

RETINOL LEVELS IN HASHIMOTO'S THYROIDITIS

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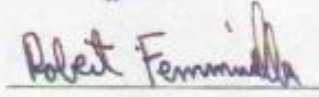

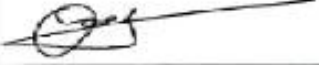
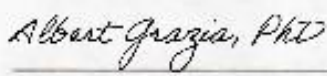
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Approval of the Dissertation

This dissertation by Audrey Folsom has been approved by the committee members below, who recommend it be accepted by the University of Bridgeport, College of Health Sciences, in partial fulfillment of requirements for the degree of Doctor of Health Sciences (D.H.Sc.)

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Abstract

Hashimoto's thyroiditis (HT) is one of the most common autoimmune diseases in the United States. Previous studies have proven that HT patients often have low vitamin D levels and benefit from vitamin D supplementation to help manage their autoimmune disease. Research is currently underway to investigate vitamin A's benefits in the management of autoimmune diseases. Both of these vitamins have immune-modulating properties, and both affect thyroid function.

This dissertation aims to establish whether HT patients had lower retinol (vitamin A) levels than participants that did not have HT. Data regarding retinol levels and thyroid function markers were gathered from a database of results from a small study conducted at Health Matters Clinic, in Northeast Arkansas.

The study participants were sorted into two groups: HT and non-HT, and then 26 participants were randomly selected for each group. The HT group had participants that were positive for either or both thyroperoxidase and thyroglobulin antibodies, and that were not on thyroid medications such as levothyroxine. The non-HT group had participants that did not have thyroid autoantibodies, and did not have any other known autoimmune disease and had normal Thyroid Stimulating Hormone (TSH) levels.

An independent sample t-test for differences in retinol levels was performed, as well as Pearson's correlation for retinol and TSH and retinol and the anti-thyroid antibodies. The results revealed no statistical differences in retinol levels between the groups and no correlation between retinol and the level of thyroid antibodies. Retinol's

tight homeostatic control, which maintains steady serum levels regardless of liver reserve status, can explain the lack of statistical difference in retinol levels between the groups. A positive correlation was found between retinol and TSH levels (high TSH with high retinol), which may indicate some novel mechanism of retinol's effect on the thyroid or retinol's involvement in the etiology of HT, therefore, needs to be validated with more data.

In conclusion, serum retinol levels do not appear to correlate with HT; in particular, serum retinol levels appear not to be decreased in patients with HT. At the same time, our data seem to indicate some involvement of retinol, or its signaling pathway, in thyroid disorders.

Keywords: Hashimoto's thyroiditis, thyroid, autoimmune, retinol, vitamin A

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List of Abbreviations

- ADH1: alcohol dehydrogenase
- AID: autoimmune disease
- AITD: autoimmune thyroid disease
- APC: antigen-presenting cell
- atRA*: *all-trans*-retinoic acid
- CRBP: cellular retinol-binding protein
- CRP: C-reactive protein
- DC: dendritic cell
- DIT: diiodotyrosine
- EAE: experimental autoimmune encephalomyelitis
- Foxp3: forkhead box P3
- fT3: free T3 or free triiodothyronine
- fT4: free T4 or free thyroxine
- GALT: gut-associated lymphoid tissue
- Ig: immunoglobulin
- IL: interleukin
- ILC: innate lymphocytic cell
- INF: interferon
- IRB: institutional review board
- HT: Hashimoto's thyroiditis
- LPL: lipoprotein lipase

LRAT: lecithin-retinol acyltransferase

MHC: major histocompatibility complex

MIT: moniodotyrosine

NIS: sodium iodide transporter

NK: natural killer

PPAR: peroxisome proliferator-activated receptor

RA: retinoic acid

RALDH: retinal dehydrogenase

RAR: retinoic acid receptor

RARE: retinoic acid-responsive elements

RBP: retinol-binding protein

RDH: retinol dehydrogenase

ROR γ t: retinoic acid receptor-related orphan nuclear receptor gamma

rT3: reverse T3

RXR: retinoid receptor X

T3: triiodothyronine

T4: thyroxine

Tbet: transcription factor T-box expressed in T-cells

TG: thyroglobulin

Th: helper T cell

TNF: tumor necrosis factor

TPO: thyroid peroxidase

Tregs: regulatory T-cells or regulatory T-lymphocytes

TRH: thyrotropin-releasing hormone

TSH: thyroid-stimulating hormone

TTR: transthyretin

CHAPTER 1: INTRODUCTION

Background

The incidence of autoimmune diseases has been increasing over the past few decades. Autoimmune disease affects approximately 8% of the population, and 78% of those that are affected are women (Fairweather & Rose, 2004). 23.5 million Americans live with an autoimmune disease, which is now a leading cause of death and disability (Goldmuntz & Penn, 2012). Hashimoto's thyroiditis (HT) is just one of the many autoimmune diseases that exist; it is the most common cause of hypothyroidism in the United States; and it affects about five people out of 100 (National Institute of Diabetes and Digestive and Kidney Diseases, 2017). Treating autoimmune diseases remains a challenge and treatments are aimed at (a) relieving symptoms; (b) replacing substances that are not made anymore, like thyroid hormones in the instance of Hashimoto's thyroiditis; and (c) suppressing the immune system (Goldmuntz & Penn, 2012). Some of these are not without side effects. Lifestyle interventions, such as a healthy diet, can go a long way towards managing an autoimmune disease (Sentenac, 2018). Additionally, restoring micronutrient balance through supplementation can also help the body restore homeostasis.

Fat-soluble vitamins, such as vitamins A and D, have emerged as nutrients of interest due to their ability to regulate the immune system. Retinoic acid (RA), the active form of vitamin A, is essential in the development and maintenance of regulatory T cells (Tregs); and it makes them more effective at regulating the immune system (Lu et al.,

2011). RA allows T cells to suppress inflammation, to maintain mucosal immunity, and to promote antibody responses to T-cell dependent antigens. RA deficiency may lead to impaired suppression of inflammation and its persistence (Ross, 2012). Additionally, RA has a role in the development of adaptive immunity. It can direct B cell immunoglobulin class switching and induce gut homing receptors in B and T cells (Vaishnava & Hooper, 2011). RA regulates innate lymphoid cell-dependent regulation of intestinal homeostasis and protective immunity (Burrows et al., 2018). It is worth noting that RA has a dual role in the regulation of the immune system. Depending on the microenvironment, it can promote either tolerance or inflammation (Hall, Grainger, Spencer, & Belkaid, 2011). RA induces tolerance by its action on peripheral regulatory T cells, and it induces inflammation by its action on the pro-inflammatory T helper cells' response to infection and vaccination (Hall, Cannons, et al., 2011).

Vitamin D also has immune-modulating properties, In the context of autoimmune thyroid disorders (AITD), and specifically HT, patients that are vitamin D deficient and are treated with vitamin D3 see an improvement in their thyroid function and thyroid antibody levels (Acıbuca, Dokmetas, Kılıçlı, Celik, & Aydemir, 2016). Women with anti-thyroid peroxidase (anti-TPO) positive subclinical hypothyroidism with vitamin D deficiency, who received vitamin D3 replacement therapy, saw their thyroid-stimulating hormone (TSH) levels and anti-TPO antibody levels drop significantly (Aminian, Bahrami, Najafipour, & Najafipour, 2019). Interestingly, Chaudhary et al. found that patients with the lowest vitamin D levels also have the highest anti-TPO antibody levels (Chaudhary et al., 2016). Moreover, a cross-sectional study of over 6000 participants

found a significant association between vitamin D deficiency or insufficiency and AITD in pre-menopausal women (Choi et al., 2014). Therefore, there is substantial agreement that normalizing vitamin D levels is beneficial in the treatment of HT. Could it be the same for vitamin A?

Research in the last decade has established Vitamin D as a therapeutic intervention in the context of autoimmune diseases. However, it is now clear that even if a patient has optimal levels of vitamin D, that patient must also have optimal levels of vitamin A to get its full benefits (Riccio & Rossano, 2018). Vitamin A, in its active form as all-trans-retinoic acid, is required for the proper binding of vitamin D to its nuclear receptor so that it can exert its anti-inflammatory effects (Riccio & Rossano, 2018). The work to investigate the potential of vitamin A as a therapeutic agent for autoimmune diseases has started, but there are still many questions that remain unanswered. Much progress has been made in the context of using vitamin A in the treatment of multiple sclerosis mostly due to the availability of the experimental autoimmune encephalomyelitis (EAE) mouse model, although human studies exist as well (Bitarafan et al., 2016; Dorosty-Motlagh, Honarvar, Sedighyan, & Abdolahi, 2016; Riccio & Rossano, 2018; Shiri-Shahsavari et al., 2016). Some work has been done to investigate the effects of retinoic acid on HT, but human studies are lacking.

This study aims to characterize the HT population from a small clinic in terms of vitamin A (retinol) levels as compared to a control group, and a potential correlation between retinol levels and some known markers of thyroid disorders, such as TSH levels and thyroid autoantibody levels. Additionally, the study aimed to assess a few nutritional

or metabolic factors that may influence retinol, such as multivitamins and food sensitivities, which may also correlate with HT. This research could lay the groundwork for interventional studies investigating retinol as another therapeutic agent for HT.

Hashimoto's Thyroiditis

According to the Mayo Clinic (2017), Hashimoto's Thyroiditis (HT) is the most common cause of hypothyroidism in the U.S (Mayo Clinic Staff, 2017). The incidence is estimated to be 1.3% in a series of 5000 children aged 11-18 years; and in adults, it is estimated to be 3.5 per 1000 per year in women and 0.8 per 1000 per year in men (Lee, 2018). It is an autoimmune disease, in which antibodies to the thyroid gland form, which causes a decrease in the gland's function. Stress, infections, certain medications, environmental, and genetic factors are all thought to be triggers of Hashimoto's. Women are more commonly affected than men are, and it often occurs between the ages of 30 to 50 years, with a rise in incidence as one ages. HT is also associated with several other autoimmune diseases such as (a) type 1 diabetes, (b) vitiligo, (c) gluten sensitivity, (d) rheumatoid arthritis, (e) Addison's disease and (f) pernicious anemia (Dorfner, 2017).

Pathophysiology

As lymphocytes invade the thyroid gland, produce autoantibodies, and slowly destroy thyroid tissue, a decrease in thyroid hormone production ensues, which results in chronic inflammation. Signs and symptoms of this process are: (a) fatigue; (b) weight gain; (c) constipation; (d) dry skin; (e) hair loss; (f) cold intolerance; (g) depression; (h) menstrual irregularity; (i) joint pain and muscle cramps. Often, the thyroid is inflamed

to the point of becoming a goiter, which is a painless enlargement of the thyroid gland. It is also possible to have nodules on the goiter. A large goiter could cause problems with swallowing and breathing and can lead to hoarseness. It is also possible to be asymptomatic in the first few years (American Association of Clinical Chemistry Board, 2017; Dorfner, 2017).

Diagnosis

The clinical laboratory assists the physician in diagnosing Hashimoto's Thyroiditis through blood testing. Usually, the findings of decreased thyroid function and increased thyroid antibody levels (thyroid peroxidase and thyroglobulin antibodies) are enough to establish a diagnosis. An elevated serum thyroid-stimulating hormone (TSH) level and a decreased level of free T4 are typically evidence of a decreased thyroid function. The total and free T3 levels could potentially be decreased but may also be normal. Additionally, a thyroid ultrasound may be performed to investigate any thyroid nodules and rule out thyroid lymphoma (American Association of Clinical Chemistry Board, 2017; Dorfner, 2017).

Treatment

There is no cure for HT, but the accepted treatment is thyroid hormone replacement therapy using levothyroxine (a synthetic form of T4) when there is evidence of decreased thyroid hormone levels. The medical staff monitors the patient's thyroid function closely and adjusts treatment as necessary. Levothyroxine has virtually no side effects with the right dosage, but dosages should be increased slowly in heart patients.

However, other substances can affect the absorption of levothyroxine, such as (a) iron supplements including multi-vitamins, (b) the cholesterol-lowering medication cholestyramine, (c) aluminum hydroxide found in some antacids, (d) the ulcer medication sucralfate, and (e) calcium supplements. Therefore, physicians recommend that levothyroxine be taken either four hours before or after any other medications (Mayo Clinic Staff, 2017).

There is some variation in therapeutic effects between brands, and there is some debate over the need to add in small amounts of T3 for patients who do not feel entirely normal on levothyroxine alone. Currently, the majority of studies have determined that the addition of T3 does not offer any advantage over treatment with T4/levothyroxine alone, but there may be some benefit to using T3 for specific subsets of people, such as people who have had a thyroidectomy. T3 can be given alone as liothyronine or in combination with T4 as liotrix, but the combination treatment can cause excessive levels of T3, which can cause symptoms of hyperthyroidism (fast heart rate, anxiety, and trouble sleeping) (Mayo Clinic Staff, 2017).

Another possible treatment option, often classified as an alternative medicine treatment, is the use of extracts that contain thyroid hormones derived from the thyroid glands of pigs. Thyroid extracts, such as Armour Thyroid, contain both levothyroxine (T4) and triiodothyronine (T3). There is concern that the T4-T3 balance in pigs is not the same as in humans, and that the hormone concentrations vary between batches and therefore lack standardization (Mayo Clinic Staff, 2017).

None of these treatments addresses the underlying autoimmune mechanism, but instead relies on replacing what is no longer produced. Therefore, HT patients need additional avenues of therapy that address the restoration of immune homeostasis as an adjunctive treatment. Over the past decade, vitamin D has emerged as such a treatment for thyroid autoimmunity, especially in patients that are either vitamin D-deficient or that have serum vitamin D levels that are at the bottom of the normal reference range (Acıbuca et al., 2016; Chaudhary et al., 2016; Simsek et al., 2016). More recently, evidence for the role of vitamin A in autoimmune disease treatment is also emerging (Erkelens & Mebius, 2017). Therefore, vitamin A might be considered as part of a potential combination therapy with vitamin D, which could restore immune homeostasis (Parastouei et al., 2018; Shiri-Shahsavari et al., 2016). One might suggest that the combined therapy could then allow the thyroid gland to heal itself, potentially allowing patients to discontinue or diminish levothyroxine therapy.

Retinol

The diet is the body's primary source of vitamin A, an essential nutrient, although the liver can store it and build years of reserve. This vitamin is crucial to many functions in the body, such as healthy vision, skin growth and integrity, bone formation, immune function, and embryonic development (American Association of Clinical Chemistry Board, 2019). Thus, its circulating levels are under tight regulation. Once in the cell, retinol converts to its active form, RA, which is used to activate retinoid-responsive genes within that specific cell. Pertinent cells that are affected by RA are hypothalamic

and pituitary cells, thyroid follicular cells, and cells of the innate and adaptive immune systems.

Absorption and Storage

Vitamin A is a fat-soluble essential nutrient that must be taken in through the diet by the consumption of plant foods containing β -carotene (pro-vitamin A) such as carrots or sweet potatoes, or animal foods containing retinol or retinyl esters such as liver, eggs or milk. The pre-formed retinol from animal sources is the most bioavailable dietary source, whereas absorption of the pro-vitamin A carotenoids from plants is influenced by various factors, one of them being thyroid hormone levels, with low levels causing a decreased rate of conversion of carotenoids into retinyl esters (Hess, 2010). Populations that rely on plant foods are at a higher risk for vitamin A deficiency (Hess, 2010). The co-ingestion of dietary fats dramatically enhances the absorption of dietary vitamin A (Harrison, 2012). The enterocytes absorb all forms of vitamin A and esterify β -carotene and retinol into retinyl esters. The enterocytes package the retinyl esters into chylomicrons, which transports them with the other lipids into the lymphatic system and then the blood.

The chylomicrons deliver the retinyl esters to the hepatocytes via lipoprotein lipase (LPL) action. LPL is the enzyme that functions to hydrolyze chylomicrons and very-low-density lipoprotein (VLDL) particles so that they may deliver their payload of monoacylglycerols, free fatty acids and retinyl esters. A complete lack of LPL results in the inability to process triglyceride-rich particles, leading to massive elevations in plasma

triglyceride and retinyl ester levels, but lack of delivery to the cells. The complete deficiency of LPL is rare in the population. However, genetic mutations in the LPL gene occurs in 2 – 4% of the Caucasian population. People with LPL gene mutations can have elevated triglyceride and retinyl ester levels, although some may have normal levels in a fasted state and elevated ones after a fatty meal, exhibiting significant disturbances in postprandial chylomicron metabolism (Pimstone et al., 1996). These mutations result in a lowered ability to process and store retinyl esters, ultimately affecting vitamin A metabolism throughout the body.

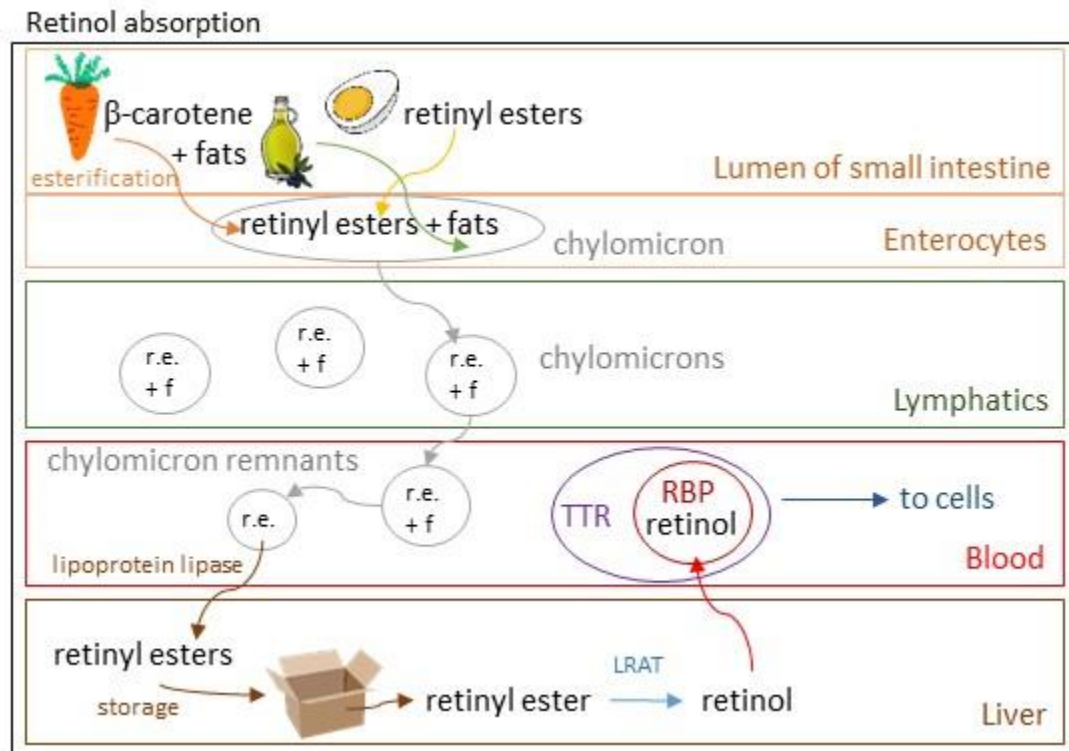


Figure 1. Retinol Absorption

Once inside the hepatocyte, lecithin-retinol acyltransferase (LRAT) hydrolyzes retinyl esters into retinol on an as-needed basis to maintain the blood supply of retinol (Harrison, 2012; Napoli, 2012; Oliveira, Teixeira, & Sato, 2018). (See Figure 1) The liver is the largest storage site for vitamin A, and it stores the retinyl ester form (retinyl palmitate or retinyl stearate) in the hepatic stellate cells (Bono et al., 2016; Hall, Grainger, et al., 2011; Napoli, 2012). Other storage sites are adipose tissue, intestine, and kidneys (Tanumihardjo et al., 2016). Healthy adults may have as much as a year's worth of vitamin A stored in the liver (American Association of Clinical Chemistry Board, 2019). Because of the liver's large storage capacity for vitamin A, dietary intake does not have a significant impact on serum retinol levels but does cause fluctuations in the liver reserves of retinyl esters (Napoli, 2012).

