the management of thyroid disorders (Okosieme *et al*., 2007). However, antibody testing is not widely available in routine clinical practice in Africa, and few studies have measured thyroid antibodies in African patients. The occurrence of thyroid autoimmunity, and hence the utility of antibody testing in African patients with thyroid disease, is therefore largely unknown. The paucity of data on the alteration of the balance between certain essential and toxic trace elements which has a strong role in the pathogenesis of autoimmune thyroid disease has stimulated the interest in this study. The study examines the contribution and relationships of this imbalance to the patterns of various thyroid disorders in Nigerian patients. Thyroid autoimmunity is uncommon in iodine-deficient areas but becomes more prevalent with improvements in iodine nutrition (Laurberg *et al*., 1998; Doufas *et al*., 1999). The majority of participants in this study were resident in Ibadan and Lagos, cities with improving salt iodization, diversified diets, and coastal access to sea foods.

The male to female ratio of thyroid disease patients in this study was found to be 1 to 4.27 (26 males to 111 females), confirming earlier report of hormonal influences (Nelson & Steinberg, 1987; Volpe, 1996). Iodine plays a key role in the manifestation of autoimmune thyroid disorder (Mariotti *et al*., 1996), as substantiated by experimental (Mooij *et al*., 1993) and epidemiological studies (Martino *et al*., 1986). Many thyroid processes are inhibited when iodine intake becomes high, and the frequency of apoptosis of follicular cells becomes higher (Laurberg *et al*., 2010). This abnormal inhibition of thyroid function by high levels of iodine is especially common in people affected by thyroid autoimmunity (Hashimoto's thyroiditis) (Laurberg *et al*., 2009; 2010). Even minor differences in iodine intake between populations are associated with differences in the occurrence of thyroid disorders.

In the present study, autoantibody positivity and titre were found to be highly significant in all forms of thyroid disorders compared with controls, this may be due to autoimmune involvement. The prevalence rates of TPOAb and TgAb were more frequent in females (48-57

years) than in males and increased with age as a result of hormonal influence; studies by Pedersen *et al*., 2006 had similar findings. The increased autoantibodies prevalence in the healthy population observed conforms with the findings of Li *et al*., 2008 and Larsen, 2003 who reported 8-27% and 5-20%, respectively but varied from the report of Okosieme *et al*., 2007 (7% and 4% for TPOAb and TgAb, respectively. Of these control groups, four were positive for TPOAb alone, while eight showed both TgAb and TPOAb activity. None was positive for TgAb without being positive for TPOAb. In Western populations, the prevalence of TgAb and/or TPOAb was estimated at about 10% of the general population (Groves *et al*., 1990), but the few available studies in Africans report a much lower prevalence (0–2.7%) in healthy individuals (Chinyanga *et al*., 2000; Njemini *et al*., 2002; Hawa *et al*., 2006). This seemingly reduced tendency for thyroid autoimmunity in Black populations is supported by studies (Hollowel *et al*., 2002) in which thyroid antibodies are less prevalent in African Americans than in Caucasian Americans (Hollowel *et al*., 2002). The discrepancy between the findings in this study and other African studies may in part be due to differences in methodology (Some of the studies in Africans measured thyroid antibodies using agglutination methods, which are less sensitive than the more recent chemiluminiscent, RIA and ELISA techniques (used in this study) as well as genetic factors (crucial for the development) regional and temporal differences between the groups studied.

The higher thyroid peroxidase TPO Ab titre found in hypothyroidism, toxic nodular goitre and hyperthyroidism compared with TPO Ab positive control subjects, agrees with the findings of Xiu *et al*., 2011 and maybe due to the presence of autoimmune thyroid disease usually manifesting as Hashimoto's and Graves' disease in hypo- and hyperthyroidism respectively.

The large number of higher thyroglobulin Tg Ab titre (agrees with the finding of Noboru *et al*., 2010) found in thyroid cancer hypothyroidism patients compared with their TgAb positive control groups maybe due to the presence of the thyroid cancer; smoking as found in some hypos and thyroid cancer patients as well as excess iodine nutrition, (since a high level of iodine, a major stimulator for TPO and Tg) increased the incidence of positive TgAb (Li *et al*., 2008, Noboru *et al*., 2011). In addition, hypothyroidism is more prevalent in populations with a high iodine intake. Probably, this is also a complication of thyroid adaptation to iodine intake (Laurberg *et al*., 2010). Although smoking was negatively associated with the presence of thyroid antibodies (TPOAb/TgAb), smoking may induce or aggravate some forms of AITD due to reactive oxygen species and the excessive iodination of proteins (particularly thyroglobulin) induced by high doses of iodine which can play important role in the development of thyroid dysfunctions and autoimmune disease (Lupachik *et al*., 2007). The following are implicated: cigarette smoke and

nicotine, CO hypoxia, and oxidative stress from oxygen radicals (Boelaert *et al*., 2010) with Graves' disease (GD) and Graves' ophthalmopathy aggreviation (Prummel *et al*., 2004). Thiocyanate, a constituent of cigarette competes with iodide for trapping by the sodium iodide symporter and for oxidation by the TPO; induces both a release of iodide from the thyroid cell and a decrease of thyroid hormone synthesis. Experimental and epidemiological studies have shown that thiocyanate overload aggravates the severity of iodine deficiency and worsens its outcome (Contempre et al., 2004; Delange, 1989). In adult humans smoking has been reported to increase thyroid hormone levels in serum independently of TSH which may be due to sympathetic activity, and subsequent cessation of smoking results in reduced T_4 and rT_3 levels, with no significant change of T_3 levels, which may explain reported weight gain as thyroid levels return to normal after cessation of smoking (Melander et al., 2009). Others have reported mild inhibition of thyroid function in female smokers with elevated T_4 and T_3 (Soldin et al., 2009).

Thyroid disorders induce heavy metabolic and enzymatic damage to cell function. The TSH values were significantly higher in patients positive for both TPOAb and TgAb than in the groups that did not have autoantibodies especially as noted with hypothyroidism when compared with controls; high iodine intake was a risk factor for developing autoimmune disease seen in hypothyroid antibody positive subjects. There is evidence, particularly in animals, that high levels of iodine can be a contributing factor in the development of autoimmune thyroid disease. How iodine produces such an effect is unclear but it may involve the production of iodine-rich thyroglobulin, which may be particularly immunogenic thereby inducing immune responsiveness. Some, but not all, studies in humans have shown that the presence of serum anti-microsomal antibodies (TPOAb) is more prevalent in areas with sufficient iodine than in areas of mild iodine deficiency (Roti & Vagenakis, 1996). Serum Tg concentration and urinary iodine concentration were closely associated not only when iodine deficiency was moderate to severe, but also when it was mild. Moreover, under conditions of moderate-to-severe iodine deficiency, serum Tg concentration was correlated with all other indicators of thyroid function except TSH. Thyroglobulin antigen used for the detection of human Tg is an aid to the diagnosis of thyroid disease but has been assayed in only a few previous studies as a requirement for determination of io dine status. This study found a high prevalence of thyroglobulin in SNTG, TNG, Hyperth, Hypoth, Thyroid cancer and Controls. Thyroglobulin, is an iodinated molecule and a template for thyroid hormone synthesis. Human and experimental data suggest that iodinated Tg is more highly immunogenic than poorly iodinated Tg (Rasooly *et al*, 1996; Rose *et al*., 1997). There is also an

enhanced proliferation response of human T lymphocytes to iodinated Tg, when compared with Tg lacking in iodine (Rose *et al*., 1997) concentration.

From the present study, Fe, Cu, Zn & Se were significantly decreased in hyperth and thyroid cancer patients. Iron decreases in HYPERTH due to hematopoietic effects that is; the increased cellular demand for oxygen in HYPERTH leads to increased production of erythropoietin and increased erythropoiesis (Williams, 2005) due to tissue hypoxia. Blood volume is usually not increased because of hemodilution and increased red cell turnover; this may result in anemia arising from decreased survival of the red blood cells. In hypoth, macrophages transfer iron (most of which comes from the destruction of old erythrocytes) to plasma transferrin, which then carries it to the bone marrow for hemoglobin synthesis; macrophages also maintain a storage pool of iron when red cell destruction exceeds the rate of production, iron accumulates within macrophages and the storage pool expands. When the balance shifts toward red cell production, macrophage releases additional iron from their store (Carpenter *et al*., 2001; Kaplan *et al*., 2003). Malignancy interferes with the release of iron from macrophages and may cause a drop in red cell production despite the presence of adequate Fe reserves. Thus, in addition to thyroid hormone deficiency, iron deficiency also. This agrees with the findings of Hawk *et al*., 2003 and Eidan *et al*., 2006.

Iron was also reduced in HYPOTH and HYPERTH when compared with the SNTG. Iron deficiency hinders the production of thyroid hormone by reducing activity of heme-dependent thyroid peroxidase. Zinc was highly reduced in HYPOTH than in the other groups; marked alterations in zinc homeostasis were observed in patients with thyroid diseases. Hyperthyroid patients excreted significantly greater amounts of zinc than controls, indicative of a higher catabolic process (Liu *et al*., 2001). Since a large proportion of Zn in serum is bound to albumin (80%) (Kaplan *et al*., 2003; Hasegawa *et al*., 2003), lowered Zn levels might accompany low albumin levels in hyperthyroidism. The increase in loss via urine could be attributed to the increased levels of serum amino acids due to catabolic effect of thyroid hormone; these ultrafilterable molecules can form complexes with this metal which can easily pass through the glomeruli resulting to their low levels.

