THYROID IMMUNE RELATED ADVERSE EVENTS FOLLOWING IMMUNE CHECKPOINT INHIBITOR TREATMENT

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Declaration

I declare that this thesis is my own work and does not contain material from any other source, except where due acknowledgement has been made.

I declare that no part of this work has been submitted previously for the purpose of obtaining any other degree or diploma.

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Abbreviations

AITD	Autoimmune thyroid disease	
AJCC	American Joint Committee on Cancer	
AML	Acute myeloid leukemia	
ANA	Antinuclear antibody	
AP-1	Clathrin adaptor protein-1	
AP-2	Clathrin adaptor protein-2	
APC	Antigen presenting cell	
CD4	Cluster of differentiation 4	
CD8	Cluster of differentiation 8	
CD28	Cluster of differentiation 28	(synonymous with CTLA-4)
CD45	Lymphocyte common antigen	
CD80	Cluster of differentiation 80	(synonymous with B7-1 protein)
CD86	Cluster of differentiation 86	(synonymous with B7-2 protein)
CD279	Cluster of differentiation 279	(synonymous with PD-1)
CI	Confidence interval	
CNS	Central nervous system	
CTCAE	Common terminology criteria for adverse events	

CTLA-4	Cytotoxic T-lymphocyte associated protein-4
ECOG	Eastern Cooperative Oncology Group
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FLT3	Fms-related tyrosine kinase-3
FLT3-L	Fms-related tyrosine kinase-3 ligand
FT3	Free triiodothyronine
FT4	Free thyroxine (tetraiodothyronine)
GD	Graves' disease
GZMB	Granzyme B
gdT	Gamma delta T-cell
GWAS	Genome wide association studies
HR	Hazard ratio
HT	Hashimoto's thyroiditis
ICI	Immune checkpoint inhibitor
IDO	Indoleamine 2,3 dioxygenase

IIF	Indirect immunofluorescence
IP	Intra-peritoneal
IQR	Interquartile range
irAE	Immune related adverse event
iRECIST	Immunotherapy response evaluation criteria in solid tumours
LDH	Lactate dehydrogenase
MHC	Major histocompatibility complex
MIA	Melanoma Institute Australia
NIS	Sodium iodide symporter
NK-cell	Natural killer cell
OR	Odds ratio
OS	Overall survival
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD-1	Programmed cell death protein-1
PD-L1	Programmed death ligand-1
PD-L2	Programmed death ligand-2
PFS	Progression free survival

PRS	Polygenic risk score
RPMI	Roswell Park Memorial Institute
SD	Standard deviation
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
T3	Triiodothyronine
T4	Thyroxine (tetraiodothyronine)
TCR	T-cell receptor
TED	Thyroid eye disease
Tg	Thyroglobulin
TgAb	Thyroglobulin antibody
TPOAb	Thyroid peroxidase antibody
TR	Thyroid receptor
TSH	Thyroid stimulating hormone
TSHR	Thyroid stimulating hormone receptor

Publications and Presentations Arising from this Candidature

Publications:

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3. ***Muir CA***, Wood CCG, Clifton-Bligh RJ, Long GV, Scolyer RA, Carlino MS, Menzies AM, Tsang VHM. Association of antithyroid antibodies in checkpoint inhibitor associated thyroid immune related adverse events. *J Clin Endocrinol Metab*. 2022; 107:e1843-1849.
4. ***Muir CA***, Menzies AM, Tsang VHM, Clifton-Bligh RJ. Immune related adverse events of the thyroid: a narrative review. *Front Endocrinol*. 2022; 13:886930.
5. Cooper B, Wijewardene AA, ***Muir CA***. Medications causing thyroid dysfunction: a guide to management. *Endocrinol Today*. 2022; 11:17-20.

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3. ***Muir CA***, Munsif A, Blaker K, Feng Y, Tewari S. Antenatal thyroid function does not increase risk of gestational diabetes mellitus in a multi-ethnic pregnancy cohort. *Int J Thyroidol*. 2020; 13(1):13-18.
4. Raven LM, Guttman-Jones M, ***Muir CA***. Hyperprolactinemia and association with prolactinoma in transwomen receiving gender affirming hormone treatment. *Endocrine*. 2021; 72(2): 524-528.
5. Raven LM, ***Muir CA***. Something to chew on: an unusual case of iatrogenic hyponatraemia exacerbated by chewing gum. *Intern Med J*. 2021; 51:1748-1749.
6. Raven LM, Greenfield JR ***Muir CA***. Glucagon-like peptide-1 receptor agonist treatment with semaglutide in type 1 diabetes. *J Clin Endocrinol Metab Case Rep*. 2022; 1:1-4.
7. Raven LM, ***Muir CA***, Macdonald PS, Hayward, CS, Jabbour A, Greenfield JR. Diabetes medications following heart transplantation: a focus on novel cardioprotective therapies, a joint review from endocrinologists and cardiologists. *Acta Diabetologia*. 2022; 1-10.

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2. Raven LM, ***Muir CA***. Gender affirming hormones: how much is too much of a good thing? Endocrine Society of Australia, 63rd Annual Scientific Meeting, virtual conference, 2020.
3. Munsif A, ***Muir CA***. Osteopetrosis with a potentially novel genetic mutation. Endocrine Society of Australia, 63rd Annual Scientific Meeting, virtual conference, 2020.
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Abstract

The pivotal role of the immune system in identification and elimination of malignant non-self tissue is well recognized. However, cancer cells have shown capacity over time to develop immune resistance mechanisms which allow evasion of normal immune-related destruction. One such resistance mechanism involves upregulation of inhibitory immune checkpoints that normally promote immune tolerance and mitigate immune related damage to collateral self-tissues. Improved understanding of immune resistance mechanisms led to development of targeted immunotherapies to overcome T-cell anergy and enhance immune responses against cancer cells. Chief among these are the immune checkpoint inhibitors (ICI) which are in widespread use for an ever-increasing number of indications and cancer subtypes.

The efficacy of ICI-treatment rivals traditional anti-tumor therapies and in most cases results in significantly less drug related toxicity. However, ICI-use can result in a myriad of immune and inflammatory side effects termed immune related adverse events (irAEs). Thyroid irAEs are the most common endocrine toxicity related to ICI-treatment. In affected patients, permanent thyroid dysfunction can result, necessitating lifelong thyroid hormone replacement and long-term clinical follow-up is required. Prior to this candidature, clinical descriptions of thyroid irAEs were predominately based on small, heterogeneous patient cohorts and little was known about the underlying pathogenesis responsible for their development.

Due to the geographic location of Australia, melanoma is a prevalent cancer and occurs at a higher rate than in most other developed countries. Melanoma was the first cancer type to be treated using ICIs, which remain standard of care as adjuvant treatment for patients with stage II-IV melanoma

as well as for patients with metastatic disease. In New South Wales, treatment of patients with melanoma is largely centralized via Melanoma Institute Australia (MIA). Through MIA, there was access to clinical data and blood samples for a large number of ICI-treated patients. This provided a unique opportunity to study thyroid irAEs on a large scale which had not previously been possible at smaller volume centres in other countries or across Australia.