Transport, Activation, and Degradation

When retinol is needed, the hydrophobic retinol leaves the liver bound to retinol-binding protein (RBP) and transthyretin (TTR) so that it can travel through the blood (Brossaud, Pallet, & Corcuff, 2017; Erkelens & Mebius, 2017). The retinol-RBP-TTR complex protects retinol and delivers it to the cells where it interacts with cellular membrane receptor STRA6 (Napoli, 2012). Subsequently, cellular RBP (CRBP) picks up retinol and delivers it for its transformation into retinoic acid (RA), which occurs via two enzymatic reactions that take place in the cytoplasm (Erkelens & Mebius, 2017; Napoli, 2012). Alternatively, α -retinol, which comes from α -carotene and does not bind to RBP, can be directly delivered to the peripheral cells via chylomicrons and other lipoproteins (Tanumihardjo et al., 2016).

Once it has entered the cell, the first reaction involves the oxidation of retinol to retinal by retinol dehydrogenase (RDH) or alcohol dehydrogenase (ADH1). In the second reaction, retinal dehydrogenase (RALDH) rapidly and irreversibly oxidizes retinal to RA (See Figure 2) (Bono et al., 2016; Napoli, 2012; Oliveira et al., 2018). There are three isoforms of RALDH, and their production is tightly regulated. This mechanism allows for the right amount of RA to be available to the cell at the right time. The presence of RALDH in a cell identifies that cell as a producer of RA (Hall, Grainger, et al., 2011; Oliveira et al., 2018). CYP26, a cytochrome P450 family 26 enzyme, also mediates intracellular RA levels by degrading RA bound to CRBP1 to prevent its accumulation (Erkelens & Mebius, 2017; Oliveira et al., 2018).

Cellular Actions

Retinoic acid can be made into several isoforms, which dictate its activity, with *all-trans* retinoic acid (atRA) being the most abundant (Oliveira et al., 2018). Once made CRBP2 ferries RA to the nucleus where it interacts with its nuclear receptors to regulate the transcription of several genes by binding to retinoic acid-responsive elements (RAREs) on DNA (Erkelens & Mebius, 2017; Taibi, Gueli, Nicotra, Cocciadiferro, & Carruba, 2014). (See Figure 2) RA-responsive nuclear receptors include the retinoic acid receptor (RAR) and the retinoid receptor X (RXR). Each of these nuclear receptors has three isoforms, which are designated α , β , and γ , and they exist as either homodimers or heterodimers. The RXR nuclear receptors mainly interact with the *9-cis* RA isoform, whereas the RAR receptors interact with all forms of RA (Oliveira et al., 2018). When RA is bound to its heterodimer nuclear receptor (usually RAR/RXR), it results in gene

transcription, whereas the heterodimer missing its RA ligand suppresses transcription. Most RA immune-related functions operate in this manner (Bono et al., 2016; Brossaud et al., 2017). Interestingly, RAR and RXR can also form heterodimers with numerous other nuclear receptors such as the vitamin D receptor, the thyroid hormone receptor, and the peroxisome proliferator-activated receptor (PPAR) (Graeppi-Dulac, Vlaeminck, Perier-Muzet, Dalle, & Orgiazzi, 2014).

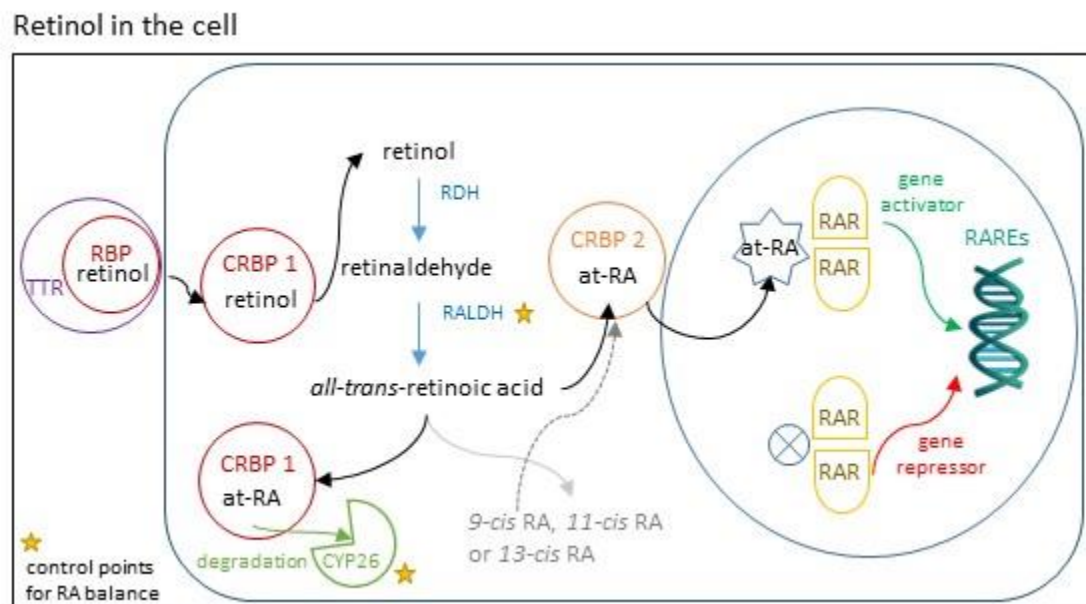


Figure 2. Retinol in the Cell

Although there are more than 500 RA responsive genes (Higdon, 2000; Tanumihardjo et al., 2016), some of the relevant roles of RA are: (a) involvement in thyroid hormone production and action, (b) coordination of the mucosal immune response, (c) the homing of innate and adaptive cells to the gut, (d) the induction of pro-inflammatory cytokines during infection, (e) immune cell differentiation, (f) directing

immunoglobulin class switching in B cells, and most importantly, (g) equilibrating immunity and tolerance (Oliveira et al., 2018; Vaishnava & Hooper, 2011). Because atRA can have such a widespread impact, there is tight control of its biosynthesis by complex interactions between retinoid-binding proteins and retinoid recognizing enzymes, which maximize its uptake and use. atRA regulates its concentrations by lowering substrate availability via conversion of retinol into retinyl esters and by enhancing its catabolism (Napoli, 2012).

Retinol Status and Testing in the U.S. Population

Deficiencies of Vitamin A are rare in the United States predominantly due to adequate nutrition at the population level, regular multivitamin intake, and the consumption of fortified foods such as ready-to-eat cereals, snack foods, beverages, margarine, and processed dairy products. Therefore, regular supplementation is not recommended at the population level in the U.S (Tanumihardjo et al., 2016).

Recommended dietary allowances are 900 micrograms (3,000 IU) for males and 700 micrograms (2,310 IU) for females 14 years or older (American Association of Clinical Chemistry Board, 2019). However, deficiencies are seen in specific populations such as those with malnutrition (food insecurity), malabsorption disorders (for example celiac disease, cystic fibrosis, gastric-bypass surgery, chronic small intestine disease or chronic pancreatitis), in the elderly, and in those with alcoholism and liver disease (American Association of Clinical Chemistry Board, 2019; Russell, 2000). The first and second National Health and Nutrition Examination Survey found that the prevalence of serum vitamin A levels below 20 µg/dL in the U.S. was low. The prevalence of levels below 30

µg/dl was higher in children. A higher prevalence of serum vitamin A levels in the low ranges was observed in blacks rather than whites and persons below poverty status than in those above the poverty level (Pilch, 1985). The first clinical sign of a vitamin A deficiency is night blindness (American Association of Clinical Chemistry Board, 2019). Additionally, other factors influence vitamin A deficiency, such as the presence of fat in the meal, which favors absorption, enough protein to manufacture RBP and to convert β-carotene to retinol, and poor zinc status, which may all negatively affect vitamin A status biomarkers (Tanumihardjo et al., 2016). Conversely, vitamin A toxicity can occur due to overuse of vitamin supplements, especially in the elderly, or in patients with type 1 hyperlipidemia (American Association of Clinical Chemistry Board, 2019; Russell, 2000).

Because vitamin A deficiency is rare in the U.S. population, there is no recommendation for widespread population screening for deficiency. A physician may order Vitamin A testing when a patient has signs and symptoms that suggest deficiency or toxicity, or when they have general malnutrition or malabsorption due to underlying conditions, especially those affecting the gastrointestinal system. Signs and symptoms of deficiency are night blindness, dry eyes, skin and hair, ulcers and damage to the cornea, skin thickening and lesions, grayish spots on the conjunctiva (Bitot spots), repeated infections, and anemia. The signs and symptoms of toxicity are headache, nausea and vomiting, double or blurred vision, fatigue, weakness, dizziness, seizures, irritability, muscle pain, bone and joint pain, weight loss, hair loss, mucous membrane dryness,

itching, liver dysfunction, cracks at the corners of the mouth and glossitis(American Association of Clinical Chemistry Board, 2019).

Retinol and the Thyroid

Retinoic acid, the active form of retinol, is involved at all levels of thyroid hormone production and action. Low levels of retinol, and therefore RA, can trigger central hyperthyroidism, whereas medically induced high levels can trigger central hypothyroidism. Epidemiologically, the main concern is the combined iodine and retinol deficiencies, which can lead to a high prevalence of goiter in endemic areas, mostly found in the developing world. However, even in the developed world, subtle deficiencies in retinol can cause health effects, such as suboptimal thyroid function, because retinol deficiency in the thyroid gland causes hypertrophy with a reduction in iodine uptake, in thyroglobulin synthesis, and in thyroid hormones synthesis (Brossaud et al., 2017).

Hypothalamus and Pituitary Actions

The control of thyroid hormone ultimately starts in the hypothalamus with the production of thyrotropin-releasing hormone (TRH), which acts on the anterior pituitary to cause the release of thyroid-stimulating hormone (TSH). Both locations contain RAR receptors, RARE-bearing genes, and retinoid-metabolizing enzymes. Studies show that retinol deprivation and retinol treatment both modulate the hypothalamic-pituitary-thyroid axis (Brossaud et al., 2017). At the pituitary level, both T4 and RA can suppress TSH synthesis because both the thyroid hormone-activated thyroid receptor and the 9-

*cis*RA-activated RXR suppress the transcription of the pituitary TSH β gene. Therefore, retinol status modulates TSH production (See Figure 3).

RXR agonists (rexinoids) can suppress the pituitary TSH β gene and the hypothalamic thyrotropin-releasing hormone gene, decreasing TSH levels independently of T3 signaling. An excess of rexinoids can lead to central hypothyroidism (low TSH, low T3, and T4) (El-Eshmawy et al., 2016; Zimmermann, 2007). However, euthyroid (normal TSH) or hypothyroid (high TSH) rats treated with RA show a spontaneous basal TSH decrease and a decreased TSH response to TRH (Graeppe-Dulac et al., 2014). Vitamin A deficiency increases pituitary TSH β mRNA levels two-fold and increases serum T4, indicating central hyperthyroidism due to pituitary resistance to thyroid hormone feedback (Zimmermann, 2007).

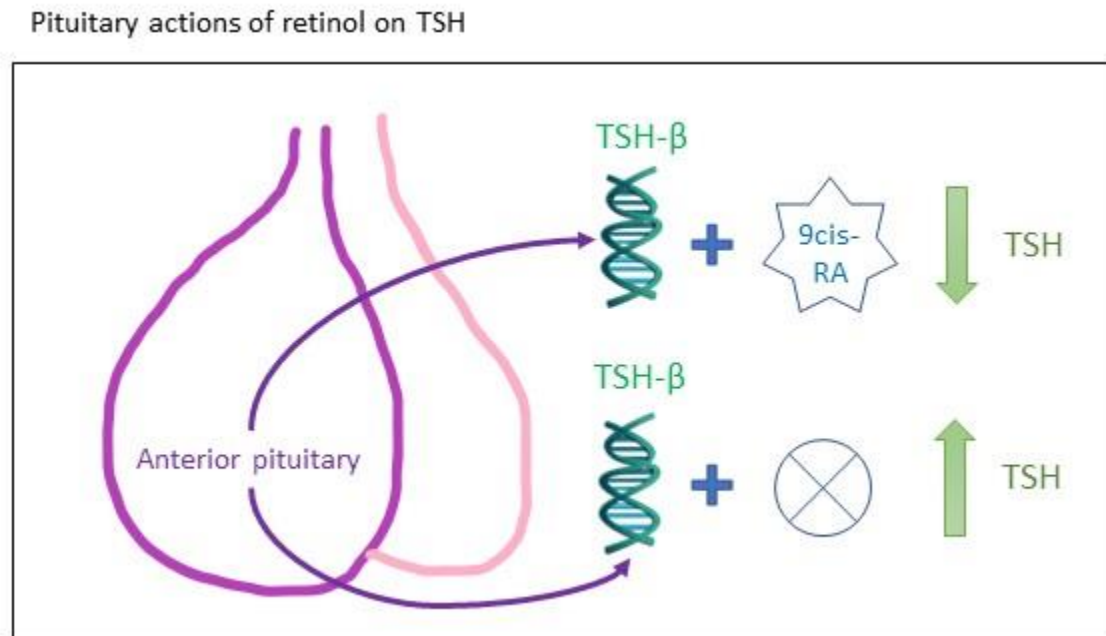


Figure 3. Pituitary Actions of Retinol on TSH

Thyroid Gland Actions

The thyroid gland produces thyroid hormone in its follicular cells by the iodination of tyrosine residues in the glycoprotein thyroglobulin. The TSH that is secreted by the pituitary in response to circulating thyroid hormone levels binds to the TSH-receptor on the thyroid follicular cells and stimulates hormone production. TSH regulates iodine uptake into the follicular cells, and retinoids decrease TSH-stimulated iodine metabolism (Brent, 2012; Frohlich & Wahl, 1999). Subsequently, thyroid peroxidase catalyzes iodide oxidation and organification, and retinoic acid is involved in many steps of this process. RA also reduces TSH receptor mRNA levels, suppresses the accumulation of TPO and thyroglobulin (TG) mRNA, and inhibits TPO and TG gene expression, which would, therefore, affect both iodine intake and thyroid hormone production (Brossaud et al., 2017; Frohlich & Wahl, 1999).

An endogenous mechanism related to the availability of thyroglobulin (TG) in the colloid of each follicle regulates thyroid hormone production. The thyroid is composed of follicles, which are an arrangement of thyroid follicular cells that face colloid on their apical surface and face a basket-like network of capillaries on their basal surface. Thyroglobulin, the precursor to thyroid hormones, is the most abundant protein stored in the follicle. Its synthesis starts when iodide from the blood is concentrated into the thyrocyte by the sodium iodide transporter (NIS) and sent to the colloid via the pendrin transporter. The TG polypeptide chain is synthesized in the rough endoplasmic reticulum of the thyrocyte and then modified via the addition of a carbohydrate chain. This dimer then is further modified by the Golgi apparatus and then transferred to the colloid where

it is iodinated by thyroperoxidase (TPO) and hydrogen peroxide. Iodine is covalently bound to the tyrosine molecules of TG to form monoiodotyrosine (MIT) and diiodotyrosine (DIT). TPO also couples these iodotyrosine residues to form thyroxine. Iodinated TG re-enters the thyrocyte, where it is degraded to release T3 and T4 from their peptide linkages. T3 and T4 enter the capillary basket and enter the circulation (See Figure 4) (Luo, Ishido, Hiroi, Ishii, & Suzuki, 2014; Sellitti & Suzuki, 2014).

Although TSH is a positive regulator of thyroid function at the basal thyrocyte surface, TG has been demonstrated to be a potent negative regulator that acts from the apical surface by suppressing the expression of thyroid-related genes in a time- and dose-dependent manner. At physiological concentrations, TG suppresses TG mRNA and protein levels, thus acting as its negative feedback loop. Additionally, TG can suppress promoter activity, mRNA transcription, and protein levels of the NIS transporter of the TPO enzyme and the dual oxidase-2 enzyme and its maturation factor (involved in hydrogen peroxide production). These actions suppress hydrogen peroxide production and thus the iodination of TG. The expression of the gene for the pendrin transporter, which transports iodine on the apical surface of the thyrocyte to promote TG iodination in the colloid, is induced by low concentrations of TG and suppressed by high TG concentrations (Luo et al., 2014; Sellitti & Suzuki, 2014).

The conflicting signals of TSH from the basal surface and TG from the apical surface affect each thyroid follicle differently, depending on which phase of the “follicular” cycle is currently active. The size, volume, and function of the follicle depends on the balance of TG and TSH action and leads to follicle heterogeneity in the

thyroid gland. TSH has a stimulatory effect on both TG synthesis and reabsorption, but the TSH-induced TG synthesis is slower than the TSH-induced TG reabsorption. TG has a strong negative feedback effect on its synthesis, which can be overcome by the stimulatory effects of TSH. Therefore, if the colloid is full of TG, the negative TG feedback effect is stronger than the TSH stimulatory effect, and it suppresses TG synthesis, while TSH only stimulates TG reabsorption from the colloid, TG degradation and thyroid hormone release in the blood. With the TG reabsorption rate higher than the synthesis rate, TG reserves slowly deplete from the colloid. This gradual drop in colloidal TG levels stops the negative feedback loop of TG and allows TSH-stimulated TG synthesis to take place anew. The low TG levels in the colloid also increase the expression of the pendrin transporter gene to facilitate the iodination of TG, whose levels will build back up in the colloid. Once colloid TG levels reach critical mass again, the negative feedback effect will take place, and the whole cycle will repeat itself (Luo et al., 2014; Sellitti & Suzuki, 2014).

Retinoic acid affects this thyroid follicular cycle by suppressing the accumulation of TPO and TG mRNA stimulated by TSH in a time- and dose-dependent manner. Additionally, it inhibits TPO and TG gene expression, interfering with the production and iodination of TG and therefore lowers thyroid hormone production (See Figure 4) (Namba et al.). In a different retinol-induced alteration of thyroid function, a deficiency of retinol can lead to an altered TG structure by reducing its mannosylation (the addition of its carbohydrate molecule) and producing monomeric TG instead of the regular

dimeric TG. This abnormal process leads to reduced MIT and DIT coupling on TG and depresses physiologically active thyroid hormone production (Ingenbleek, 2013).