Selenium is a biologically active element in the form of selenocysteine in the active centre of selenoproteins. It is a structural component of two important enzymes, glutathione peroxidase and iodothyronine deiodinase (Frier *et al*., 1999 and Duffield *et al*., 1999), which are essential for the activity of the deiodinase complex that converts T_4 to T_3 (Arthur, 1999). The increased levels of thyroid hormones and the concomitant increase in the production of hydrogen peroxide by the

thyrocytes, give glutathione peroxidase the required substrate, which may be the cause of selenium depletion in the blood in the HYPERTH.

Experimental studies have shown increased oxidative stress in hyperthyroidism as a result of increased catabolism and oxidant generation (Givelek *et al*., 2001; Konukoglu *et al*., 2001 and Seven *et al*., 2001). As a consequence, Cu-Zn-SOD thus, converts potentially toxic superoxide radical into hydrogen peroxide; as increased metabolism persists, increased toxic superoxide radical produced, require the scavenging action of superoxide dismutase which may lead to the depletion of the Cu and Zn store and thus low level of plasma Cu and Zn found in hyperthyroid subjects as demonstrated in this study. The level of Cu was markedly reduced in patients with SNTG and HYPERTH when compared with HYPOTH; Copper is known to be critical for normal brain function, and abnormal copper metabolism has been associated with some disorders involving the auditory system (Lonnerdal *et al*., 2001). The slight increase in Cu seen in HYPOS may be related to the increased ceruloplasmin levels. Furthermore, it may be explained by the elevated copper level regularly seen in females using a contraceptive since Cu homeostasis is influenced by oestrogen which induce the synthesis of ceruloplasmin. This maybe largely due to the additional (synthetic) oestrogen found in contraceptives and hormone replacement therapy. Thyroid hormone receptors interact with estrogen by specific bonds (Fischbach, 2000), oestrogen give rise to copper retention – and vice versa – ultimately leading to zinc and other nutrient depletion, due to antagonism between zinc and copper and oestrogen dominance. Once copper is in excess and too dominant in relation to zinc, it can exert an 'anti-nutrient' – or toxic metal influence, Baratosy (2005). High copper levels inhibit the absorption and utilisation of zinc (particularly), iron, magnesium, vitamins B_3, B_6, C, E and certain trace elements.

The significant reduction in copper level of patients with thyroid cancer found in this study may be as a result of the carcinogenic process which includes damage to the cancer cell by oxygen free radical more than normal thyroid cells, this low level is in agreement with the report of Hasegawa et al., (2003) but contrary to the findings of Hazim et al., 2006. Decreased expression levels of SOD in the thyroid cancer group maybe due to the possibility that cancer cells are more likely to suffer damage (Zaichick et al., 1995) by oxygen free radicals than normal or differentiated tumor cells. An insufficient intake of antioxidant vitamins and minerals such as zinc (Powell, 2000) may reduce the body's capacity to defend itself from the effect of activated oxygen derivatives on cell processes that play a role in the development of cancer and cardiovascular disease (Gallan *et al*., 2005), this may explain the low plasma Zn level demonstrated by thyroid cancer patients.

In this study, hypothyroid patients had lower levels of Fe, Zn and Se than control groups; but demonstrated higher levels of Cu. Serum selenium concentration (Type 1 T_4 deionidase contains selenium in the rat) was unchanged by Zn supplementation. Zn may play a role in thyroid hormone metabolism in low T_3 patients and may in part contribute to conversion of T_4 to T_3 in humans.

Selenium and Zinc are required for the conversion of T_4 to T_3 which prevents hypothyroidism. Zinc interacts with Se to promote the conversion of T_4 to T_3. The deiodinase enzyme contains selenium (an antioxidant and anti-inflammatory agent) which if deficient, the conversion of T_4 to T_3 is impaired leading to a functional hypothyroidism. Studies have shown that if iodine is low, selenium must also be kept low to prevent the hypothyroidism from becoming worse (from increased DI-I and T4 depletion, as explained Zinc deficiency has been shown to impair the metabolism of thyroid hormones (Kralik *et al* 1996) resulting in low levels of thyroid hormones observed in hypothyroidism and an increased Cu levels seen in these patients; since excess Zn depresses Cu resulting in possible CVD (Klevay, 1975) usually associated with hypothyroidism. Similar findings was observed by Erdal *et al*., (2008). Thyroid hormone nuclear receptors contain zinc ions, crucial for the functional properties of type I deiodinase (Olivieri *et al*, 1996). In hyperthyroidism, the increased rate of body metabolism seen may lead to the increased generation of superoxide radicals formed by univalent reduction of oxygen in the tissues. Excessive generation of these free radicals and the scavenging action of Cu-Zn superoxide dismutase can possibly lead to depletion of both copper and zinc store. Lack of zinc has been implicated in impaired DNA, RNA, and protein synthesis during brain development (Berdeimer, 1998). For these reasons, deficiency of zinc during pregnancy and lactation has been shown to be related to many congenital abnormalities of the nervous system in offspring of affected mothers.

Copper has like zinc been implicated in abnormal thyroid function, Kralik *et al.*, (1996); Elements with similar chemical and physical properties interact antagonistically by for instance competing for the same binding sites on transport proteins and enzymes. An example is the antagonism between Cu and Zn which may lead to Cu deficiency and secondary to that, Fe deficiency (Angelova, *et al*., 2014). Copper is an integral component of tyrosinase; an enzyme involved in tyrosine metabolism. Tyrosine, an amino acid, important in thyroid hormone biosynthesis (Scwhartz, 1996) maybe inappropriately metabolised. Iron deficiency impairs thyroid hormone synthesis reducing the activity of heme-dependent thyroid peroxidase (Zimmermann, 2002). Iron is critical for thyroid function, thus iron-deficiency anemia is often an important factor in hypothyroidism (Zimmermann *et al*., 2000). Iron deficiency has adverse effects on thyroid

metabolism, for example it decreases circulating thyroid hormone concentrations and blunts the efficacy of iodine prophylaxis (Zimmermann *et al*., 2007).

Selenium levels were markedly reduced in all the different groups (HYPERT, HYPOTH and thyroid cancer); **t**his perhaps is as a result of increased inflammatory activity arising from decreased activity of selenium containing antioxidative enzymes such as glutathione peroxidise (GPx) (Zimmermann & Kohrle, 2002), This is consistent with the findings of several investigators (Aliciquzel *et al*., 2001; Kucharzewski *et al*., 2002, 2003; Derumeaux *et al*., 2003). They also stated that Se deficiency is associated with AITD as shown in subjects with autoimmune thyroiditis and Graves' disease. Increasing dietary selenium or administration of selenomethionine have also been reported to diminish TPO antibody levels (Gartner *et al*., 2002; Duntas *et al*., 2003). In support of these observations, several randomized double - blind studies have shown that selenium supplementation can reduce the titre of thyroid autoantibodies (Gartner *et al*., 2002**;** Duntas *et al*., 2003; Gartner *et al*., 2003)**.** Gartner *et al*., **(**2002**)** conducted a randomized study in 70 women with autoimmune thyroiditis and demonstrated that thyroperoxidase antibody (TPO-Ab) concentrations fell significantly in subjects on selenium for three months; **i**n the 47 subjects crossed over for a further six months, TPO-Ab levels rose in subjects on placebo, yet declined further in those subjects on selenium. Interestingly, most subjects on selenium supplements also reported a subjective improvement of well-being during periods of supplementation (Gartner *et al*., 2003). Similarly, in a six month study by Duntas *et al*., (2003) in 65 subjects with autoimmune thyroiditis, TPO-Ab levels declined by 56% in those receiving T_4 and selenium versus 27% in those receiving T_4 alone. Selenium, may play an interactive role in the development of these thyroid irregularities, and in turn, cardiovascular disease (Hoption Cann, 2006). Thus, a deficiency of selenium could predispose the thyroid to oxidative injury and lead to decreased peripheral T_3 production (Sarne, 2010). An increase in iodine intake under conditions of Se deficiency may intensify the formation of hydrogen peroxides and lipid hydroperoxides (Contempre *et al*, 1996; 2003) leading to an increased the risk of AITD (Rose *et al*., 2002, Gartner *et al*., 2003). In areas with mild selenium deficiency, selenium plasma levels have been shown to be lower in subjects with autoimmune thyroiditis (Derumeaux *et al*., 2003) and Graves' disease (Kucharzewski *et al*., 2002) compared with normal controls.

In the present study, there was a negative correlation of iodine with thyroglobulin but positive with autoantibodies (TPO,Tg). The mechanism behind this may be explained by the fact that thyroglobulin combined with high iodine increases its antigenicity and promotes lymphocyte proliferation; this is partly in agreement with the works of van den Briel *et al*., 2001 and Li *et al*., 2008. Iodine is required for the formation of thyroid hormones and a deficiency results in

hypothyroidism (Ikeda *et al*., 2000). Excessive iodine intake is also known to induce hypothyroidism in people who have underlying thyroid disorders; however, few studies have been conducted on subjects with normal thyroid function without a history of autoimmune thyroid disease (Hwang *et al*., 2011). Serum TSH levels provides a good indicator of functional iodine status, since levels of the hormone increase to compensate for any inadequacy in available dietary Iodine (Arthur, 1999). Iodine deficiency induces a transient oxidative stress and oxidative DNA damage (Maier *et al*., 2007). As previously indicated, increase in iodine intake under conditions of selenium deficiency may intensify the formation of hydrogen peroxides and lipid hydroperoxides (Contempre *et al.*, 1996; 2003) leading to an increased the risk of autoimmune thyroid disease (Rose *et al*., 2002, Gartner *et al*, 2003). Iodine status, like that of iron, is a state not adequately reflected by one indicator.