To enhance the understanding of thyroid irAEs, this thesis firstly produced a detailed phenotypic report of 1246 patients with melanoma undergoing ICI-treatment. We clearly showed that prevalence of thyroid irAEs was substantially higher than previously reported, and that most patients developed subclinical disease only. We demonstrated that overt thyrotoxicosis had distinct clinical features from other thyroid irAE presentations and was uniquely associated with improvements in progression free and overall survival. We then prospectively measured anti-thyroid antibodies, TSH receptor antibodies and interleukin-6 (IL-6) levels in a subset of 122 patients from this cohort, showing that thyroid peroxidase and thyroglobulin antibodies were highly specific for identifying patients likely to experience a thyroid irAE. We also showed clearly that TSH-receptor antibodies are not involved in the pathogenesis and that IL-6 levels had no association with thyroid irAEs. Our antibody work was complemented by preclinical studies, and development of an animal model to test for additional autoantibodies against novel (non-TPOAb, non-TgAb) thyroid antigens. We performed a genetic study to test for an association of a premature stop mutation in FLT3 gene, which is strongly associated with autoimmune thyroid disease, with susceptibility to thyroid irAEs. Due to low allele frequency, we lacked power to show a clear association, although interestingly both patients homozygous for the rs76428106-C polymorphism developed a thyroid irAE. Finally, we immunophenotyped peripheral blood in patients with

thyroid irAEs to identify key immune cell populations involved in the susceptibility to and pathogenesis of thyroid irAEs.

In totality, the work reported in this thesis significantly advances understanding of thyroid irAEs and the mechanisms underpinning their development. We have shown that thyroid irAEs share similarities with autoimmune thyroiditis, but also unique differences which characterize ICI-associated thyroid disease as a distinct and novel entity within the broader classification of autoimmune thyroid disorders.

Chapter 1

Background and review of the literature

1.1 Physiology of adaptive immunity and T-lymphocyte responses

The immune system is designed to recognize and react to an enormous number of microbial and other non-self antigens. Simultaneously, there is immune unresponsiveness to self-derived antigens. These principles encompass the basic concepts of adaptive immune activation and immunological tolerance. Adaptive immune responses are induced in response to antigen exposure and changes in the cellular environment that evade control by innate immune defense mechanisms. Cellular (T-lymphocyte mediated) immunity is a key component of adaptive immunity and involves a complex set of developmental steps to direct the immune systems response to a massive array of antigenic stimuli.

1.1.1 T-lymphocyte development and survival

T-lymphocyte progenitors are derived from multipotent haemopoietic stem cells in the bone marrow (1). T-cell precursors migrate to the thymus where they are exposed to a plethora of environmental factors which commit them to the T-cell lineage (1). In the thymus, immature T-cells interact with self peptides presented by major histocompatibility complex (MHC) molecules within the thymic stroma (1). Cells which recognize MHC molecules and self peptide are positively selected and will ultimately mature into CD4+ (MHC class II presented peptides) or CD8+ (MHC class I presented peptides) T-cells (1). In this manner, T-cells become functionally segregated, and following activation are destined to become CD4+ helper T-cells or CD8+ cytotoxic T-lymphocytes (Figure 1.1) (1). Lymphocyte precursors which react

too strongly to MHC presented peptides are deleted in a process known as negative selection (1). Positive and negative selection of T-lymphocytes in the thymus ensure that the mature T-cell repertoire is both MHC-restricted and tolerant to MHC-presented self peptides.

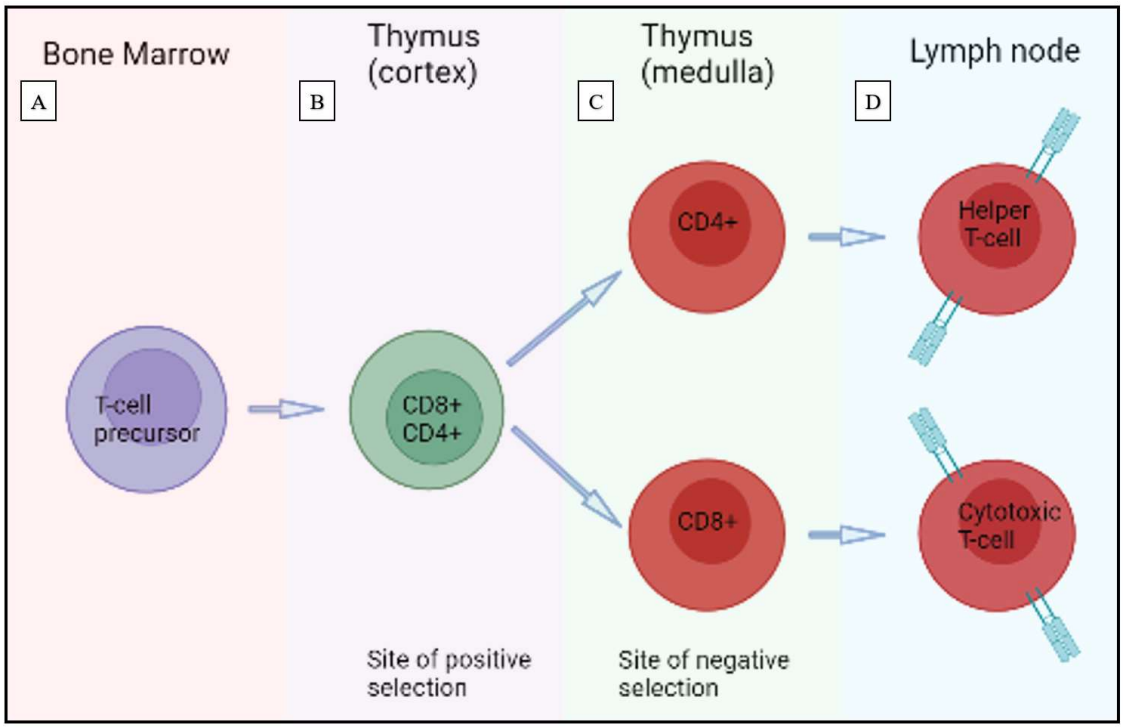

Figure 1.1: T-cell maturation

T-lymphocytes originate from hematopoietic stem cells in the bone marrow. Following differentiation into common lymphocyte progenitor cells, future T-cells migrate to the thymus, where they undergo both positive and negative selection prior to terminal differentiation into subsets of T-lymphocytes controlling distinct and specific functions within the host immune response. Figure prepared using Biorender.com.

Following thymic development, naïve T-cells travel via the blood stream to peripheral lymphoid tissues, where a multitude of non-self antigens are presented via MHC proteins expressed on the cell surface of antigen presenting cells (APC) (1). Naïve T-lymphocytes are maintained by numerous cytokines and chemokines (1). They continue to circulate through peripheral lymphoid organs in search of APCs expressing specific MHC-associated peptide antigens, which when encountered result in T-cell proliferation and terminal differentiation (1).

1.1.2 T-cell mediated immunity

Exposure to cell-specific antigen terminates recirculation of naïve T-lymphocytes and initiates differentiation and proliferation into highly efficient effector T-cells (1). Effector T-cells can bind the MHC-antigen complex with higher affinity than naïve T-cells and are functionally active on first encounter, as they do not require further differentiation to maximally engage peptide antigen (1).