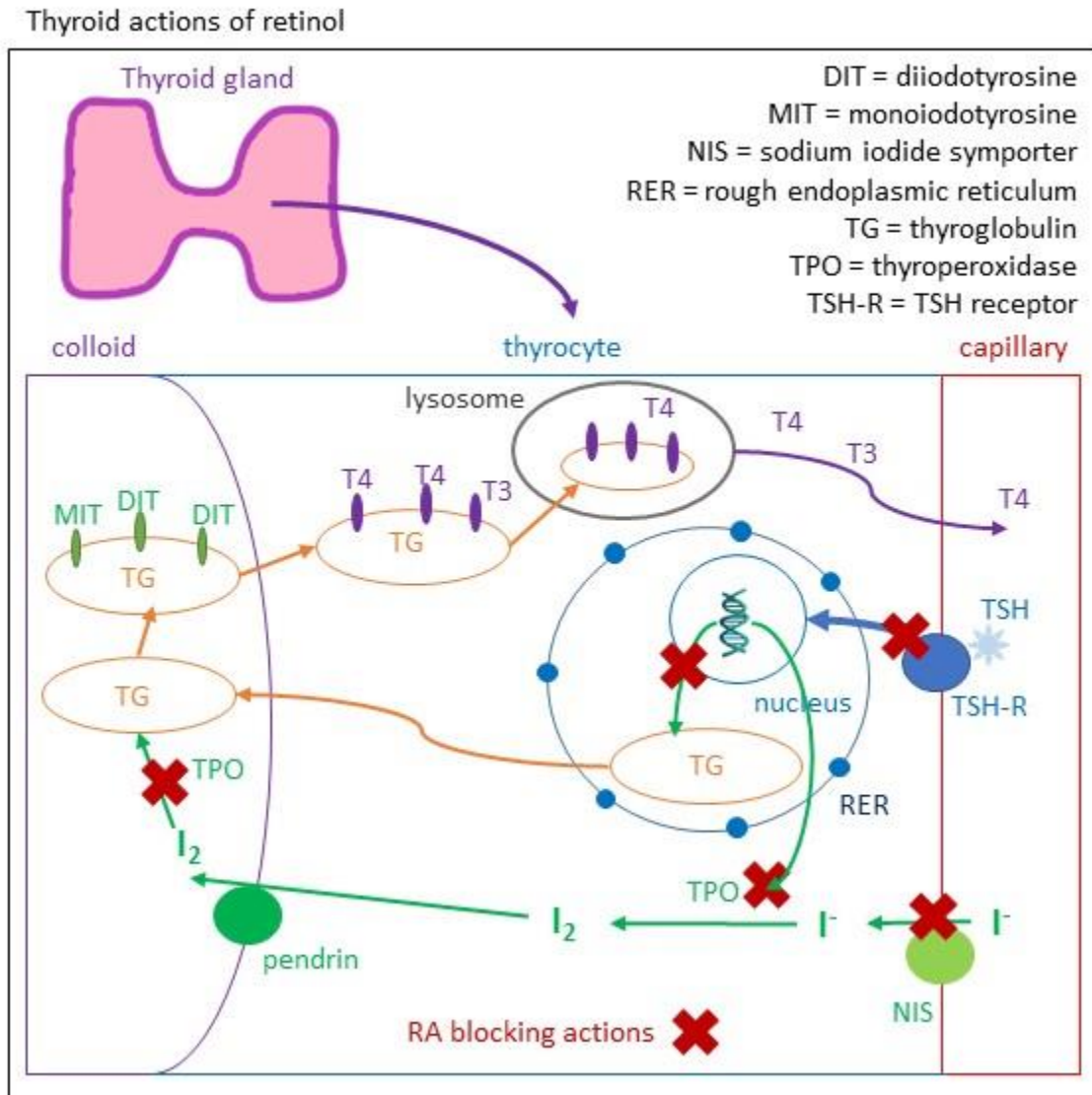


Figure 4. Thyroid Actions of Retinol

Retinol deficiency influences the thyroid gland in several ways: it causes thyroid hypertrophy (goiter), and it reduces iodine uptake, thyroglobulin, and thyroid hormone

synthesis. When retinol deficiency combines with iodine deficiency, the impact on thyroid metabolism is even greater. In children that exhibit this combined deficiency, treatment with vitamin A improves iodide efficiency and reduces thyroid gland hyperstimulation and size (Biebinger, Arnold, Langhans, Hurrell, & Zimmermann, 2007; Brossaud et al., 2017; Zimmermann, 2007; Zimmermann et al., 2007). Treatment with retinyl palmitate (vitamin A) decreases the thyroid gland size, decreases serum thyroid hormones, increases iodine uptake in the thyroid, and increases the hepatic conversion of T4 to T3. However, in the administration of retinoic acid as a treatment, the isoform given is essential. Treatment with a low dose of *atRA* decreases iodine uptake into the thyroid gland, whereas the same dose of *13-cisRA* increases iodine uptake (Brossaud et al., 2017; Muhlbauer et al., 2010).

Peripheral Actions

Thyroid hormones are hydrophobic and, therefore, are transported on proteins such as thyroid-binding globulin and transthyretin, which also ferries retinol in the blood. Transthyretin (TTR) carries retinol bound to retinol-binding protein (RBP) as one complex. The levels of both of these transport proteins correlate with serum T3 levels, indicating a relationship. If T3 levels are low, RBP and TTR levels will decrease as well (Morley, Russell, Reed, Carney, & Hershman, 1981). However, a German study demonstrated that hyperthyroid patients have lower RBP and TTR levels than euthyroid and hypothyroid patients, although retinol levels were the same in all three groups (Aktuna et al., 1993).

The production of thyroid hormones starts with the assembly of the pro-hormone thyroxine on thyroglobulin. Thyroxine, which is also known as T4, must be converted into the active hormone triiodothyronine (T3) to exert its effects. This conversion often happens at the target tissue site via deiodination. The local activation allows for the tight regulation of its tissue-specific metabolic effects. Additionally, as a secondary regulatory mechanism, T4 can be converted to the inactive reverse T3 (rT3) (Brent, 2012). Peripheral clearance of thyroid hormones by the liver also contributes to their metabolic effects. Retinoids (synthetic retinoid ligands) can induce the liver to increase the clearance of circulating thyroid hormones (Graeppi-Dulac et al., 2014).

Thyroid hormone can regulate a wide variety of cellular genes that have thyroid response elements, but it uses complex signaling pathways that interact with tissue-specific thyroid hormone transporters, uses many isoforms of the thyroid hormone receptor, which have distinctive roles, and interacts with several corepressors or coactivators. This hormone can also be involved in crosstalk with other signaling pathways, such as that of retinol (Brent, 2012). RA can modulate the effects of thyroid hormones on specific tissues by inducing the expression of the monocarboxylate thyroid hormone transporter, which is responsible for the crosstalk between RA and thyroid hormone signaling (Brossaud et al., 2017).

Vitamin A deficiency results in a decreased expression of both the thyroid and the retinoid nuclear receptor, a deficit that can be corrected by either the administration of thyroid hormone or RA (Brossaud et al., 2017). Because vitamin A deficiency increases plasma levels of TSH, T3, and T4, it also increases metabolic rate and leads to central

hyperthyroidism. Conversely, vitamin A excess decreases T3 and T4 serum levels, decreases metabolic rate and leads to central hypothyroidism (El-Eshmawy et al., 2016; Morley et al., 1981).

Retinol and the Immune System

Besides having effects on the thyroid, retinol influences many aspects of the immune response, from the innate to the adaptive response. In the most general terms, retinoic acid levels can be modulated locally to induce specific responses from the immune system. It acts as a double-edged sword with the ability to promote inflammation in the context of infection but also has anti-inflammatory actions in the context of turning down the immune response to maintain tolerance. It is also critical to maintaining the integrity of the mucosal barrier function, especially in the gut (Erkelens & Mebius, 2017). In Hashimoto's thyroiditis, as in all autoimmune diseases, there are multiple imbalances in the function of the immune system. Research has documented the following imbalances: (a) an increase in $\gamma\delta$ T-cells which enhances B-cells for antibody production (Liu et al., 2016), (b) a role for Foxp3 Tregs in the prevention of autoimmune thyroid diseases and the presence of non-functional Tregs in HT (González-Amaro & Marazuela, 2016), (c) an increase in the pathogenic Th17 cells in autoimmune thyroid diseases, especially HT (González-Amaro & Marazuela, 2016; Zaķe, Skuja, Lejnieks, Groma, & Konrāde, 2019), and (d) the involvement of Th1 cells in the pathogenesis of HT (Nielsen et al., 2007; Zaķe et al., 2019).

Innate Immune System

Retinoic acid is critical to maintaining an essential part of the innate immune response: the mucosal barrier. The loss of gut mucosal barrier integrity is associated with and thought to cause various autoimmune diseases through intestinal microbial dysbiosis. There is a documented inverse relationship between serum retinol levels and gut permeability, indicating a direct role of retinol and its metabolites in maintaining barrier functionality. RA promotes gut dendritic cells to express CD103 and to produce RA, which is a signal to promote Foxp3 regulatory T-cell differentiation and immunoglobulin A (IgA) production. Additionally, it induces the homing of innate lymphocytic cells (ILCs), regulatory and effector B, and T-cells to the gut. RA is essential for maintaining the balance between immune response and tolerance, especially at the gut mucosal barrier (Abdelhamid & Luo, 2018; Oliveira et al., 2018).

Dendritic cells and macrophages.

During an infection, RA can promote the production of pro-inflammatory cytokines by the dendritic cells (DC) and then induce the differentiation of effector T and B-cells to fight the infection at the mucosa (Oliveira et al., 2018). The antigen-presenting cells, such as DCs, maintain the balance between effector and tolerogenic responses, which is needed to prevent autoimmunity. In HT, antigen-presenting cells, especially DCs, colonize the thyroid gland and present specific thyroid antigens to T lymphocytes via the MHC complex in the lymph nodes (Zaķe et al., 2019).

Tolerogenic DCs express the CD103 marker and locate to the lamina propria and GALT of the small intestine. These tolerogenic cells can induce the differentiation of Foxp3 regulatory T-cells to maintain mucosal barrier homeostasis by secreting RA. However, not all DCs can produce RA. For example, the inflammatory CD103- DCs do not possess the enzymes necessary for RA production, and therefore, this DC population induces the differentiation of interferon-gamma (INF- γ)-producing effector T-cells and the production of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF- α) and interleukin 6 (IL-6). Inflammation can also suppress the expression of the RALDH enzyme necessary for DC to produce RA. The effects of RA are similar in macrophages, where RA inhibits the production of pro-inflammatory cytokines and favors tolerance (Oliveira et al., 2018). The DCs found in the thyroid can be similarly subdivided into two groups: immunogenic or tolerogenic depending on their activation of either autoreactive T-cells (immunogenic leading to HT) or Tregs (tolerogenic). Studies have revealed increased levels of DCs in the inflammatory infiltrate of the thyroid in HT patients (Zaęe et al., 2019).

The RA produced by the DCs is also essential to induce the expression of the gut-homing molecules $\alpha 4\beta 7$ and CCR9 on T and B-cells and innate lymphocytic cells 1 (ILC1s) and ILC3s. The expression of $\alpha 4\beta 7$ and CCR9 causes all of these immune cells to migrate to the gut, where they exert their protective effects (Bono et al., 2016). Retinoid levels in the gut directly correlate with the degree of gut-homing molecule production. If the DC-produced RA synergizes with IL-6 or IL-5, it can induce IgA class-switching in B-cells. Pathogen-induced inflammation can decrease the production

of RA by DCs and create a pro-inflammatory environment, which then facilitates the expulsion of the pathogen (Erkelens & Mebius, 2017).

Innate lymphocytic cells.

Innate lymphocytic cells (ILCs) come from the lymphoid progenitor and migrate into infected and injured tissue. They do not express acquired antigen receptors or undergo clonal selection and expansion when stimulated. They respond to the signals from infected and injured tissue and secrete cytokines that can direct the developing immune response into an adaptive one. Stress signals, microbial compounds, and the cytokine microenvironment activate ILCs. Hours after an epithelial injury, such as in the thyroid epithelial cells of HT, ILCs act in a similar manner as memory T-cells, produce effector cytokines such as $\text{INF-}\gamma$, and do so in conjunction with subsets of $\gamma\delta$ T-cells from the epithelial and mucosal compartments (Eberl, Colonna, Di Santo, & McKenzie, 2015).

Innate lymphocytic cells mirror the phenotypes and function of T cells. There are three main groups of ILCs. The natural killer (NK) cells are the counterpart to the cytotoxic CD8^+ T-cells, and the ILC1, 2, and 3 have a counterpart in the Th1, Th2, and Th17 CD4^+ T-helper cells, respectively (See Figure 5) (Eberl et al., 2015). Transcription factor T-box expressed in T-cells (Tbet) induces ILC1 cells in response to interleukin (IL)-12, IL-15, and IL-18. ILC1s, in turn, can induce a Th1 response, such as seen in the pathogenesis of HT. This response can be initiated either directly through the expression of MHC II molecules in ILC1 cells (making them act as antigen-presenting cells), or indirectly through the regulation of DCs. The main role of ILC1s is to respond to

intracellular pathogens and produce $\text{INF-}\gamma$. They accumulate during chronic inflammation in the gut and lungs. The ILC2 group activates the GATA binding protein 3 (GATA3) transcription factor in response to IL-25 and IL-33 and produces IL-5 and IL-13. They direct the response to parasite infections, allergens, and epithelial injury. The primary locations of ILC2s are the lungs, skin, and gut. They are integral to large parasite defense, such as helminths, and function in tissue repair, allergy, and asthma. They direct the Th2 response of the adaptive immune system. The ILC3 group depends on the transcription factor retinoic acid receptor-related orphan nuclear receptor gamma ($\text{ROR}\gamma\text{t}$), which is activated by IL-1 β and IL-23. ILC3s produce IL-17, IL-22, lymphotoxins, and granulocyte-macrophage colony-stimulating factors in response to bacterial and fungal infections. IL-22 is elevated in the early phases of HT, most likely in an attempt to protect the thyroid tissue through the activation of anti-apoptotic pathways. IL-23 promotes the activity of ILC3s and the differentiation of Th17 cells, which are heavily involved in the pathogenesis of HT, as well as the nitric oxide that activates B-cells, which are also involved in HT. ILC3 cells are involved in gut barrier defense and skin inflammation and can be associated with inflammatory pathology if they produce $\text{INF-}\gamma$ and IL-17, which are both also involved in the pathogenesis of HT (Eberl et al., 2015; Oliveira et al., 2018; Ruggeri et al., 2014).

Retinoic acid promotes the generation of intestinal ILC3 cells, and it is crucial for the ILC1 and ILC3's antibacterial and antiviral response in the intestines. In NK cells (ILC1), RA acts to promote tolerance and suppress NK cytotoxicity. RA participates in the intestinal immune system homeostasis by regulating ILC responses in the intestine.

(Oliveira et al., 2018) RA is necessary for the full maturation of ILC3s at the expense of ILC2s (Eberl et al., 2015). Recent evidence shows that intestinal ILCs (ILC3) express the transcription factor HIC1 in a retinol-dependent manner. In the absence of the HIC1 transcription factor, ILC3s that produce IL-22, used for tissue repair, are lost. The loss leads to an increased susceptibility to infections. *atRA* modulates HIC1 expression and thus favors the maintenance of the ILC3 population in the intestines (Burrows et al., 2018).

Adaptive Immune System

T-lymphocytes.

Once activated by antigen-presenting cells, CD4⁺ T-cells (T-lymphocytes) can differentiate into different T-helper cell subsets (See Figure 5). Classification of these T-helper subsets is according to function, transcription factor expression, and excreted cytokines; and some of these subsets include Th1, Th2, Th17 and Tregs (Bono et al., 2016). Signaling from IL-12 and INF- γ activate Tbet transcription in Th1 cells, which then produce INF- γ . The production of INF- γ allows Th1 cells to activate cellular immunity to defend against infections, such as viral infections; these cells are involved in the pathogenesis of HT. Signaling from IL-4 drives GATA3 transcription in Th2 cells, which produce IL-4 in response to parasitic infection. Th2 cells also activate the humoral response by promoting IgG antibody production in B-cells; these cells are predominant in the pathology of Graves' disease but show some involvement in HT. Th17 cells promote the control of bacterial and fungal infections at the mucosal barrier. Signaling from TGF-

β with IL-6 and IL-21 induces ROR γ t Th17 cells that produce IL-17. Th17 cells contribute greatly to the pathogenesis of HT. Signaling from TGF- β , IL-2, and RA induces Foxp3 expression and leads to the production of Tregs that secrete IL-10 and TGF- β . Tregs are tolerogenic and protect against autoimmune diseases, including HT, whereas Th1 and Th17 are often involved in the development of autoimmune diseases such as HT (Hall, Cannons, et al., 2011; Hall, Grainger, et al., 2011; Ross, 2012; Zaęe et al., 2019).

The dose of RA in the microenvironment determines its local effects. At physiological doses, RA favors a Th1/Th17 type of response, whereas, at higher doses, RA promotes a Th2/Treg response (Bono et al., 2016). High levels of retinoic acid (especially *9-cis*RA) can promote the differentiation of naïve CD4⁺ T-cells into Th2 cells by inducing the expression of GATA3 while inhibiting Th1 cell differentiation. However, RA is also essential for the stability and maintenance of the Th1 cells. Additionally, higher levels of RA can block IL-23 and IL-6 to repress ROR γ t transcription, which usually induces Th17 cell differentiation when activated. Lower levels of RA induce the generation of Th17 cells by activating ROR γ t to promote a protective response in the mucosa. Higher atRA levels enhance the differentiation of Foxp3⁺ inducible regulatory T-cells (Tregs) and induce gut-homing specificity in T-cells (Lu et al., 2011). The presence of higher amounts of Tregs suppresses the Th1/Th17 response in the steady-state, but RA is required to induce Th1 and Th17 mediated immunity during inflammatory states (Bono et al., 2016). RA can also affect INF- γ production, which is involved in the induction of the autoreactive Th1 cells in HT. If RA

and INF- γ , IL-12, or IL-4 are present, then RA increases INF- γ production from Th1 cells. If only RA is present, then INF- γ production decreases from both Th1 cells and CD8⁺ cytotoxic T-cells. Thus, the cellular microenvironment determines RA signaling and function (Erkelens & Mebius, 2017; Hall, Grainger, et al., 2011; Oliveira et al., 2018; Zaķe et al., 2019).

Gamma-delta T-cells.

Gamma-delta ($\gamma\delta$) T-cells are a subset of T-cells that express a $\gamma\delta$ T-cell receptor on their surface. The majority of T-cells express the $\alpha\beta$ T-cell receptor. The $\gamma\delta$ T-cells are unique because they function as a bridge between the innate and adaptive immune systems and, depending on the context, share attributes of the adaptive or innate system or both. They may be considered a third branch of the immune system. $\gamma\delta$ T-cells express the CD3 marker, constitute less than 5% of circulating T-cells, function mostly as Th1 cells, and develop outside of the thymus. They are thought to be a prompt first line of defense against pathogens and can increase to up to 50% of circulating lymphocytes during the first few days of a bacterial infection. Each subset of the traditional T-cells has a $\gamma\delta$ T-cell equivalent, including the Tregs (See Figure 5). They possess the ability to recognize microbial-only phosphoantigens and to react to unprocessed proteins without the need to associate with APCs. Indeed, human $\gamma\delta$ T-cells can also become phagocytes and act as professional APCs to $\alpha\beta$ T-cells (Ferreira, 2013).

Once activated, $\gamma\delta$ T-cells can proliferate, induce inflammatory cytokines, and alter cell surface phenotypes. They participate in the immune response via direct

cytolysis, antigen-presentation, and immune regulation. They are involved in autoimmune disorders, immune deficiencies, infections, and tumor diseases. Activated $\gamma\delta$ T-cells can also induce B-cell antibody production in HT. However, treatment with *atRA* can induce the apoptosis of $\gamma\delta$ T-cells in the context of thyroid autoimmunity, reducing autoantibody production (Liu et al., 2016).

Retinoic acid can enhance the production of IL-22 in $\gamma\delta$ T-cells and ILCs. IL-22 is crucial to tissue repair and the maintenance of the mucosal barrier. Excess IL-22 can lead to autoimmune issues such as psoriasis; therefore, tight control is imperative. Higher levels of serum IL-22 have been documented in the early stages of HT. RA can also induce the production of IL-22 binding protein by DCs to capture and inactivate this cytokine. Thus, RA modulates the production and the clearance of IL-22 and directs the tissue repair process (Erkelens & Mebius, 2017; Ruggeri et al., 2014).

B-lymphocytes.

Retinoic acid is essential for the humoral response, especially IgA production, which is integral to mucosal immunity. RA is an essential co-factor for the stimulation and proliferation of B-lymphocytes (B-cells). It can accelerate the maturation of human B-cells and their differentiation into plasma cells. RA can also increase IgM and IgG synthesis in human B cells during infection. In the gut, RA favors the migration and survival of B-cells and stimulates the generation of IgA in the intestine, which has an innate protective effect (Oliveira et al., 2018). B-cells produce autoantibodies, namely

anti-thyroglobulin and anti-thyroid peroxidase, during the development of HT (Liu et al., 2016; Zaķe et al., 2019).