Of special interest are the interactions between essentials and toxic trace elements. In this study, the levels of Cd, Pb, As and Ni were elevated in all the groups compared with the control. Increased levels of Cd can stimulate the formation of reactive oxygen species (ROS) and induce damage to various tissues (Alvarez *et a*l., 2007; Thijssen *et al*., 2007; Thompson et al., 2007). Cadmium enhances lipid peroxidation and DNA damage and can induce abnormal expression of the main antioxidant molecules in cells (Stohs and Bagchi, 1995). Lead & Cd decrease or impair the activity of type I 5'-deiodinase (5'-DI) in the liver (Wade *et al*., 2002) thereby inducing symptoms or clinical signs of hypothyroidism (Chaurasia et al., 1997; Gupta and Kar, 1998). With long-term exposure to Pb, it accumulates in bone, where it is stored for years; these quiescent Pb stores are mobilized when increased bone turnover occurs in endocrine disorders, such as hyperthyroidism; latent Pb toxicity may then become symptomatic. Accumulated Cd in the mitochondria of the thyroid follicular cells might disturb oxidative phosphorylation and loss of energy supply.

Arsenic, an antagonist of Se is just to the left of Se in the Periodic table of element; when As & Cd are combined, there is an increased effect of reducing Se and glutathione (Y´a~nez *et al*., 1991), this affects the antioxidative and antinflammatory properties and damages the body by oxygen free radicals. Arsenic affects the thyroid gland (Ciarrocca *et al*., 2012); it competes with the body phosphorus and may result in heart abnormalities (Frompovich, 1982). Relatively small amounts of As are consumed through the food chain from natural leaching of rocks and soils. Humans add to that by burning of coal, wood and oil (Smith *et al*., 2005; 2007). Heavy metals are known to pose a serious threat to human health (Norberg *et al.*, 2000). The toxic effects of Pb may be counteracted by essential metals like Se, Zn and in some cases Mn (Milne, 1999). Reem *et al*., 2002 showed that an increase in Cd concentration was accompanied by an equimolar increase in Zn

probably attributed to induced biosynthesis of the transport protein metallothionein by these metals. Trace elements such as Se have been known to have beneficial effects on Cd-induced oxidative stress (El-Sharaky et al., 2007) because Se is essential for Cd removal; selenium atoms combine with Cd atoms and are taken out of the body via the biliary pathway (Ghosh & Bhattacharya, 1992); this results to less Se to form the deiodinase enzymes which convert T_4 to T_3, resulting in low T_3 observed in hypothyroidism (Gupta *et al.*, 1998). There's also, less Se to form glutathione peroxidase, one of the body's prime antioxidants, resulting in greater levels of reactive oxygen species and hydrogen peroxide, which lead to an increased oxidative stress and may result in the damage to the membrane integrity and loss of membrane-bound enzymes like ATPases (Asagba *et al.*, 2004; Asagba and Obi, 2005; Galazyn-Sidorczuk *et al.*, 2009). There is also an increased production of thyroid hormone, damage to the thyrocytes, reduction in Tg producing cells and decrease in both T_4 and T_3 (Yoshizuka *et al.*, 1991). Cadmium decreases T_3 by interfering with T_4 to T_3 conversion (Nishijo *et al.*, 1994). By the selenoenzyme. Evidence indicates that acute heavy metal exposure will induce immediate hyperthyroidism (Long *et al.*, 1998), leading to preferential synthesis of T_3 and/or preferential deiodination of T_4 to T_3 (Ghosh & Bhattacharya, 1992).

Among the heavy metals, Cd an industrial pollutant from battery, plastic, fertiliser industries (Stohs and Bagchi, 1995), cigarette smoke, air, food and even water (Friberg *et al.*, 1992) is known to be abundant in tissues. It inhibits antioxidant enzymes (Asagba and Eriyamremu, 2007; Chater *et al.*, 2009) as earlier stated. Liver and kidney are considered as the main target organs in acute and chronic Cd exposure (Asagba and Obi, 2000). Other tissues involved in Cd toxicity are the testis, heart, bone, eye and brain (Eryamremu *et al.*, 2005; Asagba 2007). The possible effects in the general population of long term exposure to cadmium has been of concern (Asagba, 2009). Heavy metal toxicity has several impacts on health, they inhibit certain essential trace element absorption such as Fe and Se leading to H_2O_2 accumulation which induces cell damage and oxidative stress among others.

The significantly high level of thyroid hormones T_3, T_4, FT_4, FT_3 and simultaneously low concentration of TSH values; as well as significantly low levels of thyroid hormones T_3, T_4, FT_4, FT_3 and a very high concentration of TSH values in HYPERTH and HYPOTH subjects in this study confirmed the establishment of both hyperthyroidism and hypothyroidism respectively. Several studies reported similar finding (Al-Rubae'i and Al-Musawi, 2011). The hormonal profile of HYPERTH (elevated levels of FT_3, FT_4, reduced level of TSH) is in conformity with other studies (Swain *et al.*, 2000; Canaris *et al.*, 2007). Elevated hormone levels have been implicated in HYPERTH leading to thyrotoxicosis (Stockigt, 2003). The differences in individual thyroid

function are caused by a combination of genetic and environmental factors (Hansen *et al*., 2004). Similar considerable differences may be exhibited in thyroid function between populations when estimated by median serum TSH levels. Such variations are probably caused by a number of primarily environmental factors, of which iodine intake level seems to be of major importance (Knudsen *et al*., 2000; Laurberg *et al*., 2001). In the hyperthyroid state, the increased effect of these hormones on energy metabolism leads to accelerated oxidative activity (Seymen *et al*, 2004). The effects are accompanied by increased total oxygen consumption and an increased generation of reactive oxygen species (ROS), acceleration of free radical production and the occurrence of oxidative stress (Vcra *et al* 2004, Seymen *et al*, 2004). In this state of oxidative stress, increased activity of antioxidative enzymes is induced by excessive production of free radicals. The ROS and reactive nitrogen species (nitric oxide, nitrogen oxide) are known to attack various biomolecules in the body including, lipids, nucleic acids and proteins (Mayne, 2003), with concomitant damage to the body cellular components. In several experimental studies of hyperthyroidism, this free radical-mediated oxidative stress has been implicated in the genesis and exacerbation of degenerative diseases (e.g arthritis) and loss of cellular integrity, including the cellular defense mechanisms.

CHAPTER SIX
SUMMARY AND CONCLUSION

6.1. **Summary**

Thyroid disease may be caused by a variety of mechanisms such as different toxic trace elements interferring with the H-P-T axis at different levels. Mechanisms of action may involve the sodium–iodide symporter, thyroid peroxidase enzyme, receptors for THs or TSH, transport proteins or cellular uptake mechanisms.

The occurrence of thyroid diseases is determined by interplay between genetic and environmental factors. Among thyroid patients, specificity of antimicrosomal and antithyroglobulin antibodies were 40.1% and 24.8%. The major environmental factor that determines thyroid disease prevalence is iodine status, but other environmental factors influencing entire populations have been identified such as trace elements status, goitrogens in food and drinking water. Less focus has been on individual environmental factors and the interplay between factors. The variation in thyroid disease prevalence between the genders is well known with a higher occurrence among women (81.02% from this study). The association with age is probably dependent on certain factors such as autoantibodies and iodine status, among others, because it seems that the zenith of disease prevalence appears earlier in life the more severe trace element deficiency the population is exposed to.

In this study, there seemed to be a relationship between smoking and Graves' ophthalmopathy but may be confirmed in a larger study. The exact mechanism of the impact of cigarette smoke and nicotine on the thyroid gland remains to be elucidated (Kurdybacha et al., 2011). Studies show that in iodine-deficient areas, a strong association between tobacco smoking and goitre prevalence is found, whereas the association is less pronounced in iodine-replete areas (Knudsen *et al.* 2002). This was predictable from experimental studies showing thiocyanate (a degradation product of cyanide and a major goitrogen in tobacco smoke (Fukayama *et al.* 1992), a mediator of the goitrogenic effect of tobacco smoke acting as a competitive inhibitor of iodine uptake. Thus, tobacco smoking may induce intrathyroidal iodine depletion. The association with alcohol intake in this study followed a similar trend (a few alcohohics were recruited); it has only been investigated in few studies, but a low occurrence of goitre among alcohol consumers has been found. The mechanism of this association is not known (Nils *et al*., 2002) but may support the idea that the effect of alcohol on the formation of multiple nodules is mediated through thiocyanate.

From this study, thyroid autoimmunity appears common in the African patients. Autoantibodies were significantly associated with AITD and may help identify cases of AITD

which would otherwise have been misdiagnosed. Thyroid antibodies are an invaluable tool in the management of thyroid disorders, since most thyroid diseases are autoimmune, the combined determination of both anti-TPO and anti-Tg autoantibodies provides the most accurate diagnostic tool for thyroid autoimmunity. Circulating thyroid autoantibodies are more common in females than in males (Pedersen *et al*., 2003). The reason for this female preponderance is unclear; hormonal and genetic factors may play a role. The clinical utility of thyroid antibodies in African patients requires further evaluation in a wider population. Most of the essential trace elements were reduced in both hyper and hypothyroid patients as a result of either increased utilization or deficiency.