Effector T-cells recognize antigen via the T-cell receptor (TCR) with CD4 or CD8 as important coreceptors to assist the TCR complex to convey the initial activating signal (1). Full activation requires a costimulatory second signal, best known of which are B7 proteins (B7-1 and B7-2) expressed on the APC surface (1). B7 proteins interact with the cluster of differentiation-28 (CD28) receptor expressed on most T-cells (1). CD28-B7-protein binding enhances TCR signaling and is essential for full T-cell activation (1). Failure of activation without co-stimulation ensures harmless foreign substances and self tissue do not trigger T-cell responses, as they cannot induce B7 protein expression on the surface of APCs (Figure 1.2) (1).

Inhibitory signals are also present and are critical to terminate immune responses and prevent uncontrolled and unlimited T-cell activation (1). In large part this is accomplished via the action of immune checkpoints, which are discussed further in the following sections.

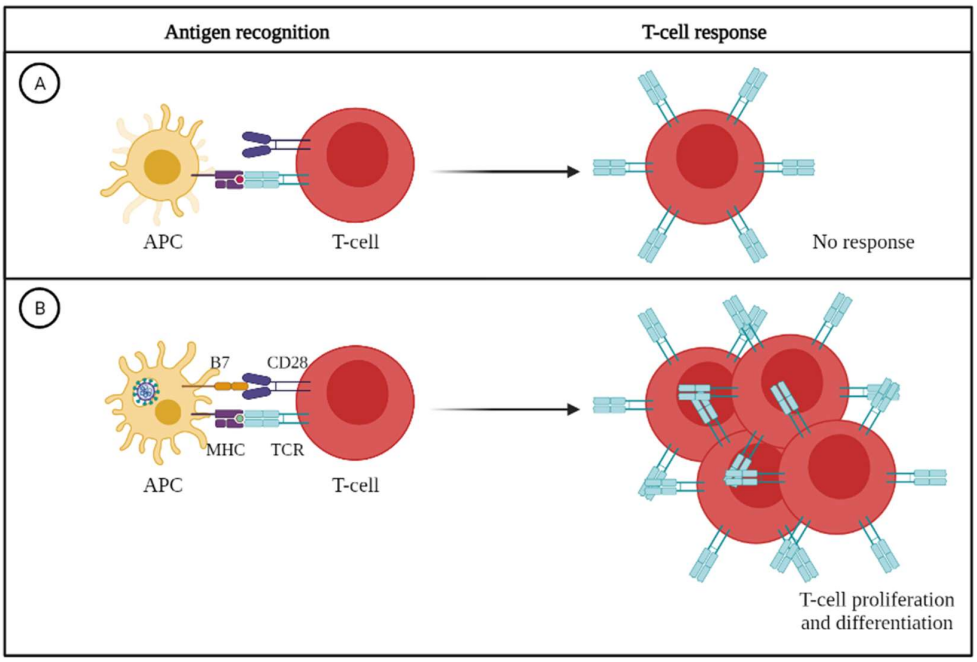

Figure 1.2: T-cell activation

T-cell activation requires two co-stimulatory signals. Firstly, non-self (i.e. tumoral) antigen is presented via MHC molecules on the APC and recognized by the TCR. (A) In the absence of a co-stimulatory signal, T-cell activation does not occur, and T-lymphocytes remain in the resting state. (B) Following CD28 binding to B7 proteins (CD80/CD86) on the APC, a co-stimulatory second signal leads to T-cell activation, proliferation, and terminal differentiation.

***Abbreviations*:** APC, antigen presenting cell; MHC, major histocompatibility complex; TCR, T-cell receptor. Figure prepared using Biorender.com.

1.2 Immune checkpoints

The duration and amplitude of immune responses are continually regulated via the integration of multiple competing stimulatory and inhibitory signals. Cytotoxic T-lymphocyte associated protein-4 (CTLA-4) and programmed cell death protein-1 (PD-1) are important inhibitory immune checkpoints expressed by T-cells which interfere with CD28 binding to B7-family ligands on the APC. The mechanism by which CTLA-4 and PD-1 regulate T-cell responses is shown in Figure 1.3.

1.2.1 Cytotoxic T-lymphocyte associated protein-4

CTLA-4, also known as cluster of differentiation 152 (CD152), is an inhibitory immune checkpoint expressed by both CD4+ and CD8+ T-lymphocytes (2). CTLA-4 along with CD28 and inducible T-cell co-stimulator (ICOS), express varying degrees of structural homology and comprise receptors within the CD28 gene superfamily (3). These receptors share a common protein structure with singular extracellular and transmembrane domains and cytoplasmic components incorporating one or more tyrosine signaling motifs (3). The structural configuration of the CD28 family of receptors is complementary to B7 proteins expressed by APCs, which act as the main receptor ligands (3).

1.2.1.1 CTLA-4 gene structure and function

The gene structure of CTLA-4 is highly conserved across mammalian species and contains four exons (4). During gene transcription, CLTA-4 can undergo alternative splicing, with four splice variants previously described (3, 4). Full length CTLA-4 mRNA contains all four exons, and the resultant protein contains a transmembrane region, cytoplasmic tail, and extracellular domain capable of binding B7 proteins (3).

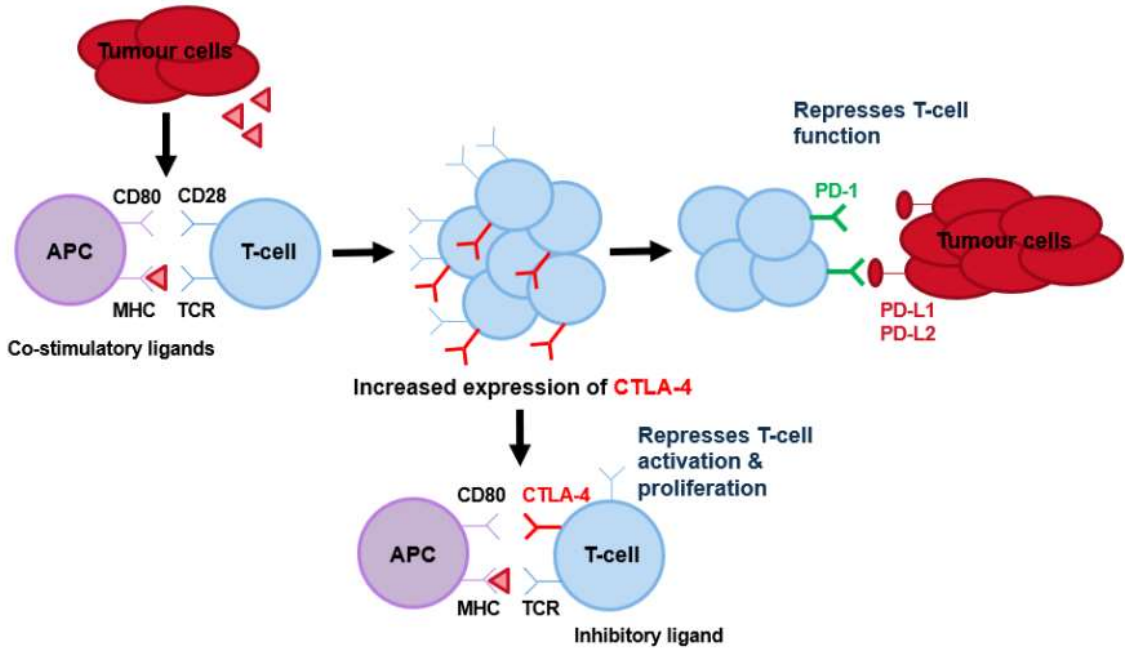

Figure 1.3: Immune checkpoints

Following T-cell activation, CTLA-4 expression is upregulated on the T-cell surface. Due to the higher affinity of CTLA-4 for B7 proteins, CD28 is displaced. By removing the costimulatory signal, further T-cell activation and proliferation is repressed. In the tumor microenvironment, cancer cells often express PD-ligands which interact with PD-1 to repress T-cell responses against tumor antigens.