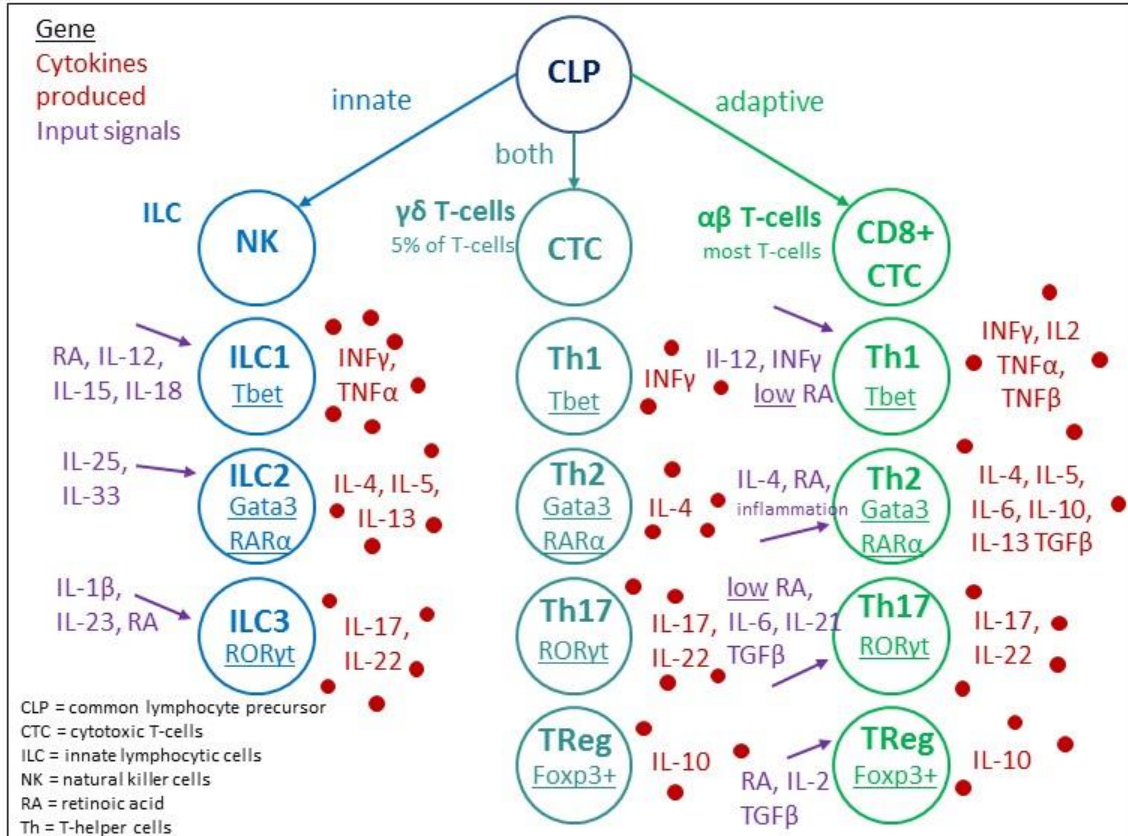


Figure 5. Lymphocyte Differentiation

Retinol and Hashimoto's Thyroiditis

In Hashimoto's thyroiditis, an autoimmune disease that affects the thyroid, there is a loss of tolerance towards self-antigens such as thyroperoxidase (TPO) and thyroglobulin (TG). A cellular autoimmune response mediates this disease process, with resulting inflammation, thyrocyte destruction, and thyroid gland failure. However, there

is also evidence of a humoral immune response due to the presence of anti-TPO and anti-TG antibodies, which are present in 90-95% and 70-80% of autoimmune thyroid disease patients, respectively (González-Amaro & Marazuela, 2016). Hashimoto's thyroiditis is the most common cause of primary hypothyroidism in iodine-sufficient areas, with a strong female bias in prevalence. The prevalence of thyroid autoimmunity also increases with age (Zaķe et al., 2019).

The immune cells involved in Hashimoto's thyroiditis include the pro-inflammatory Th1 cells, with a more substantial involvement of the pathogenic Th17 and Th22 cells, and a lack of the anti-inflammatory Tregs. The absence of the Tregs, which maintain self-tolerance, is associated with thyroid autoimmunity, especially Hashimoto's thyroiditis. If the Tregs are present in the inflammatory infiltrate of autoimmune thyroid patients, then they are usually dysfunctional and unable to downregulate the ongoing autoimmune process. The sustained exposure of Th17 cells to IL-6 and IL-23 results in their conversion to pathogenic cells, which synthesize IL-17 and INF- γ and drive inflammation. Newly diagnosed HT patients demonstrate higher levels of Th1 cells, Th17 cells (IL17+ T cells), and Th22 cells (IL-22+ T cells), with a diminished Tregs/Th17 ratio, and higher levels of INF- γ , IL-17, IL-21, IL-22 and IL-23. Additionally, IL-6 positively regulates Th22 cells and Th17 cells, which contribute to the pathogenesis of HT. Higher percentages of these cells were also negatively correlated with serum TSH levels in HT patients (Esfahanian, Ghelich, Rashidian, & Jadali, 2017; González-Amaro & Marazuela, 2016; Guo et al., 2014; Ruggeri et al., 2014; Vitales-Noyola et al., 2017).

The sequence of damage to the thyrocytes in HT starts with the infiltration of the thyroid gland by autoreactive T and B-lymphocytes, followed by the production of anti-TPO and anti-TG antibodies, and then by the induction of thyrocyte apoptosis which causes thyroid tissue destruction. Thyrocytes are mainly destroyed via a caspase-mediated apoptotic death by way of expression of the CD95/Fas death receptor. Therefore, the inflicted damage to the thyroid is in the form of cytotoxic destruction of thyroid tissue. The autoantibodies have their destructive effect, albeit minor, via complement activation, antibody-dependent cytotoxicity, and induction of oxidative stress (Stassi et al., 2000; Zaķe et al., 2019).

The $\gamma\delta$ T-cell subset is also actively involved in HT. The $\delta 1$ portion of these cells (dominant in HT patients) homes to the epithelial and mucosal surfaces as a first line of defense. The $\delta 2$ portion of these cells exists in the peripheral and lymphatic systems. Activated $\gamma\delta$ T-cells play a role in antibody production by B-cells. In HT, $\gamma\delta$ T-cells are present in increased numbers in the thyroid and contribute to anti-TPO and anti-TG antibody production. Retinol, in its active *atRA* form, inhibits autoantibody production by inducing the apoptosis of $\gamma\delta$ T-cell in the thyroid tissue of HT patients (Liu et al., 2016).

Considering all the effects of retinol and *atRA* on the cells of the immune system, the hypothalamus-pituitary-thyroid axis, other autoimmune diseases, and the consideration that *atRA* can induce $\gamma\delta$ T-cell apoptosis and a reduction in autoantibodies, how does retinol affect the development or progression of Hashimoto's thyroiditis? A recent review of the literature reveals no studies that characterize retinol levels in HT

patients. Additionally, the review found no studies that addressed whether treatments with vitamin A would be successful in decreasing auto-reactivity in autoimmune thyroid diseases. Therefore, this dissertation aims to elucidate whether there are statistical differences in retinol levels between HT patients and healthy patients. The hypothesis is that there will be a difference in retinol levels between the Hashimoto's Thyroiditis patients and the non-HT patients, with the expectation of lower retinol levels in HT patients. An additional hypothesis is that, in HT, cellular sensitivity to retinol is reduced (e.g. due to the disruption of RA signaling pathways at the receptor or secondary messenger levels) while serum retinol levels remain unchanged. However, studies that address this additional hypothesis were not found, and testing this hypothesis experimentally was beyond the scope of this study. Therefore, the focus of the study was on the serum retinol levels. In addition to correlating serum retinol levels with HT, the present study sought to find a connection between retinol levels and some known markers of thyroid disorders, such as TSH and thyroid autoantibody levels, and assessed whether additional nutritional or metabolic factors that may influence retinol, such as multivitamins and food sensitivities, may also correlate with HT.

Research Question

Is there a significant difference in retinol levels in individuals with Hashimoto's Thyroiditis, as compared to individuals without Hashimoto's Thyroiditis? In addition, is there a correlation between retinol and TSH level, or between retinol and thyroid autoantibody levels?

CHAPTER 2: REVIEW OF THE LITERATURE

Literature Review Sources

The theoretical framework for this dissertation examined the following: (a) the relationship between retinol and the thyroid considering various vitamin A statuses (deficiency, supplementation, and excess); (b) the relationship between retinol and the immune system at the innate and adaptive levels with additional consideration of its role in immune homeostasis; and (c) the involvement of retinol in Hashimoto's Thyroiditis.

Method

The OneSearch library database at Arkansas State University was searched using the Boolean AND with a combination of several keywords. Articles had to be peer-reviewed and have an accessible PDF or a complete online article to be included in this review. The search used no defined period to capture as many results as possible. Additionally, a search was conducted in Pub Med using the same terms, and an interlibrary loan request was submitted for any unavailable articles. Any duplicate results were eliminated.

The search terms used were "vitamin A and thyroid," "vitamin A assessment," "vitamin A and immune system," "vitamin A and autoimmune disease," "vitamin A and autoimmune thyroid," "vitamin A and Hashimoto's thyroiditis" and "Hashimoto's thyroiditis." This search revealed that alternative terms should be used for vitamin A. Therefore, the same searches were executed again substituting "retinol" and then

“retinoic acid” for “vitamin A” in the search parameters. Original research was preferentially selected, but meta-analysis reviews from the last decade were also included. The search included all types of research, due to the low availability of results. The search strategy yielded a mix of animal studies, human studies, histological, and cytological studies. The total amount of articles found in all categories was 101 articles. Only one was relevant to retinoic acid and Hashimoto’s thyroiditis. However, 22 articles were found that related to HT or autoimmune thyroid disease, although those emphasized the involvement of the immune system. The retinol and the immune system search resulted in 23 articles, retinol, and autoimmune disease yielded 17 articles, retinol, and the thyroid yielded another 32, and assessment of vitamin A resulted in 7 articles.

Retinol and the Thyroid

Vitamin A Deficiency

In their recent review of the effects of vitamin A on endocrine tissue, Brossaud, Pallet and Corcuff (2017) note that vitamin A deficiency in animals causes thyroid hypertrophy with a reduction in iodine uptake, of thyroglobulin and thyroid hormone synthesis (Zimmermann, Wegmüller, Zeder, Chaouki, & Torresani, 2004). Vitamin A deficiency in rats reduces the expression of retinoid and thyroid nuclear receptors in peripheral cells, thus affecting their function (Féart et al., 2005). Combined iodine and vitamin A deficiencies produce a more significant impact on thyroid metabolism than either single nutrient deficiency (Zimmermann et al., 2007). This type of deficiency is prevalent in low-income countries. This review combines animal and human evidence,

but the human evidence is exclusively from low-income country studies, which is not the population that is at risk for Hashimoto's thyroiditis.

Zimmerman (2007) reviews the combined effects of vitamin A and iodine deficiencies on the pituitary-thyroid axis. His findings reveal that this type of deficiency affects more than a third of the global population, with women of reproductive age and young children being the most vulnerable. At the level of the thyroid, vitamin A deficiency causes thyroid hypertrophy, reduces thyroidal iodine uptake, and impairs synthesis of thyroglobulin and coupling of iodotyrosine residues to form thyroid hormone (Ingenbleek, 2013; Oba & Kimura, 1980). It also decreases intrathyroidal T3 and T4. At the periphery, vitamin A deficiency increases total and free T4 and T3 and reduces the hepatic conversion of T4 to T3 (Brown et al., 2000; Ingenbleek, 2013). It also decreases cellular T3 uptake and binding (Higueret, Paillet, & Garcin, 1989). He suggests that this effect could be mediated by the shared transport protein transthyretin, which carries retinol bound to retinol-binding protein and 10 to 15% of T4 and T3. Thyroid-binding globulin transports the rest. At the pituitary level, he found that vitamin A deficiency increases pituitary TSH β mRNA levels and increases serum total T4 levels (Wolf, 2002). It also renders the pituitary thyrotropes relatively insensitive to feedback control by thyroid hormones. Vitamin A deficiency also exhibits an increased in hypothalamic thyrotropin-releasing hormone (TRH) and pituitary TSH despite the high levels of T4 and T3 (Breen, Matsuura, Ross, & Gurr, 1995; Brown et al., 2000; Haugen, Brown, Wood, Gordon, & Ridgway, 1997). Recent rat studies included in this review demonstrated that vitamin A deficiency alone was not sufficient to cause a significant change in TSH,

TSH β mRNA, and thyroid weight. The combined vitamin A and iodine deficiency is the most significant modifier of thyroid function (Biebinger et al., 2006).

Zimmerman's (2007) review of human studies included cross-sectional studies done in low-income countries such as Senegal and Ethiopia. In Senegalese adults, there is a strong negative correlation between the increasing severity of goiter and serum retinol (Ingenbleek, 1992). In Ethiopian children, those with visible goiters had significantly lower serum retinol (Wolde-Gebriel, Gebru, Fisseha, & West, 1993). The limitation of these studies is the inability to distinguish between the effects of vitamin A deficiency and protein malnutrition. However, in their cross-sectional study of 2331 Iranian children, Hashemipour et al. (2009) found no children with vitamin A deficiency in either the goitrous or the non-goitrous group. About a third of the children in each group had a low vitamin A status (meaning less than 30 $\mu\text{g/dL}$), indicating that in this iodine replete area, vitamin A status is not correlated to residual goiter in children. The weakness of this study was that they used palpation to assess goiter, whereas examination via ultrasound would have been more accurate. The goiters could be due to protein-energy malnutrition or other micronutrient deficiency, neither of which are related to the development of HT (Hashemipour et al., 2009).

Using the evidence from 76 human studies, research nutritionist Sonja Hess (2010) examined the impact of common micronutrient deficiencies (iodine, iron, zinc, and vitamin A) on thyroid metabolism. These deficiencies affect low-income countries due to a lack of access to adequate nutrition and are a significant public health concern. She concurs with Zimmerman et al. that vitamin A deficiency affects the thyroid at all

levels: pituitary, thyroid, and periphery. Vitamin A deficiency reduces iodine uptake by the thyroid, impairs thyroid hormone synthesis, which decreases intrathyroidal T4 and T3, and leads to thyroid hypertrophy (goiter). At the periphery, it increases T4 and T3 levels but decreases T4 (inactive) to T3 (active) conversion and decreases T3 uptake and binding (Hess, 2010). The weakness of this review is that this deficiency phenomenon occurs in low-income countries due to lack of nutrients, and Hashimoto's thyroiditis is more common in high-income countries that are iodine replete and well-fed.

El-Eshmawy et al. (2016) examined the vitamin A status in relationship to the thyroid axis in 112 patients with clinically stable hepatitis C-related cirrhosis, matched to 56 healthy controls, in their case-controlled study. They measured vitamin A levels via High-Performance Liquid Chromatography, used standard lab testing for liver function (albumin, total bilirubin, aspartate transferase, alanine transferase, and INR) with anti-hepatitis C testing, and thyroid function was assessed by measuring levels of free T3, free T4, TSH, rT3, TPO antibodies, and thyroid ultrasonography. Altered vitamin A and thyroid status is a common finding in patients with liver cirrhosis. They found a high prevalence of vitamin A deficiency in this population, which makes sense because the liver is the primary storage site for vitamin A. These patients also had higher levels of free T4, free T3, TSH, and thyroid volume than the healthy controls. Vitamin A deficiency was associated with Child-Pugh scores (cirrhosis severity) and TSH. They concluded that vitamin A deficiency might be linked to central hypothyroidism in this patient population. This well-done research validates the observation that vitamin A deficiency and central hyperthyroidism are common in liver cirrhosis patients. The

weakness of this study is that two-thirds of the liver cirrhosis patients were undernourished and, therefore, had inadequate intake of vitamin A as compared to the healthy controls. Interestingly, they found that thyroid autoimmunity prevalence was higher in the liver cirrhosis population (El-Eshmawy et al., 2016).

In their 1981 study, Morley et al. examined the relationship of thyroid hormones with vitamin A and zinc nutritional status in 62 clinically stable patients with chronic hepatic and gastrointestinal disorders. They treated the ten patients that had vitamin A deficiency evidenced by retinol levels below 40 $\mu\text{g/dL}$ and reassessed them afterward. Treatment consisted of retinyl palmitate at 10,000 to 30,000 IU per day. They measured total T3, T3 uptake, total T4, TSH, and calculated the free thyroxine index for thyroid function assessment and measured vitamin A, retinol-binding protein, and pre-albumin (transthyretin) in all patients. The patients with chronic hepatic and gastrointestinal disorders exhibited lower T3 levels with lower RBP and pre-albumin levels. The vitamin A therapy in the deficient patients increased their T3, T4, free T3, and RBP levels, and did not change the pre-albumin concentrations. Vitamin A therapy in healthy patients initially decreased the T4 and T3 levels and then returned them to normal a few weeks later. They hypothesized that the low T3 syndrome present in their chronically ill patients was due to a deranged retinol-RBP metabolism, which may interfere with T4 entry into the liver or with the enzymatic conversion of T4 to T3 in the liver. The weakness of this study is that it used a heterogeneous patient population affected by many different gastrointestinal and hepatic disorders, although they were all clinically stable, free of alcohol, and consumed balanced diets (Morley et al., 1981).

Interestingly, their findings are in opposition to the liver cirrhosis study done by El-Eshmawy many decades later. In this study, vitamin A deficiency (less than 40 $\mu\text{g/dL}$) was seen in patients with a hypothyroid profile, whereas in the liver cirrhosis study, vitamin A deficiency (less than 20 $\mu\text{g/dL}$ or 0.70 $\mu\text{mol/L}$) correlated with a hyperthyroid state (Morley et al., 1981). Evidently, what constitutes vitamin A deficiency has also changed over the years.

Supplementing with Vitamin A

In their thorough review of the effects of vitamin A on endocrine tissue, Brossaud, Pallet and Corcuff (2017) found that, in rats, supplementation with retinyl palmitate decreases thyroid gland size and serum thyroid hormones and increases thyroidal iodine uptake and hepatic conversion of T4 (inactive) to T3 (active). Another interesting fact they reported on is that a low-dose *atRA* treatment decreases iodine uptake, whereas the same dose of *13-cisRA* increases iodine uptake. Therefore, the RA isoform produced is essential to the cellular effect. In their review, they also note that RA can reduce TSH receptor mRNA, and suppress the accumulation of TPO and TG mRNA. RA also modulates the effects of thyroid hormones on target tissues by inducing the expression of the thyroid hormone transporter (Brossaud et al., 2017).