The difference in reduction of plasma Fe was significant. Although there appears to be no gender influence in micronutrients, plasma Fe was significantly reduced in males when compared with the females. The reason is not clear, since the balance of the 3 minerals, Cu, Fe, Zn is critically important in preventing and correcting thyroid disorders; this may be due to inadequate intake or decreased efficiency of zinc absorption. Each of these 3 elements antagonizes one another and can deplete the other 2. Many times, the antagonism and depletion effects are not due to competition in absorption, but because of their synergistic activity. These minerals work together like the corners of a triangle (Rosenzweig & Volpe, 1999) in many physiological processes and are also implicated in many pathological changes in the body (Al-Sayer *et al*, 2004). On the other hand, thyroid hormones are known to influence the metabolism of some of these trace elements (Al-Sayer *et al*, 2004). Imbalances in some of these elements such as (Cu, Fe, Zn, Se, As, Pb, Cd, Ni) from this study have been associated with the development of some thyroid pathologies. The peripheral metabolism of the THs can be affected through effects on iodothyronine deiodinases or hepatic enzymes. Even small changes in thyroid homeostasis may adversely affect human health, and especially fetal neurological development may be vulnerable (Boas *et al*., 2006).

Thyroglobulin antibody, a valuable marker for monitoring patients with thyroid cancer and an aid to the diagnosis of AITD from this study showed a high prevalence of 91.6%. From this study, a recognized measure for reducing thyroid cancer risk is to increase micronutrient consumption. Dal Maso *et al*., 2009 stated that avoiding ionizing radiation and iodine deficiency, particularly in childhood and young women is of great benefit. The presence of thyroid autoantibodies (TPO antibodies (Vadiya *et al*., 2007) is associated with an increased risk of miscarriage, premature deliveries (Negro *et al*., 2007) and the development of postpartum thyroid dysfunction (Vadiya *et al*., 2007). Euthyroid pregnant women who are positive for TPOAb may develop impaired thyroid function during pregnancy due to foetal demand and other physiological mechanisms. The results suggest that TPO-Ab and Tg-Ab predominantly develop due to a general

alteration in the immune system, whereas specific antigenic mechanisms are probably of less importance. However, further studies are needed to clarify the mechanisms involved in the development of thyroid autoantibodies. Patients with chronic renal disease may have symptoms that mimic those of hypothyroidism; in addition, free T_4 tests are not always reliable here so that Tg/TPO antibodies testing will allow a differential diagnosis.

6.2. Conclusions

The findings in this study aimed at examining the pattern and interaction of autoantibodies and trace elements in thyroid disorders are comparable with the hypothesis that toxic and altered levels of essential trace elements and autoantibodies provoke autoimmune disease leading to the increases in thyroid function indices thereby predisposing patients to thyroid disorders. These increases were more pronounced in subjects with autoimmune thyroid diseases because of the presence of autoantibodies. Increased utilization and/or deficiency of the trace elements resulted to their low levels. The prevalence of AITD is on the increase in Ibadan, Nigeria. Antimicrosomal antibody is an important and specific marker for thyroid autoimmunity. An appreciable number of patients with hyper, hypothyroidism, simple diffuse, toxic multinodular, and iodine deficiency goitres were found positive for antithyroid antibodies indicative of coexistent autoimmune phenomenon, which may remain undetected or missed unless they are investigated for antibodies. It was found that the imbalance in toxic and essential trace elements and the presence of these antibodies was associated with the development of thyroid dysfunction. This study also indicated that excessive iodine intake might promote thyroid autoimmunity, a risk factor for autoimmune-prone subjects to develop hypothyroidism. Thyroid disorders could precipitate several biochemical changes in tissue that predispose them to oxidative injury. Thus we suggest the use of trace elements as supplement with the regular therapy of thyroid disorders, in a hope to minimize or delay the chronic complications of thyroid diseases.

6.4. Contribution to knowledge

This study has contributed to an appreciation and a better understanding of thyroid pathologies, pathogenesis, certain mechanisms involved and its complications. Data from this study showed that the occurrence of thyroid autoimmunity in patients with thyroid disorders and healthy controls in South-Western, Nigeria revealed a higher prevalence of thyroid antibodies than has previously been reported in healthy Black Africans.

The prevalence of 8.8% for TPOAb and 7% for TgAb in healthy subjects is greater than the reported prevalence from other African studies. Thyroglobulin antigen appears to contribute 11.8% to thyroid disorders as seen in healthy subjects. The interactions between Cu, Fe, Se and Zn with

autoantibodies and the role of toxicants to the development of disorders in the thyroid gland. Factors other than autoantibodies and toxic trace element interaction may be responsible for some forms of thyroid disorders.

6.3. Recommendations and Further work

Since the number of hypothyroid & thyroid cancer patients recruited for this study was small, it is reconmmended that a larger population study on overt hypothyroids be done to actually confirm the biochemical status of the trace elements involved in this study.

It may be necessary to examine some other interferences such as perchlorate an endocrine disrupting chemical (EDC) found in drinking water, vitamins' (e.g vit A, E) status. Vitamin A deficiency, VAD increases the risk of goitre. Knowledge of dietary factors such as phytate which inhibit trace elements (e.g zinc) absorption and measures taken to overcome these factors is essential when designing strategies to improve the zinc nutrition of vulnerable groups. The best ratio or combination in which nutrients are best utilized which will form the basis for further research into thyroid dysfunction and auto antibodies. Thyroid volume, serum Tg concentration and urinary iodine concentration have all been suggested as useful indicators for measuring improvement in iodine status after iodine prophylaxis. However, all three have their own characteristics and limitations. The results from this study indicate the need to include a routine assay of autoantibodies, Cu, Fe, Zn, Se status in patients with thyroid disease and a screening exercise on individuals based on family history. Supplementation with these elements could be beneficial in the management of thyroid disease patients and may be useful as early intervention measures. Early detection of trace element abnormalities will be useful in overall management of thyroid disorders.

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APPENDIX I INFORMED CONSENT FORM

Title of Research: Evaluation of micronutrients and autoantibodies status in thyroid disorders

Name and Affiliation of Researcher: Aboh, Nancy A of the Department of Chemical Pathology, College of Medicine, University of Ibadan, Ibadan.

Purpose of Research: For an effective and efficient research; I would need your cooperation and assistance. Blood and urine samples will be drawn carefully from you to assay the trace e lements and thyroid function indices. The process of sample collection will not cause any har m to you. You are free to refuse to take part in the study or to withdraw at any given time. I will greatly appreciate your kind participation in this study.

Procedure of the research, what shall be required of each participant, and approximate total number of participants that would be involved in the Research: The research will recruit the patients attending the endocrinology clinics of the hospital selected. As a participa- nt, you will only be required to respond to the items on the questionnaire as honestly as possible. This is a rare disorder and from review of literature, information has been incompre hensive to enable sample size determination statistically. About 200 participants are required.

Expected Duration of Research and of Participant(s)' Involvement

The research is expected to last between three and five years. Participant's involvement in this research is once and may take about 10 – 20 minutes of your time.

Consent: Now that the study has been well explained to me and I fully understand the consent of the study process, I will be willing to take part in the study.

…… ……

Signature/thumbprint of participant/date

Signature of interviewer/date

……..

Signature/thumbprint of witness/date

APPENDIX II THYROID DISORDERS PATIENT QUESTIONNAIRE.

All the information provided shall be strictly confidential. You are implored to give accurate information to the best of your knowledge.

Good day Sir/ Madam, this questionnaire is being administered to evaluate your thyroid status and micronutrient/trace element status. This is to assist in the management of patients and help to prevent thyroid disorders in our society. Your kind cooperation in supplying the correct information to the following questions below will be highly appreciated.

SUBJECT'S IDENTITY/INFORMATION

PATIENT NUMBER. …… DATE…….

NAME OF SUBJECT……..

SECTION A DEMOGRAPHIC CHARACTERISTICS

Sex: M F

Age/Date of Birth:

Nationality:

State of origin:

Current area & address of Residence ……..

Area of Residence to include: Last 12 months……

Last 5 years……; Last 10 years……..

Marital status married / Single / Widow / Divorced

Educational Background None/Primary/Secondary/tertiary/Others (specify)

Occupation …….

SECTION B ANTHROPOMETRIC MEASUREMENT

Weight /Height /Blood Pressure /BMI

SECTION C GENETIC HISTORY

HAVE ANY OF YOUR FAMILY MEMBERS SUFFERED FROM ANY OF THESE DISEASE(S)

DIABETES MELLITUS YES / NO / NOT SURE

PERNICIOUS ANEMIA YES / NO / NOT SURE

MYASTHENIA GRAVIS YES / NO / NOT SURE

ADDISON'S DISEASE YES / NO / NOT SURE

GOITRE/THYROID DISEASE YES / NO / NOT SURE

HIGH BLOOD PRESSURE YES / NO / NOT SURE

BREAST CANCER YES / NO / NOT SURE

OTHERS, please specify ……...

When did you notice the swelling?

What other kind of treatment have you had in the past?

SECTION D MEDICATIONS

Are you currently on any Drugs ? If yes, Name

Are you currently on any herbal medications? If yes, Name

Are you currently on any Supplements? If yes, Name

Are you currently on any Contraceptives? If yes, Name

SECTION E DIET HISTORY

Vegetable intake Daily /Once a week/Occasionally/Never/ Others (specify)

Fruit intake Daily /Once a week/Occasionally/Never/ Others (specify)

Dairy foods Daily /Once a week/Occasionally/Never/ Others (specify)

Sea food consumption (Snails, shrimps etc) Daily /Once a week/Occasionally/Never/ Others (specify)

SECTION F **SOCIAL HISTORY**

Cigarette smoking Daily /Once a week/Occasionally/Never/ Others, specify

Alcohol Consumption Daily /Once a week/Occasionally/Never/ Others, specify

SECTION F WOMEN:

Parity (how many children do you have)?

Are you currently pregnant?

Form completed by ……..(self, patient relative)

Thank you for taking time to answer these questions.