***Abbreviations*:** APC, antigen presenting cell; CTLA-4, cytotoxic T-lymphocyte associated protein-4; MHC, major histocompatibility complex; PD-1, programmed cell death protein-1; PD-L1, programmed cell death ligand-1; PD-L2, programmed cell death ligand-2; TCR, T-cell receptor

In contrast, transcripts of CTLA-4 that do not contain all four exons lack transmembrane and ligand binding domains needed to interact with B7 proteins (3, 4). Full length CTLA-4 is rapidly upregulated following T-cell activation, whereas splice variants are the predominant form in resting T-cells (5). Soluble (splice variant) forms of CTLA-4 potentially inhibit regulatory T-cell (T_{REG}) function, which have been implicated in multiple autoimmune endocrine disorders (3, 6).

1.2.1.2 CTLA-4 polymorphisms

CTLA-4 polymorphisms have been implicated in the pathogenesis of human autoimmune diseases (7). In animal models, biallelic knockout of CTLA-4 in mice results in fatal multi-organ auto-inflammatory reactions within two-four weeks after birth (8). Heterozygous mutations resulting in reduced levels of CTLA-4 mRNA and protein have been described in humans (9, 10). Affected individuals develop severe immune dysregulation characterized by hypergammaglobulinemia, recurrent infection and multisystem autoimmunity, supporting the pivotal role of CTLA-4 in maintenance of immune self-tolerance (9, 10).

1.2.1.3 Regulation of CTLA-4 expression

CTLA-4 expression is upregulated following activation of CD4+ and CD8+ T-lymphocytes. CTLA-4 is also constitutively expressed by T_{REG} cells and has been documented on B-cells, dendritic cells, monocytes, granulocytes and other immune cells where its function remains less clear (3).

Regulation of CTLA-4 expression occurs via transcriptional and post-transcriptional processes (3). Post-transcriptionally, localization of CTLA-4 within T-cells is regulated and depends on the phosphorylation status of the cytoplasmic domain (3). In

resting (inactivated) T-cells, minimal CTLA-4 is present on the cell surface and is largely compartmentalized within intracellular Golgi vesicles, lysosomes, and endosomes. Although CTLA-4 is continuously trafficked to the cell surface, it is rapidly internalized by clathrin adaptor proteins AP-1 and AP-2 (11). CTLA-4 internalization is regulated through the phosphorylation status of the cytoplasmic domain. CTLA-4 is bound and internalized by AP-2 in the unphosphorylated state (12). However, phosphorylation of the CTLA-4 cytoplasmic domain occurs following T-cell activation, which inhibits internalization and promotes surface expression and localization of CTLA-4 to the immune synapse (3). Surface expression of CTLA-4 is proportional to the intensity of TCR stimulation (3). Through this mechanism and others, diversity in T-cell responsiveness can be maintained via CTLA-4 induced negative feedback to limit the amplitude and duration of immune activation (3).

1.2.1.4 CTLA-4 function

The predominant role of CTLA-4 occurs via its potent inhibitory effects on T-cell proliferation, activation, and proinflammatory cytokine production (3). However, CTLA-4 participates in other facets of the immune response via regulation of T-cell effector function and constitutive expression in T_{REG} cells (3). The importance of CTLA-4 in T_{REG} function has been illustrated in selective knockout models, wherein mice with deletion of CTLA-4 limited to T_{REG} cells display a multi-system lymphoproliferative phenotype like that observed in non-selective CTLA-4 knockout mice (13).

Relative to CD28, CTLA-4 has a greater affinity for B7 molecules expressed on the APC surface (14). CTLA-4 engages with B7 proteins in a multivalent orientation that

enhances the inhibitory function relative to CD28, which engages only a single B7 molecule (14). By binding multiple B7 molecules simultaneously, CTLA-4 promotes high avidity clustering of B7 which enhances its affinity (14). CTLA-4 expression also regulates T-cell responses via multiple cell-extrinsic mechanisms. For instance, CTLA-4 may reduce CD80/CD86 expression on APCs and can deplete tryptophan, an amino acid critical for T-cell activation, via induction of indoleamine 2,3 dioxygenase (IDO) from dendritic cells (15).

1.2.2 Programmed cell death protein-1

Programmed cell death protein-1 (PD-1), also known as cluster of differentiation 279 (CD279), binds and acts via its receptors programmed death ligand-1 (PD-L1) and programmed death ligand-2 (PD-L2) (16). The primary function of PD-1 is to regulate T-cell responses at peripheral tissues to maintain tolerance and minimize host tissue damage in the setting of T-cell activation (17).

1.2.2.1 PD-1 gene structure and function

The human PD-1 gene (PDCD1) contains five exons (18). Four splice variants of human PD-1 have been identified and result in a soluble PD-1 protein like that observed in splice variants of CTLA-4 (19). The functional significance of most splice variants remains unclear, although the splice variant lacking exon 3 has been associated with several autoimmune diseases (3). Soluble PD-1 can also be detected in multiple inflammatory and autoimmune diseases, such as rheumatoid arthritis where soluble PD-1 levels are increased in patient synovial fluid and sera, and may act as a biomarker for disease severity (20).

The PD-L1 and PD-L2 genes are structurally similar and co-located in the genome, separated by only 42 kb pairs (3). Splice variants of PD-L1 and PD-L2 have been reported, but functional implications of such variants are currently not well characterized (3).

1.2.2.2 PD-1 polymorphisms

Autoimmunity and cancer susceptibility have been associated with polymorphisms in the PDCD1 gene encoding PD-1 (3). In animal models, PDCD1 knockout promotes development of autoimmune dilated cardiomyopathy and systemic lupus erythematosus in BALB/c mice and aged C57BL/6 mice respectively (21, 22). Select PDCD1 single nucleotide polymorphisms (SNP) in humans have been associated with altered susceptibility to systemic lupus erythematosus, viral hepatitis B and multiple sclerosis (3).

1.2.2.3 Regulation of PD-1, PD-L1 and PD-L2 expression

PD-1 expression is inducible following TCR activation or in the setting of chronic inflammation, specifically by inflammatory cytokines such as interleukin (IL)-2, IL-7, IL-15, IL-21 and interferons (IFN) (3, 23). PD-1 expression occurs rapidly within 24 hours of T-cell activation, but is transient, with PD-1 levels declining in tandem as antigen is removed (3). Prolonged T-cell activation, such as occurs with chronic viral infection or some cancers, can promote continuous high levels of PD-1 expression and lead to a state of T-cell exhaustion (3).

PD ligands have distinct expression patterns. PD-L1 is constitutively expressed widely throughout the body, whereas PD-L2 expression is more restricted and limited to APCs during conditions of inflammation (3). Unlike CTLA-4, local expression of PD-L1

enables the PD-1 pathway to regulate immune responses in a tissue specific fashion (3). Induction of PD-L1 is predominately driven by type 1 and type 2 IFNs as well as tumor necrosis factor. Conversely, PD-L2 expression is driven mainly by IL-4 and granulocyte-monocyte colony stimulating factor (24).