In his review of the interactions of vitamin A and iodine deficiencies on the pituitary-thyroid axis, Zimmermann (2007) reports that in a double-blind randomized control 10-month trial of children with iodine and vitamin A deficiency, concurrent supplementation of iodized salt and vitamin A decreased the thyroglobulin, median TSH

and goiter rate in the vitamin A-treated group as compared to the placebo group. (Zimmermann, 2007) Biebinger, Arnold, Langhans, Hurrell, and Zimmermann's case-controlled rat study showed that high dose vitamin A supplementation alone, without iodine repletion, in iodine and vitamin A deficient rats is sufficient to reduce thyroid hyperstimulation and reduce the risk for goiter. Their rat study also demonstrated that the iodine and vitamin A deficient diet lead to moderate vitamin A deficiency and primary hypothyroidism with increases in serum TSH, pituitary TSH β mRNA, and thyroid size, and decreases in thyroid hormone concentrations. A single high dose of vitamin A was enough to return the serum retinol levels to normal in both the iodine-sufficient and iodine-deficient rats. Additionally, despite continuing vitamin A deficiency, providing an iodine-sufficient diet was enough to restore the normal function of the pituitary-thyroid axis. The high dose vitamin A treatment of the iodine-sufficient rats did not have any effects on the pituitary-thyroid axis, but the same treatment in iodine-deficient rats slightly reduced the pituitary production of TSH, reduced stimulation of the thyroid and had no effects on the circulating thyroid hormones. One of the study's weaknesses is that it assumed vitamin A repletion based on serum retinol levels without assessing liver stores. They suggest that vitamin A treatment could be beneficial to humans that live in areas of endemic goiter. Their finding that the provision of an iodine-sufficient diet restores the function of the pituitary-thyroid axis despite vitamin A status contradicts earlier findings that showed that severe vitamin A deficiency affects the pituitary-thyroid axis regardless of iodine status. The differences in the severity of vitamin A deficiency between the studies explain this discrepancy (Biebinger et al., 2007).

In another study by Zimmermann et al. (2007), the team conducted a 6-month randomized, double-blind, 2 x 2 interventional trial in South African children with mild to moderate vitamin A and iodine deficiency. Serum retinol levels of less than 0.70 $\mu\text{mol/L}$ (20 $\mu\text{g/dL}$) defined vitamin A deficiency, and serum retinol levels less than 1.05 $\mu\text{mol/L}$ (30 $\mu\text{g/dL}$) indicated low vitamin A status. They supplemented children with either (a) iodine as 191 mg iodine as oral iodized oil and placebo, (b) vitamin A as 200,000 IU of retinyl palmitate and placebo, (c) both iodine and vitamin A at the doses already described, (d) placebo. They measured baseline, 3-month and 6-month urinary iodine levels, thyroid volume by portable echocamera, TSH, total T4, thyroglobulin, serum retinol, and serum retinol-binding protein. They found an apparent effect of vitamin A supplementation alone on TSH, thyroglobulin, and thyroid volume. However, they also found that in the group receiving vitamin A and iodine supplementation, the effects on the thyroid axis were minimal compared to the effects of iodine supplementation alone. They concluded that iodine supplementation was sufficient to restore iodine status in vitamin A deficient areas, vitamin A supplementation was enough to treat vitamin A deficiency in areas of iodine deficiency, and that it also suppresses the pituitary TSH β gene. These findings suggest that vitamin A supplementation can reduce the risk of goiter and its sequelae, but it is not likely to have any effects on thyroid function in iodine-sufficient areas, and it even may depend on the severity of iodine deficiency. This study has a robust design, and it partially explains the inverse correlation between vitamin A status and goiter that is seen in the cross-sectional studies of developing countries (Zimmermann et al., 2007).

In their 4-month, double-blind, randomized placebo-controlled trial of the effect of vitamin A supplementation on thyroid function in obese premenopausal women, Farhangi et al. (2012) found that treatment with vitamin A significantly lowered TSH levels in obese and non-obese women. They measured baseline and 4-month concentrations of TSH, total T4, total T3, retinol-binding protein, and transthyretin. In addition to the finding of a lowered TSH, they noted that serum T3 levels also increased in both obese and non-obese vitamin A treated groups, serum T4 decreased in all treated groups, and serum RBP decreased in the obese group. They concluded that vitamin A supplementation might reduce the risk of subclinical hypothyroidism (defined as mild increases in TSH with T4 levels in the normal range) in premenopausal women. Their study included 84 women, 58 of whom were obese. They needed a total sample size of 75, which they met considering nine dropped out of the study. The supplement used was 25,000 IU of retinyl palmitate, which shows good bioavailability. Some of the women in each group had hypothyroidism, and the incidence of hypothyroidism decreased after treatment, but only the non-obese group showed significance. The authors postulate that this could be attributed to the differences in serum leptin levels between obese and non-obese women because leptin might decrease the pituitary-thyroid axis' responsiveness to therapeutic agents such as levothyroxine or vitamin A in obesity (Farhangi, Keshavarz, Eshraghian, Ostadrahimi, & Saboor-Yaraghi, 2012).

Additionally, they found that serum RBP levels decreased in vitamin A-treated obese women. They explain that RBP is overexpressed in white adipose tissue and may have a link with insulin resistance. *atRA* treatment can reduce RBP levels and suppress

insulin resistance in people with diabetes. They also postulated that because RBP is a negative acute-phase reactant, and obesity is an inflammatory condition, obesity lowers RBP levels. One of the limitations of this study was that the authors used the RBP toTTR ratio to estimate vitamin A status, and because they had some obese participants, this possibly was not an accurate assessment tool. It would have been better to use serum retinol levels, but it was too costly. They also used only women participants; therefore, findings are not generalizable to men; and neglected to assess iodine status (Farhangi et al., 2012).

Muhlbauer et al. (2010) used normal rats and treated them with different isomers of RA to evaluate thyroid iodine organification. They used the *atRA* and *13 cis-RA* isomers at 100 or 1500 µg/100 g body weight. The correct RA treatments can help increase the uptake of radioactive iodine into thyroid carcinoma cells, making the treatment more effective. The treatment effect was evaluated on healthy thyroid cells to attempt to elucidate the role of RA. The organification of iodine is catalyzed by thyroid peroxidase in the presence of hydrogen peroxide. To assess the treatment effect, they measured TPO oxidase activity, dual oxidase activity, and thyroid radioiodide content from the rat's thyroid glands, and the rats' serum total T4. The *atRA* treatment decreased thyroid iodine uptake, but the sodium-iodine transporter (NIS) function and TPO activity were unchanged, while the treatment significantly decreased the dual oxidase activity. However, the *13 cis-RA* treatment increased iodine uptake, TPO activity was unchanged, hydrogen peroxide levels increased, and serum thyroxine levels were normal. Therefore, the authors conclude that the isomer of RA used in treatment is essential and that RA

exerts its effect by regulating the activity of dual oxidase. The availability of hydrogen peroxide is the rate-limiting step in iodine organification, and the dual oxidase enzyme regulates the thyrocyte's hydrogen peroxide content. The exact mechanism of how RA regulates the dual oxidase enzyme is still unknown, and this is one of the weaknesses of this study, along with a low number of rats used (at least 6 in each group). The authors also noted that because thyroxine levels remained unchanged, there are unknown compensatory mechanisms in place to keep thyroxine levels steady (Muhlbauer et al., 2010).

Vitamin A Excess

In their cytology study, Frohlich and Wahl (1999) evaluated the effects of retinol on follicular porcine thyrocytes in culture using light- and electron microscopy. They used 0-2 μmol of *all-trans*-retinol per well of cultured thyroid cells, added on day 3, after follicle formation. Then, they treated the wells with viable thyrocyte follicles with 0, 7, 14, 27, 40, 53, 80, and 106 μM of retinol. Electron microscopy was used to assess the effects of the various retinol treatments because it allows the distinction between the membrane-toxic effects, where the membranes disappear, and the apoptotic effects, which alter the nucleus and the endoplasmic reticulum (Frohlich & Wahl, 1999).

Additionally, the TUNEL test identified apoptotic cells. This test recognizes nicked DNA. Thyrocytes cultured in anything less than 27 μM of retinol were normal, and the higher retinol concentrations affected the morphology in a dose-dependent manner. Usually, retinol modulates iodine metabolism. However, at high concentrations,

retinoids are cytotoxic to healthy thyroid cells, although the mechanism was unknown. The authors found that thyrocyte cytotoxicity was due to apoptosis. Electron microscopy was the right testing choice as it can distinguish between membrane-toxic and apoptotic effects. Evaluated effects were also dose-dependent, which validates the techniques used (Frohlich & Wahl, 1999).

Using a case report, Graeppi-Dulac et al. (2014) review the evidence for the effects of retinoids on the thyroid axis. Bexarotene (Targretin) is a retinoid (vitamin A derivative) used to treat late-stage cutaneous T-cell lymphomas, and it is known to induce significant hypothyroidism through TSH suppression, independently of T3 levels. Just as vitamin A deficiency induces central hyperthyroidism by upregulating TSH β in the pituitary via RXR γ inactivation, vitamin A excess induces central hypothyroidism by suppressing pituitary TSH β via RXR γ nuclear receptor activation. Other studies evaluated by the author provide evidence that bexarotene also increases thyroid hormone metabolism by the liver, increasing peripheral clearance, and lowering circulating thyroid hormones. Their review is thorough, including 58 studies of diverse nature (Graeppi-Dulac et al., 2014).

Retinol and the Immune System

Innate Immune System

In their histological mouse study, Burrows et al. (2018) discovered that intestinal innate lymphocytic cells (ILCs) express the Hypermethylated in Cancer 1 (HIC1) transcriptional repressor in a vitamin A-dependent manner. The authors purchased a

vitamin A-deficient diet to feed the mice. After the appropriate time frame, they removed the Peyer's patches to isolate the lymphocytes from the lamina propria. They measured absolute cell counts, performed intracellular cytokine staining, as well as fluorescent antibody staining for all the cell membrane and nuclear markers, and RNA isolation and quantitative real-time PCR. If HIC1 is absent, then the ILC3s that produce IL-22 are lost, and the loss increases susceptibility to intestinal infections. Therefore, *atRA*-dependent expression of HIC1 regulates intestinal homeostasis and protective immunity. This *atRA* is produced by intestinal epithelial cells (IECs) and CD103+ dendritic cells to maintain intestinal barrier integrity. Lack of vitamin A in the diet results in a decreased number of ILC3s and DCs but an increased number of ILC2s with enhanced type 2 immunity. Deletion of HIC1 reduces the number of $\alpha\beta$ and $\gamma\delta$ T-cells, CD103+ DCs, and ILC3s. The increased susceptibility to infection is due to the decreased number of T-bet and ROR γ t-expressing ILC3s and the decreased production of IL-22 by those ILC3s (Burrows et al., 2018). The work of Burrows et al. (2018) highlights the role of vitamin A at the front lines of innate immunity, namely barrier integrity. The molecular mechanisms are still unknown, but the authors suggest that RA induces the expression of HIC1 to promote cellular dormancy.

In their recent review article of 132 articles over ILCs, Eberl, Colonna, Di Santo, and McKenzie (2015) explore the role of ILCs as a new paradigm in immunology. They explain that the functions of the innate ILCs mirror those of the adaptive T cells. Natural killer (NK) cells mirror CD8+ cytotoxic T-cells, ILC1s mirror CD4+ T helper 1 (Th1) cells to fight intracellular pathogens, viruses, and tumors, ILC2s mirror Th2 cells to fight

large parasites, allergens, and epithelial injury and ILC3s mirror Th17 cells to fight extracellular microbes, bacteria, and fungi. Stress ligands, bacteria, and dietary compounds activate these innate cells, they do not undergo antigen-activation and clonal selection, and they act more like memory cells, enabling a prompt immune response. ILCs can regulate the activity of DCs and T-cells and can function as antigen-presenting cells, directing the adaptive immune response. Retinoic Acid (RA) favors the maturation of ILC3s at the expense of ILC2s, directing the immune system towards a type 3 immune reaction (bacteria and fungi) and inducing tissue repair via the production of IL-22 (Eberl et al., 2015).

In his meta-analysis of $\gamma\delta$ T-cells, Ferreira (2013) reviews 235 articles to characterize this novel subset of T-cells and their function in mice and men. These cells have a $\gamma\delta$ TCR instead of an $\alpha\beta$ TCR and possess innate and adaptive immune receptors. TCRs are highly homologous to antibodies produced by B-cells, and in $\gamma\delta$ T-cells, the TCR specificity correlates to function and tissue location. $\gamma\delta$ T-cells also possess a CD3 cell surface marker and comprise between 0.5 to 5 percent of T-cells. The context in which these cells operate determines if they function as innate cells, adaptive cells, or both. Some say they are the third branch of the immune system. They are thought to function as a prompt first line of defense against pathogens, in which case their numbers can increase to comprise more than 50 percent of peripheral lymphocytes. These cells have subtypes that mirror each of the $\alpha\beta$ T-cells subtypes, namely cytotoxic, Th1, Th2, Th17, and Treg. $\gamma\delta$ T-cells can act as phagocytes and function as antigen-presenting cells (Ferreira, 2013). In their histological investigation of thyroid tissue of patients with

Hashimoto's thyroiditis, Liu et al. (2015) found that treating thyroidal $\gamma\delta$ T-cells with *atRA* induced apoptosis of the $\gamma\delta$ T-cells. Analysis of those tissues also revealed that $\gamma\delta$ T-cells enhanced B-cells for antibody production, therefore contributing to the pathogenesis of Hashimoto's thyroiditis (Liu et al., 2016).

Adaptive Immune System

In their review of RA as a modulator of T-cell immunity, Bono et al. (2016) evaluate over 100 articles to elucidate the effect of RA on this branch of the immune system. Vitamin A is one of the most common micronutrient deficiency in the world, and it is associated with deficiencies in the adaptive immune response. This deficiency can lead to decreased cellular and humoral immune responses, inadequate immune regulation, a weak response to vaccines, and poor lymphoid organ development. RA induces T-cell homing to the gut via the production of $\alpha 4\beta 7$ integrin and CCR9. Gut CD103⁺ DCs from Peyer's patches and mesenteric lymph nodes provide a source of RA for these cells (Bono et al., 2016).

Additionally, Tregs are crucial to maintain gut homeostasis and tolerance to commensal microbiota and food proteins in the gut. Naïve T-cells can be transformed into Tregs in the gut via the interaction with gut DCs that produce RA, which acts on RAR α . RA also blocks the production of cytokines that block Treg differentiation. At pharmacological doses, RA has been shown to inhibit the Th1 and Th17 responses while inducing the generation of Tregs. However, lower doses of RA, even physiological ones, fail to inhibit Th17 cell differentiation and may even favor the differentiation of the

Th1 and Th17 lineages. Vitamin A deficiency can significantly impair the Th1 and Th17 responses. In the presence of IL-12 and IL-23, RA promotes Th1 and Th17 differentiation and inhibits Treg differentiation. Context determines the effect of RA, in a steady, state RA favors Treg suppression of Th1 and Th17, and in an inflammatory state, RA favors Th1 and Th17 cell-mediated immunity. Regarding Th1/Th2 balance, vitamin A deficiency contributes to INF- γ overproduction while dietary RA down-regulates the Th1 response and upregulates the Th2 response and protective IgA production. RA is an essential metabolite in the development of an appropriate immune response (Bono et al., 2016).

In their mouse model investigation of the molecular mechanisms of Treg induction, Lu et al. (2011) found that *atRA* promoted TGF- β -induced Tregs via histone modification on the Foxp3 gene locus. They assessed cell differentiation and function of the Tregs, used flow cytometry and marker specific antibodies to assess cell membrane (CD) markers, TGF- β and Foxp3, used real-time PCR to quantify Foxp3 mRNA, ran a Western-Blot analysis to assess protein composition, and assessed Foxp3 methylation. They found that the treatment of naïve cultured T-cells with *atRA* not only induced Tregs but also helped to maintain Foxp3 expression. Both increased induction and decreased apoptosis of Tregs due to the TGF- β and *atRA* treatment combination explains this finding. The *atRA* is acting on the RAR rather than the RXR signaling pathway. This study also found that *atRA*-induced TGF- β Tregs exhibited an enhanced suppressive T-cell response and ameliorated lupus syndromes in a GVHD animal model. The data suggest that *atRA* affects TGF- β signaling rather than the other way around, and RA

alone, without TGF- β , cannot induce Tregs. *atRA* significantly increases histone modification, including methylation and acetylation, but does not affect DNA demethylation in the *Foxp3* gene (Lu et al., 2011). This study is thorough and well done, but its weakness is that it is an animal cytology study.

In their histological mouse model of colitis study, Nguyen, Pearson, Kim, Kamdar, and DePaolo (2015) evaluated the effects of RA on extracted lymphocytes and dendritic cells during intestinal injury. They induced colitis in the mice selected for the study and then assessed the mice for the clinical and histological signs of colitis. They killed the mice, took colon tissue samples, and removed the spleens to get purified T-lymphocytic cells and dendritic cells. They used the purified lymphocytes and dendritic cells for either treatment with 10 nM of RA or no treatment, and subsequent evaluation for the various cytokines and cell markers using specific antibodies and flow cytometry. They found that RA treatment enhances TLR2-dependent IL-10 production from T-cells and this potentiates *Foxp3*⁺ IL-10⁺ Treg generation without the need for the activation of antigen-presenting cells, inhibiting colonic disease. In the absence of TLR2, RA signaling switches from a tolerogenic one to a pro-inflammatory one. The authors suggest that the combination of RA and TLR2 ligands should be considered in the design of therapies to treat autoimmune or inflammatory diseases, but caution against treating patients with inflammatory diseases with vitamin A alone, as it can aggravate inflammation (Nguyen, Pearson, Kim, Kamdar, & DePaolo, 2015). This in-vitro cytological mouse model gives some great insight into the molecular mechanisms of RA

action in immune system regulation, but these molecular mechanisms may be different in humans.

In their mouse model study, Hall et al. (2011) examined the role of RA in the promotion of CD4⁺ T cell effector responses. Mice were purchased and either fed a vitamin A-deficient or sufficient diet, which was also purchased. The vitamin A-deficient mice were given a total of 250 µg of *atRA* intraperitoneally every other day. Then 24 hours after the third *atRA* injection, the mice were either infected with *T. gondii* or vaccinated. The team harvested tissue samples, assessed T-cell phenotypes using flow cytometry with the appropriate fluorescent antibodies, and analyzed protein complexes using immunoblotting. They found that RA is necessary to induce pro-inflammatory CD4⁺ T cell responses to infection and vaccination. They were also able to uncover that RAR α was the critical mediator of these effects. They demonstrated that the loss of vitamin A compromised mucosal Th1 and Th17 cell responses to infection and vaccination and that treatment with RA rapidly restores mucosal Th1 and Th17 responses. The finding highlights the fundamental role of the RA-RAR α axis in mediating both the regulatory and inflammatory responses of the adaptive immune system, and the importance of nutritional status as a broad regulator of T-cell responses (Hall, Cannons, et al., 2011). The weakness of this study is that the RA-RAR α axis also exists in antigen-presenting cells and affects their function, which could have an indirect effect on T-cell function, indicating that this axis could modify both the innate and the adaptive immune systems.

In their RCT, placebo-controlled clinical trial in 84 obese and non-obese women, Farhangi et al. (2013) demonstrated that vitamin A treatment significantly reduced serum concentrations of IL-1 β (a pro-inflammatory cytokine) in obese women. Obese women were randomly allocated to receive either 25,000 IU per day of retinyl palmitate or placebo. Nonobese women also received 25,000 IU of retinyl palmitate. The team assessed anthropometric variables, and serum IL-1 β , TNF- α , IL-4, and IL-13 were analyzed at baseline and 4-months after intervention. They found a reduction in IL-1 β in vitamin A-treated obese women. The decrease in IL-1 β after vitamin A supplementation is due to its ability to reduce IL-1 β gene expression. Serum concentrations of IL-4 and IL-13 (anti-inflammatory cytokines) were also significantly reduced in obese and non-obese vitamin A-treated women, possibly due to a counterregulatory feedback mechanism in which the lowering of the pro-inflammatory IL-1 β leads to a decreased need for the anti-inflammatory cytokines. The weakness of their study lies in the fact that there is a need to establish whether IL-4 and IL-13 are increased in the white adipose tissue of obese participants to confirm this hypothesis. They suggest that because of its immune regulating effects, vitamin A could reduce the risk of autoimmune disease in obese women (Farhangi, Keshavarz, Eshraghian, Ostadrahimi, & Saboor-Yaraghi, 2013).