APPENDIX III Sample Size Determination

According to 2006 records the proportion of patients with thyroid disorders;

Sample size = $\frac{pq(z\alpha)^2}{e^2}$

where,

p = prevalence; q = 1 – p

zα = standard deviation = 1.96

e = margin of error =5% = 0.05

p = prevalence of thyroid disorders in Nigeria is still uncertain

Sample size = 42 subjects

APPENDIX IV REAGENTS PREPARATIONS AND ASSAY

ZINC

Sample preparation: Samples for plasma zinc analysis as well as their standards were diluted 1: 4 with ultra pure water (1 part of plasma plus 4 parts of ultra pure water. Blanks and standards were prepared with 10% glycerin.

REAGENTS

Zinc metal/copper metal, high purity (Fisher scientific Co. Pittsburgh).

Trichloroacetic acid (TCA) AR, 10g/dl (10% wt./vol.)

Nitric acid, AR, zinc and copper free (Fisher Scientific Co. Pittsburgh).

Hydrochloric acid, AR, zinc and copper free (Fisher Scientific Co. Pittsburgh)

Glycerol, AR, 5% and 10% vol/vol.

STANDARD

1. Zinc, stock standard, 500ug/dl.
2. Zinc, intermediary standard, 500ug/dl.
3. Zinc, working standards.
4. Ultra pure water

3.6.6. PROCEDURE

Blank: The 5% glycerol solution in deionized water served as the blank in this assay.

Standard: Five wide-mouthed plastic bottles (Fisher Scientific company, Pittsburgh, USA) were marked 50ug/100ml, 100ug/100ml, 150ug/100ml, 200ug/100ml and 300ug/100ml. Aliquots of the respective working standards were added to each tube respectively as needed.

Test Samples: Into 75mm disposable propylene test tubes (Fisher Scientific Fairlawn, New Jersey, USA) were added 0.5ml (500ul) serum, followed by 2.00ml deionized water. They were analyzed in the AAS (AGW AES Analysengerate GMbH, West Germany) at 214nm.

Results and Calculations: Values were displayed digitally in ppm, these were converted to µg/100ml as for Zinc.

COPPER

Sample preparation: Samples for plasma copper analysis as well as their standards were diluted 1: 1 with ultra pure water (1 part of plasma plus 1 part of ultra pure water. Blanks and standards were prepared with 10% glycerin.

3.7.2. REAGENTS:

1. Copper, stock standard, 500ug/dl.
2. 10% glycerin,

3. 10% TCA, 20% TCA
4. 0.1% Lanthanum chloride,
5. ultra pure water

Standard: A commercial $Cu(NO_3)_2$ standard obtainable from BDH (BDH Chemicals Ltd, Poole, England) was employed. It has a target value of 1ml-10mg copper. This is equivalent to 10,000ppm, 1.0ppm, 1.5ppm, 2.0ppm, 2.5ppm and 3.0ppm working standard were prepared from the commercial stock standard to cover the reference range of 80-155µg/dl.

Working Standards: These were prepared from the commercial stock standard using 10% glycerol as diluents. The standards were prepared in 10% glycerol to ensure similar viscosity between serum and the aqueous standard used, thus ensuring similar rates of aspiration. These standards were used to calibrate the AAS. They were also used to ensure stability of the machine during operation by rechecking the standards after every 5 samples. There was need to clean the burner head frequently because of the high protein content of the serum solution. (This could be a cause for spurious reading).

Values were displayed digitally in ppm. These were converted to µg/100ml by multiplying by 100 as for zinc, e.g reading of 0.88ppm, is equivalent to 88µg/100ml copper, 1.0ppm = 100µg/100ml copper, while reading og 1.48ppm = 148µg/100ml copper.

Another commercial standard with a target value of 200µg/100ml copper (2ppm) obtainable from Boerhinger Manheim (Boerhinger house, Sussex, England) served as quality control samples.

Lead

Sample preparation: Samples for plasma iron, cadmium, selenium and blood lead analysis as well as their standards were diluted 1: 1 with ultra pure water (1 part of plasma plus 1 part of ultra pure water. Blanks and standards were prepared with 10% glycerin).

(Prasad, 1966; Sunderman, 1973; Analytical methods for AAS, Norwalk, 1976 and Tietz 1976). The analytical conditions vary with different instruments or analytical systems. The method described below is suitable for use with different instruments and produce comparable results (Sunderman, 1973; Analytical methods for AAS, Norwalk, 1976; Tietz, 1976 and AAS instrument manual, Lexington, 1976)

3.9.2. REAGENTS:

1. Stock standard, 500ug/dl.
2. 10% glycerin,
3. 10% TCA, 20% TCA

4. 0.1% Lanthanum chloride,
5. ultra pure water

3.9.3. GENERAL PROCEDURE.

The instrument and gas flow settings were established and aspiration rate precisely set to optimize the signal and minimize background noise. The nebulizer flow adjustment in place was locked. 10% glycerin solution was aspirated into the luminescent flame and the baseline set to read 0.000±0.001 absorbance. A baseline reading was taken before and after each sample reading and reset asrequired. The working standards were sequentially sampled from the most dilute to the most concentrated, aspirating until the reading was stable (±0.001 absorbance); six successive integration reading were recorded; the readings for each sample was averaged and the resulting values were used to establish the working curve; the control and the patients' samples were aspirated and the concentration calculated from the absorbance readings by extrapolation from the working standard curve.

3.9.4. PREPARATION OF THE STANDARDS

Fischer's scientific reagent 1000ug/dl is a certified reference standard for each element from which the stock standard (100µg/dl) was prepared. Working standards for each element was then prepared from the stock standard. The concentration of working standards were as follows: 0.0, 2.0, 4.0, 6.0, 8.0 µg/dl

Calculation: concentration of the elements in µg/dl = RxD

Where, R = AAS reading; D = Dilution factor

Protein – free filtrate methods: In 5ml tubes place 1.0ml working standard (in duplicate), controls and plasma. Add 1.0ml of 10% vol/vol trichloroacetic acid. Mix for 3 s on vortex mixer.

1. All tubes were placed in 90^{0C} water both for 10mins.
2. Centrifuged (refrigerated centrifuge) at 2500-3000rpm for 15min
3. Clear supernatant was removed and transferred to a 10ml tube
4. Added 1.0ml of 10% vol/vol TCA to tube with protein precipitate; mixed and placed in 90^{0}C water bath for 10min.
5. Steps 4 was repeated twice to give a 1:4 dilution
6. AAS was set at the required wavelength for each element e.g. 214.9nm for zinc or 324.8nm for copper etc as above for cadmium, iron, selenium and lead
7. Measured standards and controls.
8. A calibration graph was prepared to find concentration of plasma and blood specimen.

IODINE

3.10.4. Materials: Heating block, calorimeter, vented time hood with perchloric and trap, thermometer, test tubes, reagent flasks and bottles, pipettes, laboratory balance.

3.10.5. Reagents:

1. $KClO_3$ (Potassium chlorate), dry powder
2. $HClO_4$ (perchloric acid, 70%)
3. As_2O_3 (arsenic trioxide), dry powder
4. NaCl (sodium chloride), dry powder
5. H_2SO_4 (sulfuric acid, concentrated – 100%, 36N, liquid).
6. $Ce(NH_4)_4 (SO_4)$**.** $2H_2O$ (ceric ammonium sulfate), dry ppowder
7. H_2O (deionized water).
8. KIO_3 (potassium iodate), dry powder

3.10.6. Procedure:

1. Urine sample were mixed to evenly suspend any sediment.
2. 250µl of each urine sample were placed into a test tube using a micro pipette; 250µl of water was added into a test tube for a blank
3. Into tubes, 250µl of each iodine standard were pipette (2,5,10,15 µg/dl) of stocks prepared, otherwise prepare standards by pipetting from the 1µg/ml KIO_3 solution into tubes (this gives iodine standards of 0 – 15 µg/dl).
4. 750µl of chloric acid solution was added to each tube (samples, blank and standards). and gently mixed
5. All tubes, were heated for 50 – 60 minutes in a heating block at 110 – 115^O in a hood with perchloric acid trap. (The exact time and temperature are not critical as long as all tubes are heated the same; there was very little volume change during heating). tubes were cooled to room temperature.
6. 3.5 ml arsenious acid solution were added to each tube, mix (by inversion or vortex); and allowed to stand for 15 minutes.
7. 350 µl of ceric ammonium sulfate solution were added to each tube and quickly mix by vortex. Using a stopwatch to keep a constant interval between additions to successive tubes, 15 – 30 seconds was convenient.
8. Exactly 20 minutes after addition of ceric ammonium sulfate to first tube, its absorbence was read at 405nm in colorimeter and successive tubes were read at the

same interval as that used for addition of the ceric ammonium sulfate (15 – 30 seconds), so that the time between addition of ceric sulfate and reading is exactly 20 minutes for each.

3.10.7. CALCULATION OF RESULTS

1. A standard curve was constructed by plotting iodine concentration of each standard X-axis(abscissa) against its spectrophotometer reading Y-axis (ordinate).
2. For each sample, its spectrophotometric absorption on the standard curve was traced and extrapolated to locate the corresponding iodine concentration on the abscissa.

This gave the urinary iodine concentration in µg/dl.

Conversion to equivalent SI units:

- µg/dL = 0.07874 µM/L
- 1.0 pM/L = 12.7 pg/dL.

Method A: Manual Acid Digestion Method (as above)

Urine samples and standards (250ul) were treated according to the method of Dunn *et al*., 1993, being digested with 750ul HCL at 110-115 C for 50-60mins.

The iodine content was estimated in the Sandell –Kolthoff reaction, in which iodine as a catalyst for the reduction of cerium IV to cerium III by argenic (III). Unknown urine samples were compared with a set of standards ranging from to 15ugld. The arsenious acid solution contained 20g As_2O_3 and 50g Nacl, dissolved in 400ml $5NH_2SO_4$. The ceric ammonium sulfate solution contained 48g Ce $(NH_4)_4$ $(SO_4)_4$. $2H_2O$ in lL.