1.2.2.4 PD-1 function

PD-1 is expressed in the setting of T-cell or B-cell activation and regulates the amplitude and duration of the local immune response via interaction with its ligands PD-L1 and PD-L2 (17). After binding its ligand, PD-1 attenuates further T-cell activation via a negative costimulatory effect which inhibits further TCR signaling (25). Additionally, PD-1 can induce dephosphorylation of CD28, thereby reducing CD28-mediated co-stimulation required for T-cell activation (17).

1.3 Cancer immunotherapy

The role of the immune system in cancer development and the potential for immune therapies targeting cancer have been scientifically evaluated for the better part of the last century. As early as the 1920's, it was recognized that increased lymphocytic infiltration of explanted tumors was predictive of improved survival in cancer patients (26). In 1943, immunization of mice with tumor antigen could block development of the same tumor in the immunized animal (27). A major breakthrough occurred in 1968, when for the first time it was demonstrated that lymphocytes could inhibit growth of multiple cancer types in vitro (28). These discoveries and others formed the bedrock for understanding modern tumor immunology and led to the discovery of multiple immune checkpoints which regulate immune cell function and participate in anti-tumor immune responses (29). As immune checkpoints are frequently overexpressed in the

tumor microenvironment, they represent targetable entities and monoclonal antibodies antagonizing these checkpoints, termed immune checkpoint inhibitors (ICI), have been developed.

1.3.1 Immune checkpoint inhibitors

Monoclonal antibodies have been developed which are capable of inhibiting CTLA-4, PD-1, and other critical immune checkpoints responsible for restraining the activation and proliferation of T-lymphocytes under normal physiologic conditions (Figure 1.4). These medications have shown durable anti-tumor responses in a wide range of malignancies. Currently, there are over 2000 clinical trials completed or in progress evaluating over 30 different checkpoint inhibitors, many of which are already in widespread clinical use (2).

1.3.1.1 CTLA-4 blockade

CTLA-4 was the first checkpoint receptor to be clinically targeted and demonstrate antitumor efficacy in preclinical studies (30, 31). Based on preclinical data, ipilimumab and tremelimumab, two antibodies targeting CTLA-4, were developed and initially tested in patients with metastatic melanoma (32). Ipilimumab, a fully humanized IgG1 antibody against CTLA-4, has a plasma half-life of approximately 14 days (2). Phase III trials of ipilimumab administered third-weekly for up to four doses in patients with metastatic melanoma demonstrated improvements in overall survival, paving the way for approval by the US Food and Drug Administration (FDA) in 2011 (33, 34). Tremelimumab failed to demonstrate improvements in overall survival in its pivotal phase III trial (32).

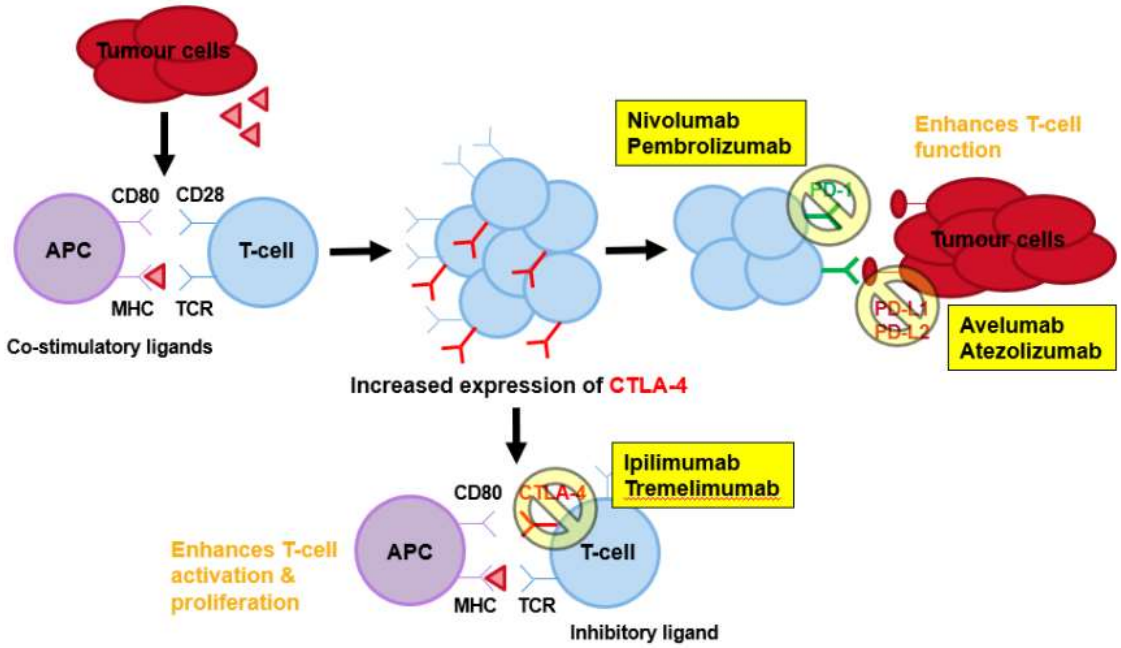

Figure 1.4: Immune checkpoint inhibitors

Monoclonal antibodies targeting specific immune checkpoints are available and in widespread clinical use as adjuvant and neoadjuvant treatment for an ever-increasing number of malignancies. At present, the most well characterized include CTLA-4 inhibitors ipilimumab and tremelimumab, PD-1 inhibitors nivolumab and pembrolizumab, and PD-L1 inhibitors avelumab and atezolizumab.

***Abbreviations*:** APC, antigen presenting cell; CTLA-4, cytotoxic T-lymphocyte associated protein-4; MHC, major histocompatibility complex; PD-1, programmed cell death protein-1; PD-L1, programmed cell death ligand-1; PD-L2, programmed cell death ligand-2; TCR, T-cell receptor

Further trials of CTLA-4 inhibitors in other cancer types such as non-small cell lung cancer, renal cell carcinoma, prostate cancer and others have failed to produce significant improvements in overall survival (32). The initial trials of ipilimumab also documented a novel class of adverse drug reactions, thought to be autoimmune in origin (33, 34). These were termed immune related adverse events (irAEs) and remain a common feature of all checkpoint inhibitor trials to date (29). Class and organ specific irAEs will be discussed further in section 1.4. As survival improvements with anti-CTLA-4 antibodies were relatively modest and associated with significant toxicity, anti-CTLA-4 monotherapy has largely been abandoned. However, CTLA-4 checkpoint inhibitors continue to be used in combination with anti-PD-1 antibodies (see 1.3.1.4) in a variety of cancers and are subject to ongoing research attempting to refine the anti-CTLA-4 effect against the tumor with less off-target toxicity (29).

1.3.1.2 PD-1 blockade

Following the clinical success of CTLA-4 inhibitors, blockade of other checkpoints became an intense focus of cancer immunotherapeutics. The PD-1 pathway has been the most extensively studied, with several agents in routine clinical use and many more undergoing further clinical investigation (17, 32). As highlighted previously, PD-1 deficient mice express a less severe autoimmune phenotype than mice deficient in CTLA-4 (8, 22). Phenotypic differences arise via mechanism of action, with CTLA-4 effect most prominent during early stages of T-cell activation, whereas PD-1 inhibits T-cell activity during the effector phase at local sites within tissues or tumors (32). This may underly the non-inferior efficacy and superior side effect profile of PD-1 and PD-L1 inhibitors relative to CTLA-4 based checkpoint inhibitors. Nivolumab, and

shortly thereafter pembrolizumab, were the first PD-1 inhibitors to undergo evaluation and shortly thereafter enter clinical practice (29).