Immune Homeostasis

Erkelens and Mebius reviewed 100 articles to evaluate the effect of retinoic acid on immune homeostasis. RA is a central molecule in the orchestration of the immune system response, and dendritic cells (DCs) and macrophages produce it. DC precursors require RA to express the gut homing molecules that allow them to reach the lamina

propria of the intestine and the GALT. Once there, RA is necessary for them to maintain a tolerogenic response. In the GALT, the RA produced by the DCs and macrophages will induce B and T cells to express their gut homing molecules. RA can also induce gut homing molecules on ILCs1 and 3, and IgA class switching in B cells. Certain combinations of inflammatory signals can induce RA production via ALDH expression in DCs and macrophages. This concept implies that the microenvironment determines the expression of the enzymes needed to produce RA. DCs and macrophages can get RA from the intestinal environment, particularly the small intestine (Erkelens & Mebius, 2017).

RA also modulates T-cell responses. During steady state, the RA produced by DCs inhibits the differentiation of naïve T-cells into Th17 cells by blocking certain interleukins, allowing them to mature into Tregs instead. During infection, other molecular signals counter the inhibition of this differentiation and, thus, produce Th17 cells. Additionally, under the condition of infection, RA can induce a pro-inflammatory phenotype in DCs. When IL-6 and TGF- β stimulate a naïve T-cell, it becomes a Th17 cell. RA can block IL-6 and prevent this conversion. If RA and TGF- β stimulate a naïve T-cell, it becomes a Treg. If RA and IL-4 stimulate it, it becomes a Th2 cell, and if it is RA, INF- γ , and IL-12 stimulate it, it becomes a Th1 cell. Therefore, RA is essential for both a tolerogenic response and for a proper Th1, Th2, and Th17 response (Erkelens & Mebius, 2017).

Oliveira, Teixeira, and Sato (2018) review 185 articles to elucidate the effect of RA on innate and adaptive immunity with an emphasis on inflammatory conditions. DCs

mediate the balance between tolerogenic and effector immune responses. The CD103⁺ DCs are the tolerogenic ones, and they respond to RA to induce Foxp3⁺ Tregs that produce the anti-inflammatory IL-10, and B and T-cell migration to the gut. The CD103⁻ DCs are the inflammatory DCs that do not require RA, promote the differentiation of INF- γ -producing T cells, and produce inflammatory cytokines such as TNF- α and IL-6. RA also favors the homing of ILCs 1 and 3 to the gut. In NK cells, RA also acts to favor tolerance and suppress NK cell cytotoxicity activated by INF- α . RA plays a vital role in the humoral response and is essential for B-cell production of IgA. RA is a cofactor that is important for the stimulation and proliferation of B-cells; it accelerates the maturation of human B-cells and their conversion into plasma cells. RA may also increase IgM and IgG synthesis. In the T-cell line, high levels of RA promote the differentiation of Th2 cells and inhibit the Th1 phenotype. However, RA is essential for the stability and maintenance of Th1 cells, repressing ROR γ t which induces the Th17 phenotype (Oliveira et al., 2018).

During inflammatory states, RA can attenuate inflammation by increasing IL-22 production by ILC3 and $\gamma\delta$ T-cells in the gut. In ulcerative colitis, upregulation of the RA degrading enzyme CYP26 reduces colonic RA levels and leads to tumorigenesis. Treatment with *at*RA decreases the tumor burden. Ulcerative colitis and Crohn's patients often have low serum retinol levels, likely due to a lack of absorption. The authors note that RA administration should be an adjuvant treatment for inflammatory diseases. They also note that RA administration in Multiple Sclerosis patients shifts the T-cell balance towards tolerance and away from inflammation. There is also evidence that RA has

neuroprotective effects in those patients. Topical retinoids are also used in the treatment of psoriasis due to their anti-inflammatory effects (Oliveira et al., 2018).

Retinol and Hashimoto's Thyroiditis

Liu et al. (2016) evaluated the function of $\gamma\delta$ T-cells in the thyroid tissue of patients with Hashimoto's thyroiditis (HT). They enrolled 148 participants: 99 with HT, five with simple goiters, and 44 healthy controls. The team obtained peripheral blood and thyroid samples from all the participants. They used flow cytometry to analyze peripheral blood and thyroid mononuclear cells. They tested thyroid tissues via immunofluorescent staining and immunohistochemistry for $\gamma\delta$ T-cells and anti-thyroid antibody detection. The team used an ELISA and an automated chemiluminescent assay Antibody production. They measured the activation and apoptosis of peripheral $\gamma\delta$ T-cells and B-cells by flow cytometry. They found that the percentage of $\gamma\delta$ T-cells was higher in the thyroid tissue of HT patients than that of goiter patients. The proportion of $\gamma\delta$ T-cells in the CD3+ T-cell population reached 55%, indicating that they play an essential role in HT (Liu et al., 2016).

Additionally, the $\gamma\delta$ T-cells of HT patients expressed the activation markers CD69 and HLA-DR, and the CD40L (which regulates production of B-cells, Ig class switching and antibody production) and ICOS cell markers with significantly higher frequency than the healthy controls. Moreover, the $\gamma\delta$ T-cells of HT patients enhanced B-cells for antibody production. *at*RA treatment of those cells inhibited the antibody production

effect by inducing apoptosis of the $\gamma\delta$ T-cells but had no direct effect on the B-cells (Liu et al., 2016).

Interestingly, *atRA* targets the activated HT $\gamma\delta$ T-cells for apoptosis but not the non-activated ones. However, it is still unclear whether $\gamma\delta$ T-cells also help mediate autoantibody production in HT. The authors conclude that $\gamma\delta$ T-cells might play an essential role in the development of anti-thyroid autoantibodies in HT patients and *atRA* administration could be used to manipulate $\gamma\delta$ T-cell activation and apoptosis (Liu et al., 2016). The weakness of this study is that they incubated the culture of $\gamma\delta$ T-cells with *atRA*, a method that may not reflect the finely-tuned local control of *atRA* production. The study does give a direction for future research, opening the door for exploring vitamin A as a potential dietary treatment modality for HT.

In conclusion, retinol has diverse effects on the immune system and thyroid metabolism, effects that are under tight regulation at the tissue level, and which vary depending on the immunological microenvironment. It could potentially modulate the progression of HT and could play a role in its treatment. However, the evidence is sparse, and more research is needed. Therefore, the goal of this study is to establish, as groundwork, whether serum retinol levels in HT patients are significantly different from patients who do not have HT. The hypothesis is that the retinol levels in HT participants are not lower than that of healthy (non-HT) participants. Additionally, a secondary goal of the study is to explore any relationships between retinol and TSH levels in the entire sample, and retinol and thyroid antibody levels in the HT participants.

CHAPTER 3: METHODS

Study Design

This retrospective case-controlled study was executed using a database of patient information gathered from the Health Matters Clinic in Jonesboro, AR. This database was obtained during a previous IRB-approved study conducted at Arkansas State University in 2014. Approval was granted by Dr. Stacy Walz, the chair of the Clinical Laboratory Sciences Department at Arkansas State University, for the use of that database for this dissertation project. First, database entries were screened for retinol levels, then for inclusion and exclusion criteria for each group. Then, randomization was executed separately for each group using the number randomizer found at this URL: <https://andrew.hedges.name/experiments/random/>. The 26 charts that were randomly selected for each group were then used for the study. The University of Bridgeport Institutional Review Board approved this study, and it was also approved by the Arkansas State University Institutional Review Board in August 2019 (Exemption 45 CFR 46, 104(d)(4) from UB).

Database Selection Criteria

This database included 229 non-pregnant female patients between the ages of 18 and 65. The database did not contain retinol levels in all the patient entries. Therefore, all entries that did not contain a retinol level were first excluded from the study. The elimination of those entries reduced the number from 229 to 103 entries.

For the Hashimoto's Thyroiditis group (HT), inclusion criteria were database patient entries with either or both positive thyroid peroxidase (TPO) antibody and positive thyroglobulin (TG) antibody. The application of these criteria allocated 41 patients to that group. The exclusion criteria included any patient on documented thyroid medication, such as levothyroxine or Synthroid. The rationale behind using thyroid medication as an exclusion criterion is that it increases the conversion of β -carotene to retinol (Aktuna et al., 1993). The application of this criterion excluded nine patients, leaving 32 entries in the HT group. Twenty-six entries were randomized from those entries. (See Figure 6)

For the control group (non-HT), the inclusion criteria were database patient entries that were negative for TPO and TG antibodies (62 entries) and had a normal thyroid-stimulating hormone (TSH). The application of the inclusion criteria resulted in 59 entries remaining in the control group. The exclusion criteria for the control group included any database patients that have thyroid issues, take thyroid medication, or were diagnosed with an autoimmune disease. Both thyroid dysfunction and autoimmunity can affect retinol levels (Abdelhamid & Luo, 2018; Aktuna et al., 1993). The application of this criterion excluded seven patients that either had thyroid issues or were on documented thyroid medication and three patients that had a documented autoimmune disease. These were Celiac Disease, Myasthenia Gravis, and Multiple Sclerosis. Twenty-six entries were randomized from the remaining forty-nine database entries for the control group. (See Figure 6)

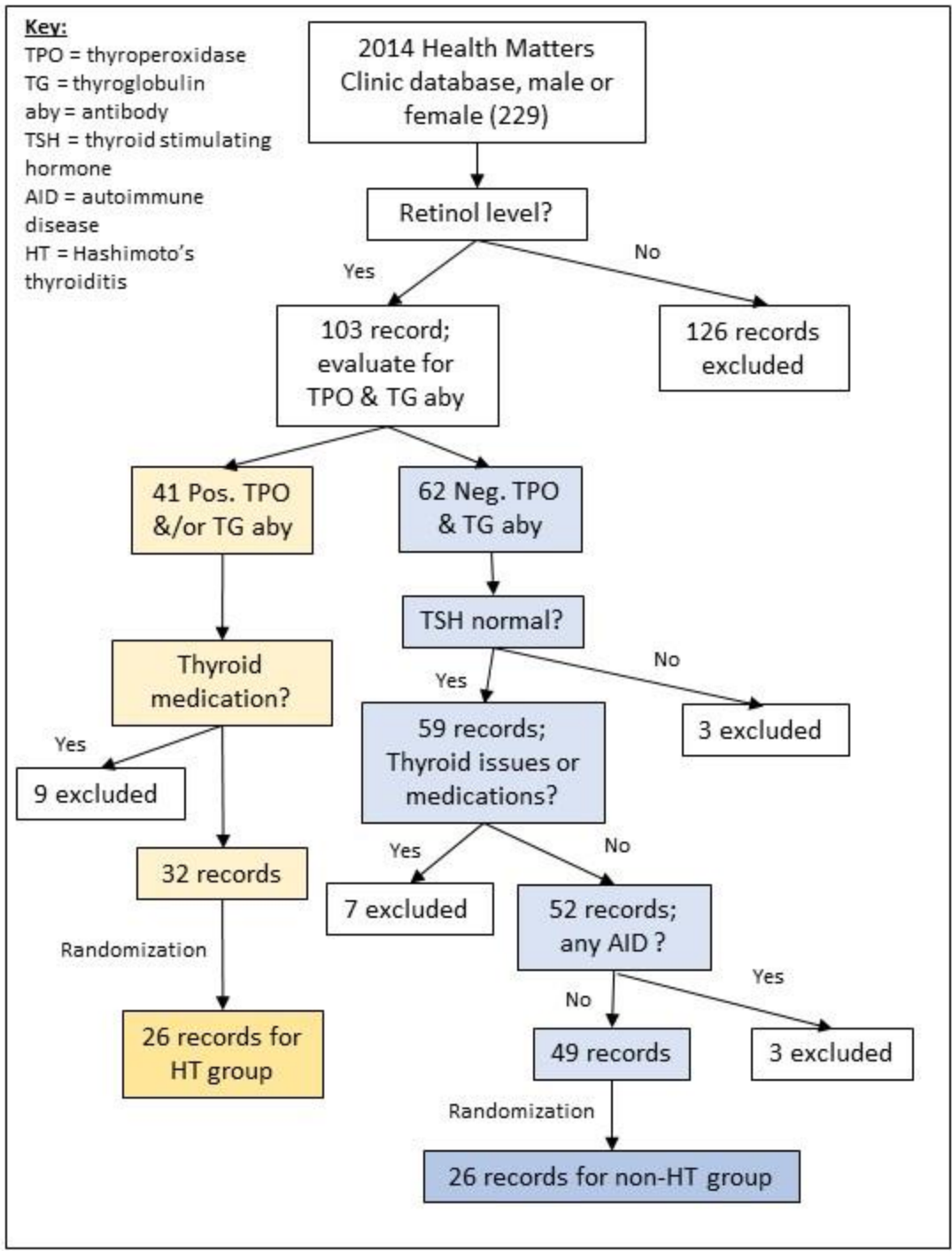


Figure 6. Selection Flow Chart

Data Collection

The data collected from all selected charts were: (a) retinol levels in mcg/dL; (b) age in years; (c) TSH in μ IU/mL; (d) T4 in mcg/dL; (e) T3 in ng/dL; (f) free T4 in ng/dL; (g) free T3 in pg/mL; (h) reverse T3 ng/dL; (i) TPO antibodies in IU/mL; (j) TG antibodies in IU/mL; (k) vitamin D in ng/mL; (l) gluten IgG in mcg/mL; (m) dairy IgG in mcg/mL; (n) multivitamin use. The aim of collecting all these data was to characterize each group and potentially ascertain differences in gut permeability issues (as evidenced by the presence of food antibodies), which could affect retinol levels. Retinol levels were the independent variable used to compare the HT and non-HT groups. Retinol and TSH levels were used to determine the potential correlation between thyroid function and retinol levels within the entire sample (all 52 patient entries), using retinol as the independent variable and TSH as the dependent variable. Retinol levels (independent variable) and TPO antibody levels (dependent variable) were used to determine if any correlation existed between these variables in the HT group in one analysis. Additionally, retinol levels (independent variable) and TG antibody levels (dependent variable) were used to determine any correlation between those variables within the HT group, in a separate analysis.

Statistics Analysis

Intellectus Statistics ® was used for all statistical calculations. Firstly, each group was characterized as to their average value (group mean) or percentage for each of these variables: (a) retinol levels in mcg/dL; (b) age in years; (c) TSH in μ IU/mL; (d) T4 in

mcg/dL; (e) T3 in ng/dL; (f) free T4 in ng/dL; (g) free T3 in pg/mL; (h) reverse T3 ng/dL; (i) TPO antibodies in IU/mL; (j) TG antibodies in IU/mL; (k) vitamin D in ng/mL; (l) gluten IgG in mcg/mL; (m) dairy IgG in mcg/mL; (n) multivitamin use. The independent sample t-test was used to determine if there were any statistically significant differences between each group for each of these variables. There were some expected differences between the groups, such as in the thyroid function tests. Some of these data were included in the group characterization because they could be confounders, such as vitamin D, food antibodies, and multivitamin use.

Secondly, for the independent sample t-test evaluating the difference in retinol between HT and non-HT groups, a power analysis was conducted in G*Power to determine a sufficient sample size using an alpha of 0.05, a power of 0.80, a large effect size ($d = 0.8$), and two tails (Faul, Erdfelder, Buchner, & Lang, 2013). There was an equal allocation of participants into each group. Based on the mentioned assumptions, the desired sample size was 52 (26 in each group). For the various correlation calculations (retinol and TSH in the sample, retinol and TPO antibodies in HT group and retinol and TG antibodies in HT group), a power analysis for a Pearson correlation was conducted in G*Power to determine a sufficient sample size with an alpha of 0.05, a power of 0.80, a large effect size ($p = .5$), and two tails (Faul et al., 2013). Based on the mentioned assumptions, the desired sample size was 26, which is the number of randomly selected entries in the HT group.

Statistical calculations from the data entries were executed with Intellectus Statistics ®. These were: (a) an independent sample t-test evaluating the difference in retinol between HT and non-HT groups; (b) a Pearson's correlation evaluating the relationship between retinol and TSH in the entire sample; (c) a Pearson's correlation evaluating the relationship between retinol and TPO antibodies in the HT group; and (d) a Pearson's correlation evaluating the relationship between retinol and TG antibodies in the HT group.

Outcomes Measured

The first outcome measured was the database retinol levels in mcg/dL for each participant, after allocation into their respective groups. The next outcome measured was the database TSH levels in μ IU/mL for all participants, and its correlation to their retinol levels. The third and fourth outcomes measured were the database TPO and TG antibody levels, respectively, in IU/mL, for the HT group and its correlation to their retinol levels.

CHAPTER 4: RESULTS AND FINDINGS

Introduction

Due to its immune-modulating properties, there is a potential therapeutic role for retinol in autoimmune diseases. Therefore, the following statistical analyses aim to ascertain whether serum retinol levels in Hashimoto's thyroiditis patients are significantly different from patients who do not have Hashimoto's thyroiditis. The hypothesis is that the retinol levels in HT participants are lower than that of healthy (non-HT) participants. The study and the following statistical analyses aim to establish some groundwork for the potential use of vitamin A supplementation in the treatment of HT. Additionally, a secondary goal of the study is to explore any relationships between retinol and TSH levels in the entire sample, and retinol and thyroid antibody levels in the HT participants, as they each could potentially be influenced by retinol status. All statistical calculations were performed with Intellectus Statistics ®, and their provided interpretations and commentaries on each were used to interpret the results. The detailed descriptive statistics for each group, the retinol independent samples t-test, and the various Pearson correlations are in the paragraphs that follow. The database used was in an Excel file, and the data were extracted and formatted as needed for the calculations.

Summary of Collected Data

Data collected from the database for the Hashimoto's thyroiditis group and the non-Hashimoto's thyroiditis group were (a) retinol levels in mcg/dL; (b) age in years; (c) TSH in μ IU/mL; (d) T4 in mcg/dL; (e) T3 in ng/dL; (f) free T4 in ng/dL; (g) free T3 in

pg/mL; (h) reverse T3 ng/dL; (i) TPO antibodies in IU/mL; (j) TG antibodies in IU/mL; (k) vitamin D in ng/mL; (l) gluten IgG in mcg/mL; (m) dairy IgG in mcg/mL; (n) multivitamin use. The database used came from the Health Matters Clinic in Jonesboro, AR, a small integrative health clinic in a town of 60,000 inhabitants, that is also serving a large rural area. The original data was collected in 2014 and used for a previous study regarding the predictive value of reverse T3 measurements.

Descriptive Findings

Each group was characterized by the previously-mentioned parameters and compared using a two-tailed paired samples *t*-test. Table 1 displays the findings from the descriptive analysis. Normal ranges for each test are also indicated in Table 1, where appropriate, as are the calculated *p*-values.

The observations for age had an average of 40.77 years for the HT group (*SD* = 12.80, range = 18.00 - 61.00) and 40.92 years for the non-HT group (*SD* = 11.83, range = 19.00 - 65.00). The result of the two-tailed paired samples *t*-test for the age of the participants was not significant based on an alpha value of 0.05 and a *p*-value of .967, which suggests the difference in the means of the age in years was not significantly different from zero. The observations for retinol had an average of 54.04 mcg/dL for the HT group (*SD* = 19.54, range = 30.00 - 96.00) and 53.81 mcg/dL for the non-HT group (*SD* = 14.82, range = 37.00 - 95.00). It is worth noting that the variation around the mean (*SD*) is slightly larger in the HT group. The result of the two-tailed paired samples *t*-test for retinol was not significant based on an alpha value of 0.05 and a *p*-value of .956,

which suggests that the difference between the means was not significantly different from zero.