The intraassay and interassay CVs were less than 6.7%.

Quantitative assay for Thyroid Peroxidase IgG antibodies by ELISA method (TPO Ab)

Materials /Reagent

Microplate: 96 wells in 12 X 8 break-apart strips, pre-coated with recombinant human TPO, with holder in a foil bag with desiccant

Reagent 1: **Sample Diluent** 10mM Tris-buffered saline, pH 7.2 with antimicrobial agent, 50ml, (blue), ready to use

Reagent 2: Wash Buffer 100mM Tris-buffered saline with detergent, pH 7.2, 100 ml, **concentrate** (x10)

Reagent 3: Conjugate rabbit anti-human IgG conjugated to horseradish peroxidase in protein stabilising solution and antimicrobial agent, 12 ml, (red), ready to use

Reagent 4: TMB Substrate aqueous solution of TMB and hydrogen peroxide, 12 ml, ready

Reagent 5: Stop Solution 0.25M sulphuric acid, 12 ml, ready to use

Standards: 0, 33, 100, 330, 1000, 3000 U/ml, 1ml of 10mM Trisbuffered saline containing human serum antibodies to TPO, ready to use

Positive Control: 1ml of 10mM Tris-buffered saline containing human serum antibodies to TPO, ready to use

Negative Control: 1ml of 10mM Tris-buffered saline containing normal human serum, ready

3.11.4. Equipment

Test tubes for dilution • graduated cylinder for preparing wash buffer • precision pipettes and disposable tips to deliver 10µl, 100µl, 1ml • EIA microplate washer or multi-channel pipette or wash bottle • de-ionised water • absorbent paper • EIA microplate reader with 450nm and optional 620nm reference filter.

3.11.5. Preparation of reagents

1. Wash Buffer (Reagent 2) was diluted 1: 9 in distilled water to make sufficient buffer for the assay run (ie. 50ml wash buffer concentrate was added to 450ml water)

3.11.6. Assay Procedure

Duplicate of all standards and specimens, were run at the same time to keep testing conditions the same.

1. The number of strips required for the assay was assembled
2. Patient samples was diluted 1:100 in Sample diluents (Reagent 1)(10µl serum+1ml diluent)
3. 100 µl of each standard, the diluted patient samples the negative and positive Controls were dispensed into appropriate wells labeled standards, samples, negative and positive controls and incubated for 30 minutes at room temperature.
5. After 30 minutes, the well contents was aspirated and the wells washed 3 times using automated washing wash procedure.
6. 100µl of Conjugate (Reagent 3) was dispensed into each well and the wells was incubated for 15 minutes at room temperature.
7. After 15 minutes, the well contents were carefully discarded and the wells washed 4 times with wash buffer ensuring that the wells were empty but not allowed to dry out.
8. Using a repeating dispenser, I rapidly dispensed 100µl of TMB Substrate (Reagent 4) into each well and incubated the plate for 15 minutes.
9. 100µl of stop solution (Reagent 5) was added to each well. To allow equal reaction times, the stop solution was added to the wells in the same order as the TMB Substrate.

10. The optical density (OD) of each well was read at 450nm in a microplate reader within 10 minutes using 620nm filter as a reference wavelength.

11. Quality control: The QCs were ran alongside with the samples in duplicates

Quality control data supplied on the lot-specific QC certificate included in the kit and were used to monitor for substantial reagent failure.

3.11.7. Interpretation of Results

The OD of each standard was plotted against its concentration and the curve of best fit was drawn through the points. The unknowns were read off this curve.

Values below 75 U/ml are considered normal (values above 75 U/ml are consistent with the presence of autoimmune thyroid disease) while values above 3000 U/ml were repeated at a higher dilution.

3.12. Quantitative assay for Thyroglobulin IgG antibodies by ELISA (TgAb)

3.12.3. Materials /Reagents

Microplate: 96 wells in 12 X 8 break-apart strips, pre-coated with purified human thyroglobulin, with holder in a foil bag with desiccant.

Reagent 1: Sample Diluent 10mM Tris-buffered saline, pH 7.2 with antimicrobial agent, 50ml, (blue)

Reagent 2: Wash Buffer Concentrate (X 10), 100mM Trisbuffered saline with detergent, pH 7.2, 100ml

Reagent 3: Conjugate rabbit anti-human IgG conjugated to horseradish peroxidase in protein stabilising solution and antimicrobial agent, 12 ml, (red) , ready to use

Reagent 4: TMB Substrate aqueous solution of TMB and hydrogen peroxide, 12 ml, ready

Reagent 5: Stop Solution 0.25M sulphuric acid, 12 ml

• Standards: 0, 100, 300, 1000, 3000, 9000 IU/ml, 1ml of 10mM Tris-buffered saline containing human serum IgG antibodies to thyroglobulin.

• Positive Control: 1ml of 10mM Tris-buffered saline containing human serum antibodies to thyroglobulin.

• Negative Control: 1ml of 10mM Tris-buffered saline containing normal human serum,

3.12.4. Equipment

1. Test tubes for dilution • graduated cylinder for preparing wash buffer • precision pipettes and disposable tips to deliver 10µl, 100µl, 1ml • EIA microplate washer or multichannel pipette or wash bottle • distilled or de-ionised water • absorbent paper • EIA microplate

reader with 450nm and optional 620nm reference filter. Alternatively, a suitable automated system may be used.

2. Instrumentation, whether manual or automated, should meet the following criteria: pipettes with better than 3% imprecision with no carry over between pipetting steps; microplate washers should remove 99% of fluid; automated machines should minimise time between washing and adding the next reagent.

3.12.5. Preparation of reagents

1. The Wash Buffer (**Reagent 2) was diluted** 1: 9 in distilled water to make sufficient buffer for the assay run (50ml wash buffer concentrate was added to 450ml water).

3.12.6. Assay Procedure

Duplicate of all standards and specimens, were run at the same time to keep testing conditions the same.

1. The patient samples were diluted 1:100 in sample diluent (10µl serum plus 1ml diluent).

2. 100 µl of each standard, the diluted patient samples, the negative and positive controls were dispensed into the appropriate wells labelled standard, patient sample, negative and positive controls and incubated for **30** minutes at room temperature.

3. After 30 minutes, I aspirated the well contents and washed the wells 3 times using automated washing carefully

4. 100µl of conjugate (**Reagent 3)** was dispensed into each well and I incubated the wells for **15** minutes at room temperature.

5. After 15 minutes, the well contents were discarded and the wells were washed 4 times with wash buffer ensuring that the wells are empty but not allowed to dry out.

6. Using a repeating dispenser, I rapidly dispensed 100µl of TMB substrate (**Reagent 4)** into each well and incubated the plate for **15** minutes.

7. 100µl of stop solution (**Reagent 5) was added** to each well in the same order as the TMB Substrate; this was to allow equal reaction times.

8. The optical density (OD) of each well was read at 450nm in a microplate reader within 10 minutes using a 620nm filter as a reference wavelength.

9. Interpretation of Results

The optical density of each standard was plotted against its concentration and the best-fit curve was drawn through the points. The unknowns were read off this curve. Values above 100 IU/ml were considered positive for autoimmune disease. Values below 100 IU/ml were considered negative.

3.13. Quantitative assay for the detection of human Thyroglobulin Antigen by ELISA method (Tg)

3.13.3. Materials /Reagent

• **Microplate:** 96 wells in 12 X 8 break-apart strips, pre-coated with rabbit anti-thyroglobulin antibodies, with holder in a foil bag with desiccant.

• **Reagent 1**: **Sample diluent** 10mM Tris-buffered saline, pH 7.2 with antimicrobial agent, 10ml, (blue), ready to use

• **Reagent 2: Wash buffer** 100mM Tris-buffered saline with detergent, pH 7.2, 75 ml, **concentrate** (X 10)

• **Reagent 3: Conjugate** rabbit anti-human thyroglobulin IgG conjugated to horseradish peroxidase in protein stabilizing solution and antimicrobial agent, 12 ml, (red) ready to use

• **Reagent 4: TMB Substrate** aqueous solution of TMB and hydrogen peroxide, 12 ml, ready to use

• **Reagent 5: Stop Solution** 0.25M sulphuric acid, 12 ml, ready to use

• **Standards:** 10, 50, 100, 200, 400 ng/ml, 1ml of 10mM Trisbuffered saline containing human thyroglobulin, ready to use

• **Elevated control:** 1ml of 10mM Tris-buffered saline containing thyroglobulin, (green), ready to use

• **Normal control:** 1ml of 10mM Tris-buffered saline containing normal human serum, (turquoise), ready to use

3.13.4. Equipment

1. Test tubes for dilution • graduated cylinder for preparing wash buffer • precision pipettes and disposable tips to deliver 50µl, 100µl, 1ml • EIA microplate washer or multichannel pipette or wash bottle • distilled or de-ionised water • absorbent paper • EIA microplate reader with 450nm and optional 620nm reference filter.

2. Instrumentation, whether manual or automated, should meet the following criteria: pipettes with better than 3% imprecision with no carry over between pipetting steps; microplate washers should remove 99% of fluid; automated machines should minimise time between washing and adding the next reagent.

3.13.5. Preparation of reagents

The Wash Buffer (**Reagent 2) was diluted** 1: 9 in distilled water to make sufficient buffer for the assay run (50ml wash buffer concentrate was added to 450ml water)

3.13.6. Assay Procedure

1. Duplicate of all standards and specimens, were run at the same time to keep testing conditions the same.

2. 100 µl of sample diluent was dispensed as the 0 U/ml standard; 50 µl each standard, control and patient sample was dispensed into the wells, then 50 µl of sample diluent was dispensed into all wells except those containing the 0 U/ml standard.