Nivolumab, a fully humanized IgG4 antibody against PD-1, was the first PD-1 inhibitor to undergo clinical testing starting in 2006 (35). Complete or partial responses were observed in multiple cancer types, which were then advanced to large scale phase III clinical studies. The results of phase III trials led to FDA approval of nivolumab for metastatic melanoma in 2014, and for non-small cell lung cancer and renal cell carcinoma in 2015 (17). It has gone on to be approved for use in multiple other cancer types and has over 1000 trials currently registered evaluating the safety and efficacy of nivolumab in a wide range of tumors (36).

Pembrolizumab, a recombinant humanized IgG4 antibody against PD-1 was the first PD-1 inhibitor to be trialed in humans, and gained FDA approved for treatment of unresectable or metastatic melanoma in 2014 (17). It has now been approved for use in 10 different cancer types with over 1300 clinical trials completed or in progress to evaluate safety and efficacy in an expanded range of human cancers (36).

1.3.1.3 PD-L1 blockade

Increased expression of PD-L1 has been demonstrated in multiple cancer types such as melanoma, lung, and ovarian (37). Additionally, immune cells located within the tumor microenvironment frequently overexpress PD-L1 (37, 38). Preclinical research suggested benefit of PD-L1 blockade in numerous animal and cell culture models of malignancy, paving the way for human trials and approval of multiple PD-L1 inhibitors starting with atezolizumab in 2016 for urothelial carcinoma, followed by avelumab in 2017 and durvalumab in 2018 (17, 36).

PD-L1 inhibitors produce responses that are phenotypically similar to those observed with PD-1 blockade, although PD-L1 antagonism may induce additional responses via antibody dependent cellular cytotoxicity and natural killer cell responses, which are not a major component of PD-1 inhibitors (17). PD-L1 may also interact directly with CD80, such that PD-L1 inhibitors effectively block this interaction, whereas PD-1 inhibitors leave the PD-L1-CD80 inhibitory signal intact (39). PD-L1 tumor expression can be measured using immunohistochemistry and has utility as a predictive biomarker for selecting patients likely to respond to PD-1 or PD-L1 checkpoint inhibitor treatment in select malignancies (40).

1.3.1.4 Combined CTLA-4 and PD-1/PD-L1 blockade

Trials combining CTLA-4 and PD-1 checkpoint inhibitors began in 2009 (29). Improved outcomes were postulated on the assumption that anti-CTLA-4 and anti-PD-1 antibodies would act complementarily via inhibitory effects on distinct, non-redundant pathways regulating T-cell and other immune cell responses. It quickly became evident that impressive clinical responses were possible, leading to the first large phase III trial of combined ipilimumab and nivolumab versus either alone (41). Results were impressive, and combined ipilimumab plus nivolumab treatment resulted in superior long-term survival compared to nivolumab or ipilimumab monotherapy in patients with advanced melanoma (42). Survival benefits from combination treatment were also sustained during prolonged follow-up, although 55% of patients treated with ipilimumab and nivolumab experienced adverse events of grade 3 severity or higher, double the rate experienced with nivolumab monotherapy and triple that with ipilimumab monotherapy (42). Combination treatment with ipilimumab and

nivolumab was FDA approved for treatment of melanoma in 2015, and combined CTLA-4 and PD-1 blockade has since been approved for management of multiple cancers including renal cell carcinoma, non-small cell lung cancer, colorectal cancer, hepatocellular cancer and pleural mesothelioma (29). Subsequent studies have trialed lower doses of ipilimumab, or extended dosing intervals to mitigate drug related toxicity and irAEs, which remain a frequent and clinically significant complication of treatment using ICIs from both CTLA-4 and PD-1/PD-L1 classes.

1.4 Immune related adverse effects

As characterized in the previous sections, immune checkpoints act physiologically to constrain T-cell activation and promote appropriate immune responses against foreign antigens. As such, immune checkpoints are part of an intricate system which can identify and destroy non-self entities whilst minimizing host tissue damage and providing a safeguard against development of autoimmune disease. Immune checkpoint inhibitors antagonize CTLA-4 and PD-1 pathways, which strongly enhance anti-tumor immune responses, but are associated with off target inflammatory and autoimmune effects known as irAEs (43).

IrAEs are highly prevalent and effect the majority of patients treated with ICIs (44). IrAEs range in severity from mild to life threatening and are characteristically graded using the Common Terminology Criteria for Adverse Events (CTCAE) (Table 1.1). They can occur in any organ system with unique class-specific and tumor-specific differences (Figure 1.5) (43, 45, 46). IrAE onset is usually within 4 months of the first administered ICI dose, although hyperacute (within days) and delayed onset (>1-year) cases have been reported (45). Quantitatively, there is a threefold increased risk for

irAE onset during the first 4-weeks of treatment relative to the period between 4-weeks and treatment cessation (45, 47).

Table 1.1: Common Terminology Criteria for Adverse Events

Grade	Definition
1	Mild; asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated.
2	Moderate; minimal, local, or noninvasive intervention indicated; limiting age-appropriate instrumental activities of daily living.
3	Severe or medically significant; hospitalization or prolongation of hospitalization indicated; disabling; limiting self-care activities of daily living.
4	Life-threatening consequences: urgent intervention indicated
5	Death related to adverse event

1.4.1 Class-specific differences in irAE presentation with CTLA-4 checkpoint inhibitors compared to PD-1 checkpoint inhibitors

CTLA-4 and PD-1 based ICIs exhibit distinct patterns of irAEs, consistent with their unique and non-redundant targets within the immune system. Serious (CTCAE grade 3 or higher) irAEs occur more frequently with CTLA-4 inhibitors than with PD-1 or PD-L1 inhibitors (48). Following treatment with ipilimumab, 31% of irAEs were classified as CTCAE grade 3 severity or higher (48). However, following PD-1 inhibitor treatment with nivolumab or pembrolizumab, only 10% of irAEs were classified as severe (48). Combination ICI treatment with CTLA-4 and PD-1 inhibitors

increases the toxicity profile further (45). Following treatment with ipilimumab and nivolumab, 90% of patients experienced an irAE, two-thirds of which were classified CTCAE grade 3 or above (49). Organ specific irAE affinity differs between CTLA-4 and PD-1 ICIs. Following treatment with CTLA-4 inhibitors, patients are more likely to develop colitis (odds ratio [OR] 8.7) and hypophysitis (OR 6.5) (48). Following PD-1 inhibitor treatment, patients experience a different pattern of irAEs, with pneumonitis (OR 6.4), thyroiditis (OR 4.3), and arthralgias (OR 3.5) predominating (48).