The observations for TSH had an average of 9.45 $\mu\text{IU/mL}$ for the HT group ($SD = 37.87$, range = 0.68 - 195.00) and 1.56 $\mu\text{IU/mL}$ for the non-HT group ($SD = 0.78$, range = 0.49 - 3.04). The variance around the mean is much larger in the HT group than the non-HT group, with a higher SD and a wider range. The result of the two-tailed paired samples t -test was not significant based on an alpha value of 0.05 and a p -value of .300, which suggests the difference in the TSH means was not significantly different from zero. The observations for T4 had an average of 8.41 mcg/dL for the HT group ($SD = 1.59$, range = 6.60 - 14.00) and 8.20 mcg/dL for the non-HT group ($SD = 2.16$, range = 5.20 - 16.60). The result of the two-tailed paired samples t -test was not significant based on an alpha value of 0.05 and a p -value of .687, which suggests the difference in the T4 means was not significantly different from zero. The observations for T3 had an average of 110.08 ng/dL for the HT group ($SD = 21.79$, range = 75.00 - 182.00) and 104.19 ng/dL for the non-HT group ($SD = 37.75$, range = 58.00 - 249.00). The result of the two-tailed paired samples t -test was not significant based on an alpha value of 0.05 and a p -value of .517, which suggests the difference in the T3 means was not significantly different from zero. The observations for fT4 had an average of 1.12 ng/dL for the HT group ($SD = 0.12$, range = 0.90 - 1.40) and 1.15 ng/dL for the non-HT group ($SD = 0.14$, range = 0.90 - 1.50). The result of the two-tailed paired samples t -test was not significant based on an alpha value of 0.05 and a p -value of .349, which suggests the difference in the fT4 means was not significantly different from zero. The observations for fT3 had an average of

3.07 pg/mL for the HT group ($SD = 0.28$, range = 2.40 - 3.80) and 3.03 pg/mL for the non-HT group ($SD = 0.34$, range = 2.60 - 3.90). The result of the two-tailed paired samples t -test was not significant based on an alpha value of 0.05 and a p -value of .600, which suggests the difference in the fT3 means was not significantly different from zero. The observations for rT3 had an average of 20.31 ng/dL for the HT group ($SD = 6.29$, range = 13.00 - 36.00) and 23.92 ng/dL for the non-HT group ($SD = 8.59$, range = 10.00 - 45.00). The result of the two-tailed paired samples t -test was not significant based on an alpha value of 0.05 and a p -value of .112, which suggests the difference in the rT3 means was not significantly different from zero. The observations for TPO had an average of 81.96 IU/mL for the HT group ($SD = 223.28$, range = 0.00 - 1000.00) and 0.00 IU/mL for the non-HT-group ($SD = 0.00$). A t -test could not be calculated because one of the group's TPO means is zero. The observations for TG had an average of 5.85 IU/mL for the HT group ($SD = 15.21$, range = 0.00 - 51.00) and 0.00 IU/mL for the non-HT-group ($SD = 0.00$). A t -test could not be calculated because one of the group's TG means is zero.

The observations for vitamin D had an average of 36.04 ng/mL for the HT group ($SD = 12.75$, range = 17.00 - 67.00) and 33.27 ng/mL for the non-HT group ($SD = 11.09$, range = 12.00 - 55.00). The result of the two-tailed paired samples t -test was not significant based on an alpha value of 0.05 and a p -value of .438, which suggests the difference in the vitamin D means was not significantly different from zero. The observations for gluten antibodies had an average of 16.20 mcg/mL for the HT group ($SD = 39.82$, range = 0.00 - 200.00) and 10.17 mcg/mL for the non-HT group ($SD = 23.77$,

range = 0.00 - 119.00). The result of the two-tailed paired samples *t*-test was not significant based on an alpha value of 0.05 and a *p*-value of .220, which suggests the difference in the gluten antibody means was not significantly different from zero. The observations for dairy antibodies had an average of 20.86 mcg/mL for the HT group (*SD* = 6.86, range = 10.60 - 30.00) and 16.77 mcg/mL for the non-HT group (*SD* = 6.68, range = 9.18 - 30.00). The result of the two-tailed paired samples *t*-test was significant based on an alpha value of 0.05 and a *p*-value of .036, indicating the null hypothesis can be rejected. This finding suggests that the difference in the means of dairy antibodies was significantly different from zero. The mean of the non-HT group was significantly lower than the mean of the HT group. The observations for multivitamin intake had an average of 12% for regular intake for the HT group (*SD* = 33) and 38% for the non-HT group (*SD* = 50). The result of the two-tailed paired samples *t*-test was significant based on an alpha value of 0.05 and a *p*-value of .016, indicating the null hypothesis can be rejected. This finding suggests that the difference in the means of multivitamin intake was significantly different from zero. The mean of the non-HT group was significantly higher than the mean of the HT group.

The groups were similar in almost all parameters. Because it was a selection criterion, the HT group had elevated levels of both thyroid antibodies, whereas the non-HT group's levels were zero for both. The only other significant differences found between the groups were dairy antibodies and multivitamin use. The HT group had significantly higher levels of dairy antibodies, and the non-HT group had significantly

higher multivitamin use. TSH levels were higher in the HT group, but not significantly so. Gluten antibodies were also higher in the HT group, but again, not significantly.

Table 1. HT and non-HT Group Characteristics

Parameters	Normal ranges	HT		Non-HT		p-values
		Mean	SD	Mean	SD	
Age (years)	N/A	40.77	12.80	40.92	11.83	.967
Retinol (mcg/dL)	38-98	54.04	19.54	53.81	14.82	.956
TSH (μ IU/mL)	0.465-4.68	9.45	37.87	1.56	0.78	.300
T4 (mcg/dL)	5.1-11.9	8.41	1.59	8.20	2.16	.687
T3 (ng/dL)	76-181	110.08	21.79	104.19	37.75	.517
fT4 (ng/dL)	0.8-1.8	1.12	0.12	1.15	0.14	.349
fT3 (pg/mL)	2.3-4.2	3.07	0.28	3.03	0.34	.600
rT3 (ng/dL)	8-25	20.31	6.29	23.92	8.59	.112
TPO aby (IU/mL)	\leq 9	81.96	223.28	0	0	.*
TG aby (IU/mL)	\leq 1	5.85	15.21	0	0	.*
Vitamin D (ng/mL)	30-100	36.04	12.75	33.27	11.09	.438
Gluten IgG (mcg/mL)	<2.0	16.20	39.82	10.17	23.77	.220
Dairy IgG (mcg/mL)	<0.15	20.86	6.86	16.77	6.68	.036**
Multivitamin (% of group taking)	N/A	12%	.*	38%	.*	.016**

*not calculated

** $p < 0.05$

Out-of-range values are in **bold**

Results

The two-tailed independent samples t-test was conducted to examine whether the mean level of retinol was significantly different between the subjects classified as having Hashimoto's thyroiditis (HT group) and those classified as not having it (non-HT group). The analysis revealed that the difference was not significant based on an alpha value of 0.05 and a *p*-value of .962. (see Figure 7).

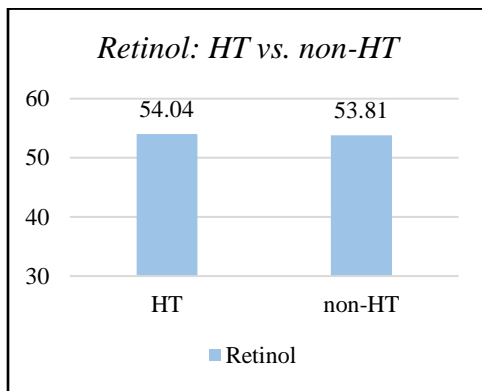


Figure 7. Retinol: HT vs. non-HT

Pearson's correlation between retinol and TSH levels for the entire sample was calculated next. Cohen's standard was used to evaluate the strength of the relationship, where coefficients between .10 and .29 represented a small effect size, coefficients between .30 and .49 represented a moderate effect size, and coefficients above .50 represented a large effect size (Cohen, 1988). The correlation was examined based on an alpha value of 0.05. A significant positive correlation was observed between retinol and TSH, with a correlation coefficient of 0.44, indicating a moderate effect size. This correlation indicates that as retinol increases, TSH tends to increase (see Figure 8).

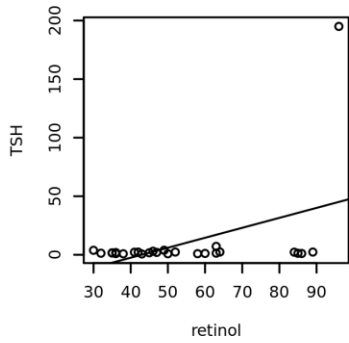


Figure 8. Correlations Between Retinol and TSH

Then, Pearson’s correlation analysis was conducted between retinol and TPO antibody levels in the HT group only. Cohen’s standards, as described in the previous paragraph, were used again to evaluate the strength of the relationship. The correlations were examined based on an alpha value of 0.05. There were no significant correlations found between retinol and TPO antibody levels (see Figure 9). The correlation coefficient calculated at -0.19. The same analysis, with the same parameters, was repeated for retinol and TG antibody levels in the HT group. Again, there were no significant correlations found between retinol and TG antibody levels (see Figure 10). The correlation coefficient calculated as 0.08.

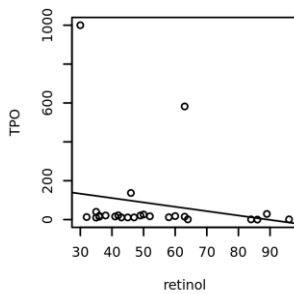


Figure 9. Correlations Between Retinol and TPO Antibody Levels

CHAPTER 5: DISCUSSION, CONCLUSION AND RECOMMENDATIONS

Introduction

This study aimed to establish whether there was a significant difference between retinol levels in patients with Hashimoto's thyroiditis (HT) as compared to patients that do not have Hashimoto's thyroiditis. Some patients with autoimmune disease demonstrate low levels of retinol, such as Multiple Sclerosis patients, and benefit from retinol supplementation (Dorosty-Motlagh et al., 2016). Therefore, it would be beneficial to examine the retinol status in other autoimmune diseases. This study used an already established database of patients' lab results from the Health Matters Clinic in Jonesboro, Arkansas, which had an appropriate number of patients with positive thyroid autoantibodies indicative of HT, to examine retinol levels and its relationship to a few HT markers.

The statistical analysis revealed no significant difference in retinol levels between patients with HT and patients who do not have HT. However, it revealed a moderate positive correlation between retinol and TSH levels in the entire sample, with high TSH levels correlating to higher (but still within normal range) retinol levels. Additionally, the examination of the HT group for a correlation between retinol and thyroid antibodies levels found no correlations between retinol and either thyroid antibody levels (anti-thyroid peroxidase and anti-thyroglobulin antibodies). The analysis of the groups' characteristics failed to reveal a statistically significant difference in the TSH levels between the groups, although the HT group did demonstrate higher levels of TSH than

the non-HT group, as expected. Additionally, this analysis revealed higher levels of gluten antibodies in the HT group (but not significantly so), significantly higher levels of dairy antibodies in the HT group, and that significantly more participants took a multivitamin in the non-HT group.

Interpretation of the Findings

Even though the initial expectation of this study was that HT patients would have lower retinol levels than non-HT patients, the findings did not prove the hypothesis. Researching the literature and thinking through this process did reveal some interesting information. The first point is that serum retinol levels do not accurately reflect liver vitamin A storage status. Homeostatic mechanisms keep serum retinol levels constant and only drop when liver stores are near depletion (Tanumihardjo et al., 2016). Additionally, the tissue microenvironment is more likely to determine the local retinol availability and vary by organ or tissue location. The second point is that retinol status did not correlate with autoantibody levels in HT; other mechanisms might be at play in this process. The third point is that there are several levels, or classifications, of HT and the representation of each level in the HT group could be a significant factor in the relationship between retinol and HT. The fourth point is that higher serum retinol levels are correlated to higher TSH levels, but that relationship is not consistent in this data set, meaning that some data points have low retinol and high TSH. The fifth point is that taking a multivitamin may be protective against HT. Lastly, retinol's involvement in the HT disease process might be from the relationship between retinol, gut health, and the autoimmune process.

To the point that serum retinol does not accurately reflect vitamin A liver reserves, a study by Olsen et al. (2018) examined the relationship between liver stores of vitamin A (VA) and serum biomarkers and found that serum retinol concentrations of patients that had hypervitaminosis A (evidenced by a total liver reserve of $\geq 1.0 \mu\text{mol VA/g}$) were not different from those who had hypovitaminosis A (evidenced by a total liver reserve of $< 0.1 \mu\text{mol VA/g}$). This University of Wisconsin-Madison team examined 27 cadavers and found a wide variety of total liver vitamin A reserves. The distribution was approximately 22% vitamin A deficient ($< 0.1 \mu\text{mol VA/g}$), 44% vitamin A adequate ($0.1\text{-}0.7 \mu\text{mol VA/g}$) and 37% vitamin A excess ($\geq 1.0 \mu\text{mol VA/g}$ with one patient between 0.7 and 1.0 $\mu\text{mol VA/g}$) (Olsen et al., 2018). The homeostatic mechanism that regulates serum retinol levels is tightly regulated to keep those levels constant across a range of liver vitamin A reserves, and therefore, they are not the best choice to assess liver vitamin A status. Serum retinol levels only correlate to liver vitamin A stores when those storage levels are deficient (Tanumihardjo et al., 2016).

The study team did find that although they did not correlate to serum retinol, total liver reserves of vitamin A did positively correlate to serum retinyl esters. Therefore, it is possible that serum retinyl esters, as a percent of total vitamin A, is a better biomarker of liver vitamin A reserves. This test has been used to evaluate vitamin A toxicity, but it has not been validated against total liver reserves in human studies (Olsen et al., 2018). Stable retinol isotope dilution (RID) can provide an accurate estimate of total liver retinol, but it is not suitable for population evaluations because it involves the administration of ^{13}C -labeled retinyl acetate and its subsequent measurement 14 to 21

days later (Tanumihardjo et al., 2016). Retinol binding protein (RBP) levels can also serve as a biomarker of vitamin A status but, although their synthesis by the liver is constant, they are negatively affected by inflammation. The relative dose-response test (RDR) and the newer modified relative dose-response test (MRDR) rely on the hepatic accumulation of apo-RBP during vitamin A inadequacy. Hepatic RBP synthesis is independent of vitamin A status, but its release from the liver is dependent on its status. When vitamin A intake is inadequate, the liver accumulates apo-RBP. The administration of a dose of vitamin A during a deficiency state leads to the administered retinol being quickly bound up by apo-RBP in the liver, which then becomes holo-RBP and causes a rise in plasma retinol levels. The RDR test involves getting blood samples before and five hours after an oral dose of retinyl esters and calculating the RDR value as a percentage based on those results. The MRDR uses a challenge dose of vitamin A₂ acetate, directly followed by a fatty snack to ensure absorption, and a single blood draw for serum retinol and 3,4-didehydroretinol (DR, the vitamin A₂ metabolite) four to seven hours later. Then, those two values are used to calculate the ratio of retinol to DR (Tanumihardjo et al., 2016). These are time-consuming and not practical for population screening either.

Physiologic methods for vitamin A status assessment include the classical dark adaptation test, which uses a cumbersome and expensive adaptometer and requires a controlled clinical setting, but the results will be abnormal in subclinical vitamin A deficiency before symptoms of night blindness occur. There is a version of the standard dark adaptation test that is easier to administer. The test is called the rapid dark

adaptation test, and it requires simple equipment but needs further validation. The rapid dark adaptation test might prove more valuable and cost-effective than serum retinol levels, which are only low when liver reserves are near depletion. Although not widely used, pupillary threshold testing is another option. It is an objective test of the dark-adapted pupillary reflex (Russell, 2000; Tanumihardjo et al., 2016).

Another factor to consider is that serum retinol levels do not reflect retinoic acid (RA) activity at the cellular and tissue level. Once retinol has entered into the cell, it can be converted into retinoic acid and activate more than 500 retinoid-responsive genes in any given cell (Bono et al., 2016; Napoli, 2012; Oliveira et al., 2018). However, the intracellular availability of the RALDH enzymes strictly regulates this conversion to active RA (Hall, Grainger, et al., 2011; Oliveira et al., 2018). Additionally, cytochrome P450 enzymes can degrade RA (Erkelens & Mebius, 2017; Oliveira et al., 2018). Therefore, the fate of retinol once it enters any given cell is subject to control mechanisms from its microenvironment, and serum retinol levels are unable to provide that information.

To address the observation that retinol status did not correlate with autoantibody levels in HT, one could refer back to the previous paragraph about cellular and tissue level regulation of RA, but there are a few things worth mentioning. Vitamin A does affect antibody production and the regulation of B-cell activity. Adequate vitamin A levels support the production of IgG, IgM, and IgA and enhance the immune response during infection, but retinol acts in the context of other cell-signaling cues, such as those of cytokines (Huang, Liu, Qi, Brand, & Zheng, 2018; Mora, Iwata, & von Andrian,

2008). Therefore, the immune system does not solely react to cellular retinol levels, but takes the whole cytokine “picture” into account. Because retinol can support antibody production, one could then argue that adequate retinol levels support autoantibody production. However, no relationship was found between retinol levels and autoantibody levels, therefore, retinol levels alone could not predict a rise or drop in autoantibodies, in the context of HT.

Additionally, antigen stimulation of immune cells through specific IgE antibodies results in a hypersensitivity response that is involved in most autoimmune diseases, and experiments have shown that *atRA* has an IgE repressive effect through action on $RAR\alpha$ with the help of IL-10. More specifically, *9-cisRA* can modulate established IgE responses by downregulating them and increasing specific IgA responses, which provide homeostasis to the immune system (Huang et al., 2018). RA can also enhance immune homeostasis and self-tolerance by enhancing the Treg and Breg response. However, the mechanism by which it regulates Breg activity is still unknown at this time (Huang et al., 2018).

Moreover, RA inhibits B-cell apoptosis, favoring their proliferation (Mora et al., 2008). In the context of HT, Liu et al. (2016) found that *atRA* treatment inhibited the effects of HT specific $\gamma\delta$ T-cells on B-cells for antibody production by inducing the apoptosis of those $\gamma\delta$ T-cells. Therefore, the influence of serum retinol on antibody and autoantibody production is complex and dependent on multiple cues that are specific to the tissue microenvironment and disease process. These cannot be ascertained by simple

measurement of serum retinol levels, as they do not reflect the HT, thyroid-specific microenvironment.

The HT group composition after randomization might also have been an issue. The criterion that was used to include patients in the HT group was the presence of either, or both, anti-thyroid peroxidase or anti-thyroglobulin antibodies, which are diagnostic for HT. Some of these patients had normal TSH levels with elevated levels of one or both thyroid autoantibodies, while others had elevated TSH levels with elevated thyroid autoantibodies. This group heterogeneity reflects the various levels of thyroid hypofunction that can be seen in HT. Clinical manifestations of HT can range from diffuse or nodular goiter with euthyroidism, or subclinical hypothyroidism (mild TSH elevation with T4 levels in the normal range), to overt hypothyroidism (TSH elevation with low thyroid hormones) (Farhangi et al., 2012; Liontiris & Mazokopakis, 2017). The current study group did not have enough participants to allow further subclassification by HT clinical manifestation and still achieve statistical significance in the analyses. Additionally, euthyroid participants seem to be overrepresented. In the future, the work could be replicated with a higher number of participants allowing for statistical analysis by HT classification.

The positive correlation between retinol and TSH appears novel and important, and needs validation with more data. Only a few participants had both a retinol value on the high end of normal and a higher than normal TSH, and one had an extremely high TSH. It is a possibility that those values drove the correlation, which is why it needs validation with a bigger study. Additionally, some patients had a retinol value on the

high end of normal and a normal TSH. Farhangi et al. (2012) found that supplementing with vitamin A downregulates TSH- β gene expression at the pituitary, lowering serum TSH levels, raising serum T3, and lowering serum T4. The Farhangi randomized, double blind, placebo-controlled study did not measure serum retinol levels pre- and post-intervention, but instead they measured all the serum thyroid markers. Their study did also not include patients with autoimmune thyroid disorders such as HT. Additionally, Aktuna et al. (1993) remarked that retinol deficiencies could be present in both hyper- and hypothyroid patients; but their study revealed that serum retinol levels were not significantly different between their hypothyroid, euthyroid and hyperthyroid patients. Taken all together, these observations suggest that there might be a novel mechanism at play here, possibly a retinol resistance mechanism similar to that of insulin resistance in the context of type II diabetes.