3. They were incubated for **60** minutes at room temperature.

4. After 60 minutes, the well contents were aspirated and the wells washed 3 times using automated washing.

5. 100µl of Conjugate (**Reagent 3) was dispensed** into each well and incubated the wells for **30** minutes at room temperature.

6. After 30 minutes, the well contents were discarded and carefully washed the wells 4 times with Wash Buffer ensuring that the wells are empty but not allowed to dry out.

7. Using a repeating dispenser, I rapidly dispensed 100µl of TMB Substrate (**Reagent 4)** into each well and incubated the plate for **10** minutes.

8. 100µl of stop Solution (**Reagent 5) was added** to each well in the same order as the TMB Substrate; to allow for equal reaction times.

9. The optical density (OD) of each well was read at 450nm in a microplate reader within 10 minutes, using a 620nm filter as a reference wavelength.

3.13.7. Interpretation of Results

The OD of each standard was read against its concentration and the best-fit curve drawn through the points; the unknowns were read off this curve.

In normal individuals, circulating Tg values range from 2 – 50 ng/ml.

Values above 400 ng/ml were repeated at a dilution of 1:5 in sample diluent.

3.14. Enzyme-Immunoassay (EIA) for the detection of Thyroxine (T_4) in human serum (Pathozyme T_4 assay)

3.14.3. Materials/Reagents

Microtitre Plate 12 x 8 wells x 1 Breakable wells coated with specific antibody contained in a resealable foil bag with a desiccant.

Cal A 0 ng / ml 1 ml Reference Standard: Human serum free of T_4.Ready to use.

Cal B 20 ng / ml 1 ml Reference Standard: T_4 diluted in human serum.Ready to use.

Cal C 50 ng / ml 1 ml Reference Standard: T_4 diluted in human serum.Ready to use

Cal D 100 ng / ml 1 ml Reference Standard: T_4 diluted in human serum. Ready to use

Cal E 150 ng / ml 1 ml Reference Standard: T_4 diluted in human serum. Ready to use

Cal F 250 ng / ml 1 ml Reference Standard: T_4 diluted in human serum. Ready to use

Washbuf 20X 50ml Wash Buffer concentrate: Tris based buffer containing detergents.

Conj 11X 1.3 ml T_4 HRP Conjugate concentrate: T_4 conjugated to Horseradish Peroxidase.

DIL Conj 12 ml Conjugate diluent. Phosphate based buffer containing stabilising proteins. working strength (Green)

Subs TMB 11 ml Substrate Solution: 3,3', 5,5' Tetramethyl Benzidine in a citrate buffer.

Soln Stop HCl 1 M 11 ml Stop Solution: Hydrochloric Acid diluted in purified water. Ready

Micropipettes: 50µl, 100µl, 200µl and 1000µl;

Disposable pipette tips, thoroughly clean laboratory glassware, absorbent paper, Microplate reader fitted with a 450nm filter, Graph paper

3.14.4. ASSAY PROCEDURE

Duplicate of all standards and specimens, were run at the same time to keep testing conditions the same.

1. All the kit components and the test serum were brought to room temperature (25°C) prior to the start of the assay.

2. The desired number of coated wells in the holder was first secured. At each run with each batch of the test serum, one set of the standards was run alongside and the position of the standards and the test serum on the EIA data was recorded on the recording sheet provided.

3. 25µl of standards and test serum was dispensed into the assigned wells.

4. 100µl of working strength conjugate was then dispensed into each well; thoroughly mixed for 30 seconds and incubated for 60 minutes at room temperature (25^0 C).

5. During machine washing; 300µl of wash buffer was dispensed per well and disinfectant was added to the waste collection bottle. The empty wells were washed 5 times and all residual water droplets was removed by striking the wells sharply onto absorbent paper.

6. 100µl substrate solution was dispensed into each well, mixed gently for 5 seconds and incubated in the dark for 20 minutes at room temperature.

7. The reaction was stopped by adding 100µl stop solution to each well, gently mix for 30 seconds to ensure that the blue colour changes completely to a yellow colour and the optical density read immediately (not later than 10 minutes) using a microplate reader with a 450nm filter.

3.14.5. CALCULATION OF RESULTS

The mean absorbance value (A450) for each set of standards and specimens was calculated. A standard curve was constructed by plotting the mean absorbance from each standard against its concentration in ng/ml on the graph paper, with absorbance values on the Y-axis and concentrations on the X-axis. The mean absorbance values for each specimen was used to determine the corresponding concentration of T_4 in ng/ml from the standard curve.

3.15. Enzyme Immunoassay for the quantitative determination of free thyroxine (FT_4) in human serum

3.15.3. Materials / Reagents

Microtitre Plate 12 x 8 wells x 1

Breakable wells coated with specific antibody contained in a resealable foil bag with a desiccant.

Cal A 0 pg / ml 1 ml: Reference Standard: Human serum free of Free T_4.

Cal B Level as stated on vial 1 ml: Reference Standard: Free T_4 diluted in human serum.

Cal C Level as stated on vial 1 ml: Reference Standard: Free T_4 diluted in human serum.

Cal D Level as stated on vial 1 ml: Reference Standard: Free T_4 diluted in human serum.

Cal E Level as stated on vial 1 ml: Reference Standard: Free T_4 diluted in human serum.

Cal F Level as stated on vial 1 ml: Reference Standard: Free T_4 diluted in human serum.

Conj 11 ml:T_4 HRP Conjugate: T_4 conjugated to Horseradish Peroxidase.

Subs TMB 11 ml: Substrate Solution: 3,3', 5,5' Tetramethyl Benzidine in a citrate buffer.

Soln Stop HCl 1M 11 ml: Stop Solution: Hydrochloric Acid diluted in purified water. Micropipettes: 100µl, 200µl and 1000µl

Disposable pipette tips, Absorbent paper, Microplate reader fitted with a 450nm filter

Graph paper, Thoroughly clean laboratory glassware.

3.15.4. REAGENT PREPARATION

All reagents were brought to room temperature (25 ^{o} C) and mixed gently prior to use, avoiding foaming.

3.15.5. ASSAY PROCEDURE

Duplicate of all standards and specimens, were run at the same time to keep testing conditions the same.

1. All the kit components and the test serum were brought to room temperature (25°C) prior to the start of the assay.

2. One set of standards were ran with each batch of test serum. The desired number of coated wells in the holder was secured and the position of the standards and the test serum recorded on the EIA data recording sheet provided.

3. 50µl of the standards, controls and test serum were dispensed into the labelled wells.

4. 100µl of Thyroxine enzyme conjugate were added to all wells; the micro plate was swirled gently for 30 seconds to mix and incubated for 60 minutes at room temperature (25 ^{o} C).

5. At the end of the incubation period, machine washing was carried out by dispensing 300µl of distilled water per well. Excess fluid was removed by by striking the wells sharply onto absorbent paper towel to remove all residual water droplets.

6. 100µl of Substrate was added to all the wells and incubated for 20 minutes at 25 ^{o} C.

7. The reaction was stopped by adding 100µl stop solution to each well; then it was gently mixed for 30 s making sure that the entire blue colour changed completely to a yellow colour.

15. Absorbance was read at 450 nm with a microtitre well reader within 10 minutes.

3.15.6. CALCULATION OF RESULTS

The mean absorbance value (A450) for each set of reference standards and samples was calculated; a standard curve was then constructed by plotting the mean absorbance obtained for each reference standard against its concentration in pg/ml on graph paper, with absorbance values on the Y-axis and concentrations on the X-axis. The mean absorbance values for each specimen was used to determine the corresponding concentration of fT_4 in pg/ml from the standard curve.

3.16. Enzyme Immunoassay for the quantitative determination of L- Triiodothyronine (FT_3) in human serum.

3.16.2. Materials / Reagents

Microtitre Plate 12 x 8 wells x 1

Breakable wells coated with specific antibody contained in a resealable foil bag with a desiccant.

Cal A 0 pg / ml 1 ml: Reference Standard: Human serum free of Free T_3.

Cal B Level as stated on vial 1 ml: Reference Standard: Free T_3 diluted in human serum

Cal C Level as stated on vial 1 ml: Reference Standard: Free T_3 diluted in human serum.

Cal D Level as stated on vial 1 ml: Reference Standard: Free T_3 diluted in human serum.

Cal E Level as stated on vial 1 ml: Reference Standard: Free T_3 diluted in human serum.

Cal F Level as stated on vial 1 ml: Reference Standard: Free T_3 diluted in human serum.

Conj 11 ml: T_3 HRP Conjugate: T_3 conjugated to Horseradish Peroxidase.

Subs TMB 11 ml: Substrate Solution: 3, 3', 5,5' Tetramethyl Benzidine in a citrate buffer.

Soln Stop HCl 1M 11 ml: Stop Solution: Hydrochloric Acid diluted in purified water. Micropipettes: 100µl, 200µl, 1000µl and 5000µl

Disposable pipette tips, Absorbent paper, Microplate reader fitted with a 450nm filter

Graph paper, thoroughly clean laboratory glassware.

PATHOZYME FREE T_3 Reagents do not contain dangerous substances as defined by current UK Chemicals (Hazardous Information and Packaging for Supply) regulations. All reagents should, however, were treated as potential biohazards in use and disposal.

Pathozyme Free T_3 Stop Solution is dilute Hydrochloric Acid

3.16.3. Reagent Preparation: All reagents should be brought to room temperature (20 o C to 25 o C) and mixed gently prior to use. Do not induce foaming.