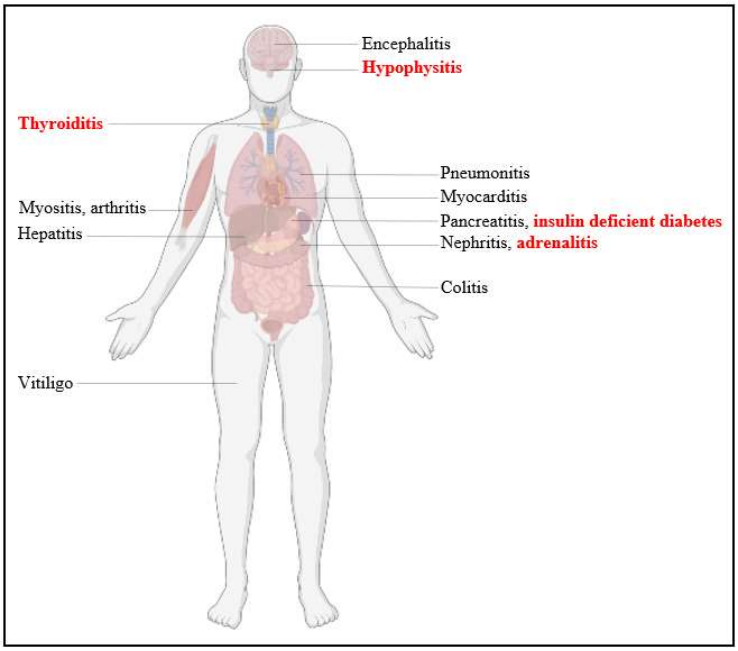

Figure 1.5: Immune related adverse events

Immune checkpoint inhibitor treatment can provoke inflammation and autoimmunity in any organ system. The most frequently encountered irAEs occur within dermatologic, endocrine (highlighted in red), and gastrointestinal systems. They can vary in severity from asymptomatic to life threatening. Figure prepared using Biorender.com.

1.4.2 Risk factors for irAE development

Definitive risk factors have not been reliably identified, although tumor-specific and patient-specific factors likely impact the probability of irAE development (50). From a tumor perspective, two studies have demonstrated clonal T-cell populations with shared TCR sequences in tumors and irAE affected organs (51, 52). In a similar vein, patients with melanoma are prone to development of vitiligo following ICI treatment, suggesting shared epitopes between melanoma cancer cells and normal melanocytes that become indistinguishable to T-cell populations activated against the tumor (53). Further mechanistic examples include pituitary CTLA-4 expression as a mediator of ICI-induced hypophysitis and analysis of intestinal microbiota has utility in identifying patients at heightened risk of ICI-induced colitis (54, 55). Patient factors have less robust evidence to support an association with irAE development (56). Male sex, renal impairment and elevated body mass index have been suggested as risk factors in small observational studies (57, 58). However, findings have not been confirmed in larger clinical trials, thus rendering the clinical significance of such demographic factors uncertain (50).

Patients with pre-existing autoimmune disorders are vulnerable to disease reactivation following ICI-treatment (59-61). Patients without a personal history, but who have a family history of autoimmune disease also appear to have increased likelihood of developing irAEs (45, 62). It is possible that many patients who develop irAEs could have subclinical pre-existing autoimmunity which is manifest following ICI treatment and resultant immune activation. This is suggested by multiple studies which have identified pre-treatment autoantibodies in patients who later developed organ-specific

irAEs (45). Examples include anti-thyroid antibodies in patients who develop thyroiditis, acetylcholine receptor antibodies in patients who develop myocarditis, anti-nuclear antibodies in patients who develop colitis, and pneumonitis in patients with anti-CD74 antibodies (63-66). Other patient-specific immune factors, such as treatment related changes in B-cell populations and pro-inflammatory cytokines may also have utility in predicting patients destined to develop ICI-related irAEs (67, 68).

1.4.3 Survival implications of irAEs

Although irAEs are an unintended and off-target effect of ICI treatment, early studies suggested patients who developed an irAE paradoxically experienced better outcomes than patients who remained unaffected (69-73). These findings were subsequently validated by analyses of large, prospective randomized clinical trials, which also found irAE development to be associated with improvements in tumor response and progression free survival (69, 74, 75). Recent meta-analyses of real-world observational data across multiple cancer types and ICIs continue to support an association between irAE development and improvements in cancer related survival (76, 77). Data also suggests that endocrine irAEs, particularly thyroid irAEs, may exert a particularly beneficial association with overall and progression free survival in cancer patients (71, 72).

1.5 Thyroid immune related adverse effects

Thyroiditis, and resultant thyroid dysfunction, is one of the most common occurring irAEs following ICI treatment (78). Despite their high prevalence, little was known about thyroid irAEs at the commencement of my doctoral studies in 2019. As such, my

thesis project was designed to fully characterize thyroid irAEs and better define their clinical and prognostic significance as an adverse effect of ICI treatment.

In the following sections of this chapter, I briefly review the anatomy and physiology of the thyroid gland, followed by a systematic review encompassing knowledge of thyroid irAEs prior to my own research. To complete my introductory chapter, I present data from a second narrative review, authored recently, which updates the original review with data we generated over the past three years as well as research from other groups that enhanced the knowledge and understanding of thyroid irAEs.

1.5.1 Anatomy of the thyroid gland

The thyroid is a butterfly shaped organ located in the anterior neck, inferior to the thyroid cartilage. It consists of two lobes connected by a central isthmus. The thyroid is highly vascular and adult weight typically ranges between 15-25 grams (79). The thyroid gland is organized into follicles, which vary in size and contain abundant colloid. Colloid acts as a bank of thyroglobulin, which is required for thyroid hormone biosynthesis. Follicles are lined by a single layer of thyroid epithelial (follicular) cells, which are highly specialized to accept iodine from the bloodstream and utilize it for thyroid hormone biosynthesis (79). Follicles are supported by thyroid parenchyma containing parafollicular c-cells and a highly vascularized capillary network (79).

1.5.2 Physiology of the thyroid gland

The thyroid is under the direct control of pituitary thyrotrope cells via the action of thyroid stimulating hormone (TSH) (79). TSH secretion is pulsatile and occurs in response to stimulation by hypothalamic thyrotrophin releasing hormone (79). Surface receptors on the thyroid gland bind TSH, which stimulates the release of thyroid

hormones thyroxine (T4) and triiodothyronine (T3) (79). Thyroid hormones exert negative feedback on the pituitary and hypothalamus to maintain T4 and T3 within a defined range (Figure 1.6). Disease states can arise which effect the thyroid, pituitary, or hypothalamic regulation of thyroid hormone synthesis and secretion. When severe and/or prolonged, dysregulation of thyroid function can precipitate states of hypothyroidism and hyperthyroidism, which then manifest as multi-system metabolic abnormalities. Thyroid irAEs arising after ICI treatment represent a discrete example of thyroid pathology and will be reviewed in detail throughout the remainder of this thesis.