Graeppi-Dulac et al. (2014) examined the effects of retinoids, used as anti-cancer drugs, on the thyroid axis. They found that bexarotene (a retinoid X receptor-selective ligand, brand name Targretin) induced significant hypothyroidism through TSH- β gene suppression at the pituitary. The patient had an extremely low TSH level, with low thyroid hormones, indicating central hypothyroidism. All other pituitary hormones were within the normal range. The patient started with normal-range thyroid values before bexarotene treatment. While she received treatment, which acted as a high dose of retinoic acid at the cellular level, her TSH levels were undetectable. The Farhangi and the Graeppi-Dulac studies showed that the more retinoic acid is active in the pituitary thyrotropes, the lower the serum TSH values are. Neither studies relied on retinol levels

to make their determinations. These observations further reinforce the need to repeat this study with more participants with a greater diversity of TSH levels to confirm or refute this correlation and to find a substitute marker that is more indicative of retinoic acid activity at the cellular level.

Another interesting find was that the non-HT group had a significantly higher daily multivitamin intake than the HT group. From this, the assumption would be that this daily intake in the non-HT group would lead to higher liver reserves of vitamin A, in the form of retinyl esters, than the HT group, and this could potentially be protective against HT. The liver will store the fat-soluble vitamins, such as vitamin A, even to the point of toxicity if there is steady supplementation in addition to fortified food intake. Liver reserve levels may potentially be more indicative of the retinol availability at the cellular level throughout the body. Lower liver vitamin A reserves could mean less retinol delivered to each cell throughout the body even though the amount of retinol in transit is steady.

Vitamin D levels were not significantly different between the two groups, but it is interesting to note that both groups had vitamin D levels on the low end of the normal range of 30-100 ng/mL, with 36.04 ng/mL as the mean for the HT group and 33.27 ng/mL as the mean for the non-HT group. Because the non-HT group had a significantly higher daily multivitamin intake, one would expect that their mean vitamin D levels would be higher but instead, they are lower. Multivitamin intake is not the only factor that influences serum vitamin D levels; sun exposure is another significant factor.

Although most multivitamins are formulated to meet the RDA of 600 IU/day for adults,

correlating dietary and supplement intake with serum levels is problematic because it cannot account for the sun exposure factor (NIH Office of Dietary Supplements, 2019).

The non-HT group had lower TSH levels than the HT group (although not significantly), which correlates nicely with the findings from the Farhangi and the Graeppi-Dulac studies, indicating that higher levels of vitamin A (or more RXR stimulation) correlate with lower levels of serum TSH. The fact that both groups had similar retinol levels speaks to the fact that serum retinol is under tight homeostatic control and that it takes extreme liver depletion of stored vitamin A to lower it. Various foods in the U.S. food supply chain are fortified with vitamin A (Tanumihardjo et al., 2016), making vitamin A deficiency with low retinol levels an unlikely finding.

The HT group demonstrated significantly higher levels of dairy antibodies and marginally higher, but not significantly so, levels of gluten antibodies than the non-HT group. The notion that all diseases begin in the gut supports that observation. Celiac disease (CD), or gluten-sensitive enteropathy, is a permanent dietary intolerance to gluten leading to permanent mucosal damage in the proximal small bowel. Lontiris and Mazokopakis (2017) suggest screening all autoimmune thyroid disease patients for CD due to their association, which is evidenced by the deficiency of critical elements such as selenium and iodine due to malabsorption, or due to antibodies that affect both target tissues. They further suggest that HT patients, with or without CD, can benefit from a gluten-free diet. Ch'ng, Jones, and Kingham (2007), who note an increased prevalence of CD in patients with autoimmune thyroid disease, type I diabetes mellitus, autoimmune liver disease, and inflammatory bowel disease further support these ideas (Ch'ng, Jones,

& Kingham, 2007). The pathogenesis that links HT and CD is unknown, but both conditions share similar HLA haplotypes and are associated with the gene encoding cytotoxic T-lymphocyte-associated antigen-4. Celiac patients also exhibit higher levels of thyroglobulin and thyroid peroxidase antibodies and show a higher incidence of hypothyroidism and autoimmune thyroid disease with euthyroidism. Additionally, they note that symptomatic celiac patients might only be the tip of the iceberg and that most cases in adult life have milder symptoms and atypical features (Ch'ng et al., 2007).

Ongoing inflammation has been supposed to contribute to the development of both diseases (HT and CD). Both exhibit an increase in Th1 cells with secretion of IL-18 and INF- γ . The presence of IL-18 within the epithelium correlates with enterocyte damage, and INF- γ is a critical factor in gut permeability alteration and inflammation. IL-18 is an essential inducer of INF- γ , and it increases after gluten intake in CD. IL-18 and INF- γ are also implicated in the thyrocyte destruction of HT (Mormile, 2016). Additionally, Santaguida et al. (2018) found that the association of HT and CD was characterized by an increase in the number of Bregs that are possibly defective, and a reduction in Breg memory subsets (Santaguida et al., 2018).

Asik et al. (2014) evaluated the effect of dairy restriction in HT patients with lactose intolerance on serum TSH levels and found that avoidance of dairy products in those patients significantly decreased TSH levels. One of the factors in consideration was that all the patients enrolled in the study were taking levothyroxine (supplemental T4). The HT patients with lactose intolerance had mildly elevated TSH at baseline, and all patients were on a steady dose of levothyroxine. Avoiding all dairy products for eight

weeks significantly decreased TSH levels in the lactose intolerant HT patients, while the TSH levels in the HT patients without lactose intolerance, who avoided dairy products in the morning, remained unchanged. They supposed that the ingestion of dairy in dairy-sensitive HT patients was causing intestinal villus injury and subsequent malabsorption, which was interfering with the absorption of the levothyroxine medication (Asik et al., 2014). The connection between gastrointestinal inflammation and nutrient malabsorption, present in both CD and lactose intolerant patients and autoimmunity, such as HT, could explain the significantly higher levels of dairy antibodies and the higher gluten antibodies found in the HT group. C-reactive protein (CRP) levels would have been helpful in further confirming the increased inflammation involved in this relationship; however, the database used in this study did not contain CRP levels.

Significance of the Study

Considering the finding of no significant difference in retinol levels between the HT and non-HT groups, and the review of the literature, which indicates a lack of fluctuation in serum retinol levels despite vast differences in hepatic vitamin A storage levels, testing serum retinol levels is currently not recommended for HT patients, as it provides little clinical value. It appears that most of the studies which assessed the clinical response to vitamin A supplementation in various pathological conditions did not assess retinol levels pre- and post-intervention, but instead focused on markers of the effects of such supplementation, such as TSH levels. The observation indicates that if clinical value is provided to HT patients from vitamin A supplementation, markers other than serum retinol will have to be used to assess the effectiveness of such an intervention

(Rubin, Ross, Stephensen, Bohn, & Tanumihardjo, 2017). This study was not an interventional one; therefore, the results of this study cannot either support or reject the recommendation to use vitamin A supplementation in HT. However, the finding of higher multivitamin use in the non-HT group does potentially support the simple recommendation of a daily multivitamin for HT patients. Moreover, this finding probably indicates that there are some, potentially unknown/missed factors that may control or stabilize immune and/or thyroid systems, worthy of further studies.

Because retinol does have immune-modulating effects, it could still be involved in the pathogenesis of HT, but it could also be an innocent bystander in a similar manner as cholesterol is in cardiovascular disease. Retinol, and its active metabolite retinoic acid, are indeed signaling molecules that modulate more than 500 different retinoid-responsive genes, but their effects are modulated locally and act in conjunction with other signaling molecules so that, for example, the immune system can use retinoic acid to increase the effectiveness and stability of the immune response during infection but also to promote immune tolerance and downregulate the immune response once infection resolves (Bono et al., 2016). Another point worth noting is that Rubin et al. (2017) found that acute inflammation can induce a temporary drop in serum retinol levels due to the immediate decrease in RBP and transthyretin during that process, even before CRP levels rise. Serum retinol and RBP concentrations inversely correlate to serum IL-6 levels, which is the principal regulator of the acute phase response because it induces the expression of many acute-phase protein genes. Once the infection resolves, the serum retinol levels return to normal. The rapid, subsequent return to normal suggests that during the acute

phase reaction, retinol redistributes to other body compartments, from which it can re-enter blood circulation when the acute phase process is complete. A study using labeled retinol indicated that the body compartment in which retinol sequesters is the liver and that there is no appreciable irreversible loss of retinol (Rubin et al., 2017). The temporary drop in serum retinol indicates that, in clinical practice, the finding of low serum retinol levels should be interpreted in conjunction with indicators of infection and acute inflammation such as CRP. Unfortunately, no CRP levels were available in the database used for this study, and thus, no statistical analyses could be done to elucidate the inflammatory status of each group and its relationship to serum retinol levels in the context of HT. The work should be repeated using a new collection of data that includes CRP or high sensitivity CRP levels.

It is possible that the correlation that was found between TSH and retinol depicts some kind of cellular resistance mechanism. The rise in retinol, which parallels the rise in TSH, could potentially be an attempt to downregulate the immune response and restore homeostasis but the signals sent by retinol are not being acted on. This could indicate that the retinol-signaling pathway is disrupted in HT, or in a subset of patients with HT. This correlation warrants a deeper investigation, as it may reveal some yet unknown cellular mechanisms in the development of HT.

Limitations

The limitations of this study were that serum retinol levels don't reflect the hepatic vitamin A reserve, the non-HT group was potentially not reflective of an average

healthy population, there were not enough overt hypothyroid HT patients in the HT group, it used a small sample size, it was composed of all female participants, and it reflects a limited geographical area. The first internal validity problem was that serum retinol levels are not indicative of hepatic vitamin A reserves, which is the gold standard for vitamin A status. However, assessing hepatic vitamin A reserves requires a liver biopsy, which is an invasive procedure and not suitable for population testing. The second internal validity problem was that the independent t-test assumes a normal distribution, and the serum retinol measurements were unlikely to have been produced by a normal distribution, violating the normality assumption.

The first external validity problem was that the non-HT group was potentially not reflective of a healthy control group. The entire dataset used the clinic patients' lab values and chart data for both groups. Patients come to this clinic because they have chronic health issues. Therefore, although the non-HT group did not have Hashimoto's thyroiditis, they could have had another chronic inflammatory condition. The lack of CRP values, as a marker of inflammation, in the dataset makes it impossible to account for this confounding factor. The second external validity problem was that there were not enough overtly hypothyroid HT patients in the HT group. The lack of overtly hypothyroid participants could cause the HT group not to be genuinely reflective of the fully developed condition and could have confounded some of the statistical measurements, especially those of TSH levels and anti-thyroid antibodies. The third external validity problem was the small sample size. Although it was sufficient to achieve statistical significance, the size of the original dataset limited the sample size.

The last two issues were that the dataset was composed of all female patients, which were included in the original dataset because they have more thyroid issues than males, and that all patient data came from a single clinic that serves a limited geographical area in rural Arkansas.

These issues limit any broad external validity claims. The study should be repeated with more patients, of both sexes, and ideally over a broader geographical area or in a different geographical area. The broader geographical coverage and the higher sample size would also allow for the HT group to have more overt hypothyroid HT patients, which would more accurately reflect the pathology of the disease. It could also be repeated with only overt hypothyroid patients in the HT group. Finding an actual healthy control, non-HT, group may still prove problematic because physicians rarely order serum retinol levels in healthy people. Including CRP measurements in a new study would also allow some control over this confounding factor. Lastly, finding a more accurate but non-invasive marker of liver vitamin A status is essential for accurate measurement of vitamin A status in a repeat study.

Delimitations

Hashimoto's thyroiditis was selected as the disease to investigate in this study because it is a common autoimmune disease, and there were enough patients in the dataset that had positive thyroid antibodies to achieve statistical significance. Research teams all over the world are investigating vitamin A for its potential therapeutic benefits in various autoimmune diseases. Most of the published work uses the mouse model for

Multiple Sclerosis (MS), but some of the research with MS does include human interventional studies. There is very little research involving HT and vitamin A (or retinol). With HT being one of the most common autoimmune diseases, vitamin A affecting both the thyroid and immune systems, it is only logical to investigate any connections between these and add to the existing body of research. It also seemed logical first to establish whether a deficiency of vitamin A existed in HT patients, albeit the marker chosen was inadequate to reveal that information.

This study was designed as a retrospective case-controlled study using an existing dataset from a previous study because IRB approval was not granted for an original study, which would have established a brand-new dataset. Using the already established dataset limited the variables that were available for analysis and limited the study population to non-pregnant females aged 18 to 65 years. All the patients in the dataset had all the usual thyroid function tests on record, including the independent variable, TSH, but some did not have thyroid antibodies on record. The dataset contained enough patients with serum retinol levels to be able to apply selection criteria and get two groups of 26 participants each.

The first selection criterion was that all participants must have retinol levels on record because retinol was the dependent variable. Then any participants that had either or both TPO and TG antibodies on record were selected into the HT group because these are diagnostic for HT. Participants that were on thyroid replacement medications were removed from the HT group because these can increase the conversion of β -carotene to retinol and thus might be a confounding factor. For the non-HT group, participants had

to have a normal TSH and no thyroid antibodies, because any thyroid abnormalities would be confounders in the statistical analysis. From those participants, anyone with a diagnosed autoimmune disorder was eliminated, due to the immune abnormalities that are common to many autoimmune diseases, and which could also act as confounders. Finally, randomization was used in each group of participants to eliminate selection bias and allow for an equal number of participants in each group.

Each group's characteristics were analyzed to uncover any unexpected differences. Gluten and dairy antibodies were included due to the connection between gut health and autoimmunity. All the available thyroid lab values were compared to evaluate group heterogeneity. Vitamin D analysis was also included on the basis that it is a fat-soluble vitamin that can modulate the immune response and attenuate the symptoms of certain autoimmune diseases, as well as modify thyroid function. Those characteristics make it a sort of cousin to vitamin A, and therefore, its group characterization seemed essential. Lastly, multivitamin use was included in the group characterization due to its potential impact on nutrient status differences between the groups.

Conclusion

This study did not find a difference in serum retinol levels between patients with HT and patients without HT. Although the hypothesis was that the serum retinol levels would be lower in HT patients due to the autoimmune process, the data did not support this hypothesis. The additional hypothesis that, in HT, cellular sensitivity to retinol is reduced while serum retinol levels remain unchanged remains to be explored, although

our finding of positive correlation of retinol levels with higher TSH fits well with this idea and may even support it. This study did reveal a moderate positive correlation between retinol and TSH levels in the entire sample, with high TSH levels correlating to higher (but still within normal range) retinol levels. Additionally, the examination of the HT group for a correlation between retinol and thyroid antibodies levels found no correlations between retinol and either thyroid antibody levels. This study also revealed that the HT group demonstrated higher levels of gluten antibodies (but not significantly) and significantly higher levels of dairy antibodies and the non-HT group had significantly more participants that took a multivitamin.

This study did find a few points to consider when conducting human research on retinol. The first point to consider is that this study revealed an important new aspect, namely a relationship between retinol and TSH. It is possible that under normal physiological conditions, an increase in retinol causes a decrease in TSH, but in HT, when TSH increases because of the disease mechanism, it may cause an increase in retinol as a compensation for a possible reduced cellular sensitivity to retinol uptake or a less effective retinol to retinoic acid metabolism pathway.

The second point is that a minimally invasive, convenient marker of actual liver vitamin A reserves is needed to assess vitamin A status. The percentage of retinyl esters in total circulating vitamin A may be a promising candidate, but it would need validation as a tool to assess vitamin A deficiency. Currently, this test is only used and validated to assess hypervitaminosis A. Although, one study reported the normal percentage of retinyl esters in fasting blood plasma was reported to be 11% of the total circulating

vitamin A in people aged 19-59 years and 13% in people older than 60 years old (Tanumihardjo et al., 2016). Additionally, the use of the rapid dark adaptation test is a possible candidate for the assessment of vitamin A deficiency, but it needs evaluation as an efficient in-office tool to differentiate patients that have liver vitamin A reserves low enough to cause the first symptoms of night blindness.

The third point to consider is that several of the interventional vitamin A studies reviewed did not use serum retinol levels pre- and post-intervention. They instead used other markers of disease activity, specifically thyroid function or immune function, but omitted to establish low vitamin A status. Therefore, it is still possible that VA plays an important role, and a vitamin A supplementation may affect the markers of Hashimoto's thyroiditis. This idea could be validated with an interventional study. Lastly, the study uncovered a significantly lower multivitamin use in the HT group. This finding strongly indicates that there are some, potentially unknown or at least missed, molecular factors that may control or stabilize the immune and/or thyroid systems, which warrants further investigations.

Future Research

One avenue of future research is investigating alternate, minimally invasive ways to assess liver vitamin A reserves or finding a marker that reflects the cellular activity of retinol or retinoic acid. One suggestion would be to study the rapid dark adaptation screening technique in Hashimoto's thyroiditis, and other autoimmune diseases, to identify patients that are low in vitamin A. The researcher could then subsequently

implement supplementation and reevaluate both dark adaptation and disease activity assessment. Another suggestion would be to use the SpectraCell micronutrient test from SpectraCell Laboratories to assess functional vitamin A status in the context of HT. This test uses white blood cells to measure functional deficiencies at the cellular level (SpectraCell Laboratories, 2020).

The second avenue of future research is to design a study based on the observation that several of the reviewed interventional vitamin A studies did not use serum retinol levels pre- and post-intervention. They instead used other markers of disease activity specifically thyroid function or immune function but omitted to establish low vitamin A status. Therefore, it would be possible to design a study that evaluated the effect of vitamin A supplementation on the markers of Hashimoto's thyroiditis, namely TSH levels and thyroid antibodies.

Additionally, a new study with a higher number of participants might shed some light on the issues encountered in the current study. Screening more participants, to find those with overt hypothyroidism due to HT, could allow more of them to be subcategorized from the HT group and compared to non-HT participants. Alternatively, the HT group could have a higher number of overt hypothyroid patients, likely allowing the group to represent the pathology of HT better.

Because this study revealed a relationship between retinol and TSH that seemed to be an important one, repeating this analysis with a larger sample size would confirm or refute this relationship, or may even uncover new patterns of association between retinol

and TSH in the context of HT. Having more participants with increased TSH values in the HT group would serve to confirm or refute the relationship between TSH and retinol in the context of HT. Because injections of high doses of synthetic retinol as a second-line treatment for late stage T-cell lymphoma in patients with previously normal thyroid function, are known to decrease TSH and not increase it (Graeppi-Dulac et al., 2014). The repeat study could also include the capture of CRP levels, an indicator of inflammation, or high sensitivity CRP levels, an indicator of low-grade inflammation, in the data to characterize each group better and evaluate how inflammation might affect the results. Acute inflammation is known to decrease serum retinol levels by decreasing RBP production (retinol's transport protein) in the liver (Tanumihardjo et al., 2016). It would be interesting to find out if a persistent low-grade inflammation affects serum retinol levels, to characterize the CRP levels in HT patients and to investigate any correlation between CRP/hsCRP and serum retinol in the context of HT.

It would also be interesting to design the study as a prospective case-controlled study, allowing for more control over confounders such as autoimmune disease and celiac disease status screening, dietary patterns, type and frequency of multivitamin use, and the incorporation a more representative selection for the HT and normal control groups.

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