3.16.4. ASSAY PROCEDURE

Duplicate of all standards and specimens, were run at the same time to keep testing conditions the same.

1. All the kit components and the test serum were brought to room temperature 25°C prior to the start of the assy.

2. One set of standards was run with each batch of test serum. A holder was used to secure the desired number of coated wells in the holder. The position of the standards and the test serum were recorded on the EIA data recording sheet provided.

3. 50µl of standard, control or test serum was dispensed into the assigned well.

4. 100µl of Triiodothyronine conjugate solution was dispensed to all wells.The microplate was gently swirled for 20 to 30 seconds to mix covered, incubated for 60 minutes at room temperature 25^{o}.

5. At the end of the incubation period, 300µl of distilled water was dispensed per well and the wells machine washed 5 times.

6. After washing excess fluid was removed by striking the wells sharply onto absorbent paper or paper towel to remove all residual water droplets.

7.100µl of substrate solution was dispensed to all wells and incubated in the dark for 20 minutes at room temperature (25 ^{o} C).

9. The reactionwas stopped by adding 100µl stop solution to each well and gently mixed for 30 seconds making sure that all the blue colour changed completely to a yellow colour.

11. The absorbance was read at 450 nm with a microtitre well reader within 10 minutes.

3.16.5. CALCULATION OF RESULTS

The mean absorbance value (A450) was calculated for each set of reference standards and specimens and a standard curve was constructed by plotting the mean absorbance obtained from each reference standard against its concentration in pg/ml on a graph paper, with absorbance values on the Y-axis and concentrations on the X-axis.

The mean absorbance values for each specimen was used to determine the corresponding concentration of fT_3 in pg/ml from the standard curve.

3.17. Enzyme-Immunoassay (EIA) for the detection of Total Triiodothyronine (T_3) in human serum

3.17.3. Materials /Reagents:

Micropipettes: 100µl, 200µl and 1000µl, 5000µl

Disposable pipette tips, Absorbent paper, Microplate reader fitted with a 450nm filter

Graph paper, Thoroughly clean laboratory glassware.

Microtitre Plate 12 x 8 wells x 1

Breakable wells coated with specific antibody contained in a resealable foil bag with a desiccant.

Cal A 0 ng / ml 1 ml: Reference Standard: Human serum free of T_3.

Cal B 0.75 ng / ml 1 ml: Reference Standard: T_3 diluted in human serum.

Cal C 1.5 ng / ml 1 ml: Reference Standard: T_3 diluted in human serum.

Cal D 3.0 ng / ml 1 ml: Reference Standard: T_3 diluted in human serum.

Cal E 6.0 ng / ml 1 ml: Reference Standard: T_3 diluted in human serum.

Cal F 10 ng / ml 1 ml: Reference Standard: T_3 diluted in human serum.

Washbuf 20X 50ml: Wash Buffer concentrate: Tris based buffer containing detergents. **Conj 11X 1.3 ml:** T_4 HRP Conjugate concentrate: T_4 conjugated to Horseradish Peroxidase.

DIL Conj 12 ml: Conjugate diluent. Phosphate based buffer containing stabilising proteins. Working strength

Subs TMB 11 ml: Substrate Solution: 3,3', 5,5' Tetramethyl Benzidine in a citrate buffer.

Soln Stop HCl 1 M 11 ml: Stop Solution: Hydrochloric Acid diluted in purified water. Instruction Leaflet and EIA Data Recording Sheet **1 + 1**

3.17.4. ASSAY PROCEDURE

Duplicate of all standards and specimens, were run at the same time to keep testing conditions the same.

1. All the kit components and the test serum were brought to room temperature (25°C) prior to the start of the assy.

2. One set of Standards were run with each batch of test serum. The desired number of coated wells in the holder was secured and the position of the standards and the test serum was recorded on the EIA data recording sheet provided.

3. 25µl of standards and test serum was dispensed into the assigned wells.

5. 100µl of working strength conjugate was dispensed into each well, thoroughly mixed completely for 30 seconds and incubated for 60 minutes at room temperature (25 ^{o} C).

8. 300µl of wash buffer was dispensed per well and that an appropriate disinfectant was added to the waste collection bottle. Wash the empty wells 5 times. After washing remove excess fluid by striking the wells sharply onto absorbent paper or paper towel to remove all residual water droplets.

12. 100µl Substrate Solution was dispensed into each well and mixed gently for 5 seconds and incubated in the dark for 20 minutes at room temperature (25 ^{o} C).

14. The reaction was stopped by adding 100µl Stop Solution to each well and gently mixed for 30 seconds to ensure that the blue colour changes completely to a yellow colour.

16. The optical density was read immediately (no later than 10 minutes) using a microplate reader with a 450nm filter.

3.17.5. CALCULATION OF RESULTS

The mean absorbance value (A450) was calculated for each set of standards and specimens. Construct a standard curve was constructed by plotting the mean absorbance from each standard against its concentration in ng/ml on graph paper, with absorbance values on the Y-axis and concentrations on the X-axis. The mean absorbance values for each specimen was determined and the corresponding concentration of T_3 in ng/ml from the standard curve.

Enzyme Immunoassay for the quantitative determination of Low Levels of TSH in human serum.

Pathozyme Ultrasensitive TSH is an Enzyme Immunoassay (EIA) for the quantitative determination of Thyroid Stimulating Hormone (TSH) in human serum.

3.18.2. Materials/Reagents

Micropipettes: 100µl, 200µl, 1000µl and 5000µl

Disposable pipette tips, Absorbent paper, Orbital Motion microtitre well shaker, capable of shaking at 175 +/- 25 RPM, Microplate reader fitted with a 450nm filter, Graph paper, Thoroughly clean laboratory glassware.

Microtitre Plate 12 x 8 wells x 1

Breakable wells coated with specific antibody contained in a resealable foil bag with a desiccant.

Cal A 0 µIU / ml 1 ml: Reference Standard: Human serum free of TSH. Lyophilised.

Cal B 0.1 µIU / ml 1 ml: Reference Standard: TSH diluted in human serum. Lyophilised.

Cal C 0.5 µIU / ml 1 ml: Reference Standard: TSH diluted in human serum. Lyophilised

Cal D 2 µIU / ml 1 ml: Reference Standard: TSH diluted in human serum. Lyophilised

Cal E 5 µIU / ml 1 ml: Reference Standard: TSH diluted in human serum. Lyophilised

Cal F 10 µIU / ml 1 ml: Reference Standard: TSH diluted in human serum. Lyophilised

Washbuf 20X 50 ml: Wash Buffer concentrate: Tris based buffer containing detergents.

Conj 11 ml: TSH HRP Conjugate: TSH conjugated to Horseradish Peroxidase.

Subs TMB 11 ml: Substrate Solution: 3,3', 5,5' Tetramethyl Benzidine in a citrate buffer.

Soln Stop HCl 1 M 11 ml: Stop Solution: Hydrochloric Acid diluted in purified water.

3.18.3. Specimen Collection and Preparation: A sample of venous blood was obtained from the patient/subject in a heparinized tube and centrifuged, the clear plasma was colledcted; haemolysed, contaminated or lipaemic serum were not used for testing as this will adversly affect the results. Thawed samples were mixed prior to testing. The use of sodium Azide as a preservative inhibits the peroxidase enzyme system.

3.18.4. Reagent Preparation All reagents were brought to room temperature (25 ^{o} C) and mixed gently prior to use avoiding foaming

Standards: To reconstitute the lyophilised standards 1 ml of distilled water was added to each standard vial and allowed to stand for 20 minutes before use.

Wash Buffer: I diluted the concentrated wash buffer using 1 part wash buffer concentrate with 19 parts distilled water. For every 8-well breakable strip, I prepared 25ml of diluted wash buffer by adding 1.25ml of concentrated wash buffer to 23.75ml of distilled water. I prepared fresh diluted wash buffer prior to every assay run.

3.18.5. ASSAY PROCEDURE

Duplicate of all standards and specimens, were run at the same time to keep testing conditions the same.

1. All the kit components and the test serum were brought to room temperature (25°C) prior to the start of the assy.

2. One set of standards were run with each batch of test serum.

The desired number of coated wells in the holder was secured, while the position of the standards and the test serum were recorded on the EIA data recording sheet provided.

3. 100µl of Standards and test serum were dispensed into the appropriate wells.

4. 100µl of Anti-TSH Conjugate was dispensed into each well mixed thoroughly for 30 seconds and the plate was incubated for 120 minutes at room temperature (25 ^{o} C) shaking at 175 +/- 25 RPM.

5. At the end of the incubation period, the well contents were discarded by flicking plate contents into a Biohazard container. Then the wells were striked sharply against absorbent paper ensuring that adequate disinfectant is contained in the Biohazard container.

6. The wells were filled with a minimum of 300µl of wash buffer per well. Flick plate contents into a Biohazard container and machine washed the empty wells 5 times.

7. The wells were striked sharply onto absorbent paper or paper towel to remove all residual water droplets.

8. 100µl substrate solution was dispensed into each well; mixed gently for 5 seconds and incubated in the dark for 20 minutes at room temperature (25 ^{o} C).

9. The reaction was stopped by adding 100µl stop solution to each well and gently mixed to ensure that the blue colour changes completely to a yellow colour.

15. The optical density was read immediately (no later than 10 minutes) using a microplate reader with a 450nm filter.

3.18.6. CALCULATION OF RESULTS

The mean absorbance value (A450) was calculated for each set of standards and specimens. A standard curve was constructed by plotting the mean absorbance obtained from each standard against its concentration in µlU/ml on graph paper, with absorbance values on the Y-axis and concentrations on the X-axis. The mean absorbance values for each specimen were used to determine the corresponding concentration of TSH in µlU/ml from the standard curve.