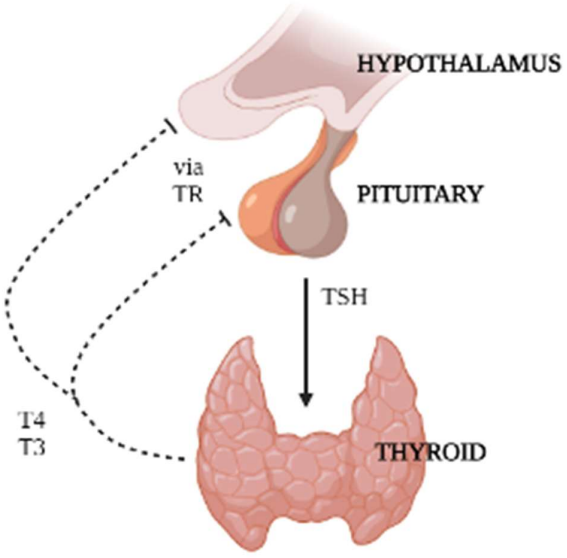

Figure 1.6: Hypothalamic-pituitary-thyroid axis

Classic endocrine negative feedback loop whereby TSH stimulates thyroid hormone synthesis and secretion, which is then monitored by the pituitary and hypothalamus to regulate TSH and maintain thyroid hormone concentrations within a controlled range.

1.5.3 Peer-reviewed systematic review of thyroid immune related adverse events.

Published in *Thyroid*® at commencement of this candidature (see Appendix 1 for print version)

Targeted cancer immunotherapy to enhance T-cell activation has revolutionized the management of cancer. ICIs are the best studied of currently available cancer immunotherapies. In multiple cancer types such as melanoma, non-small cell lung cancer, renal cell cancer and others, ICIs have become standard of care with multiple large randomized controlled trials confirming benefit in terms of tumor response, progression free and overall survival (80). Immune checkpoint antagonists primarily enhance anti-tumor immune responses via inhibition of the negative regulatory effects of CTLA-4 and PD-1 pathways, although other pathways are also under investigation (37). The CTLA-4 and PD-1 pathways are non-redundant and regulate immune activation via unique mechanisms (37). Features of different immune checkpoint inhibitors are presented in Table 1.2.

Table 1.2: Characteristics and properties of single agent immune checkpoint inhibitors

	Ipilimumab	**Nivolumab**	**Pembrolizumab**
Trade name	Yervoy	Opdivo	Keytruda
FDA approval	2011	2014	2014
Antibody target	CTLA-4	PD-1	PD-1
Antibody type	Recombinant human IgG1 kappa monoclonal antibody	Fully humanized IgG4 kappa monoclonal antibody	Recombinant human IgG4 kappa monoclonal antibody
Molecular weight	148 kDa	146 kDa	149 kDa
Adult dosing schedules	3 – 10 mg/kg q3-weekly x 4 doses	240 mg q2-weekly 480 mg q4-weekly	200 mg q3-weekly
Elimination half life	15 days	25 days	22 days

Cytotoxic T-lymphocyte associated protein-4

Immune responses by way of T-cell activation, differentiation and proliferation are dependent on co-stimulatory signals between the T-cell and the antigen presenting cell. The first signal is through recognition of antigen derived peptides presented via major histocompatibility complex proteins to T-cell receptors on CD8+ and CD4+ positive T-cells. The second signal amplifies the effect of the first, and occurs following recognition of B7 proteins (CD80, CD86) by CD28, also present on the T-cell membrane (81).

CTLA-4 is a CD28 homolog (37, 80). Both CTLA-4 and CD28 are expressed on the cell surface of CD4+ and CD8+ T-lymphocytes. They have opposite effects on T-cell activation and as such CTLA-4 competes with CD28 for binding to B7 proteins expressed by antigen presenting cells (37). Once bound, CD28 promotes T-lymphocyte activation and clonal expansion. In contrast, CTLA-4 dampens T-cell signaling and thereby suppresses immune activation (37). The affinity of CTLA-4 for B7 proteins on antigen presenting cells is greater than that of CD28 (37). As such, surface expression of CTLA-4 is inducible and increases following T-cell activation to prevent uncontrolled T-cell activity (37). The primary role of CTLA-4 appears to be maintenance of central tolerance, as evidenced by CTLA-4 knock-out mice who develop systemic immune activation and fatal multi-organ autoimmunity (8).

Programmed cell death protein-1

In comparison, PD-1 is a primary regulator of peripheral tolerance and limits the activity of effector T-cells at a tissue level (37). PD-1, unlike CTLA-4, exerts its effect via interaction with its own specific ligands PD-L1 and PD-L2 (37). Once bound, PD-

1 inhibits CD28 co-stimulatory signaling and dampens T-cell activation (78). Similar to CTLA-4, PD-1 expression is very low in resting T-lymphocytes of healthy individuals (78). Following T-cell activation, PD-1 surface expression is induced to limit uncontrolled proliferation, effector function and survival (78). In cancer, tumor cells often express PD-L1 which may directly lead to T-cell inactivation and avoidance of anti-cancer immunity (37). As might be expected, animal PD-1 knockout models result in a less severe phenotype than CTLA-4 knock-outs (21).

Immune related adverse events

The improvements in cancer outcomes observed with ICIs come with unintended off-target effects, namely new onset autoimmunity termed irAEs. Immune related complications occur in up to 80% of CTLA-4 and PD-1 treated patients (81). Anti-tumor efficacy is improved with combined CTLA-4 and PD-1 blockade compared to single agent monotherapy, but the risk of irAEs increase in tandem (81). Manifestations range from mild to life-threatening and the spectrum of irAEs differ between CTLA-4 and PD-1 inhibition (43). Immune adverse effects have been reported in multiple organ systems, with the endocrine system appearing particularly susceptible. Hypophysitis and thyroiditis are the most common endocrine manifestations, with the former more likely following anti-CTLA-4 treatment and the latter with anti-PD-1-based therapy (48). Endocrine irAEs are often irreversible, unlike irAEs that occur in other organ systems. This is particularly interesting in the setting of thyroid irAEs, as autoimmune thyroiditis normally requires years to decades of immune mediated lymphocytic infiltration before hypothyroidism results, whereby

progression to overt hypothyroidism following ICI treatment can develop within weeks to months (82).

Thyroid dysfunction is the most common irAE following ICI treatment. Description of thyroid irAEs across studies is not standardized, and the true prevalence and mechanism of this phenomenon is not well understood. Thyroiditis following ICI treatment provides a new model to study the origin and pathogenesis of autoimmune thyroid disease more generally. As such, we endeavored to assess the incidence, etiology, clinical course and other features of thyroid dysfunction developing as an adverse effect of ICI-based treatment.

Search strategy

We performed a review of published articles reporting irAEs affecting the thyroid in patients with advanced cancer who underwent ICI treatment. A search of Medline, Embase and PubMed databases identified relevant articles published prior to November 15, 2019. Additionally, we manually reviewed references of included studies for further literature of relevance. In PubMed, we searched keywords “safety” OR “side effects” OR “adverse events” AND “ipilimumab” OR “nivolumab” OR “pembrolizumab” OR “CTLA-4” OR “PD-1” with article type restricted to clinical trial, controlled clinical trial, multicenter study, observational study and randomized controlled trial. We searched the same keywords in Medline and Embase with results restricted to human and English language studies.

Selection criteria included studies that (a) investigated the use of ICIs in the treatment of cancer; (b) reported on irAEs related to thyroid function; and (c) were published in the English language. Phase 3 randomized trial data were assessed to determine the

incidence of thyroid irAEs. To further characterize thyroid irAEs, all study types (including case reports) which described specific effects of immune checkpoint blockade on thyroid function were included. Exclusion criteria included (a) animal studies; (b) review articles, systematic reviews, and meta-analyses; (c) editorials and letters to the editor; and (d) phase I/II trials without a specific focus on thyroid-related outcomes.

Study characteristics

The search strategy identified 3086 studies. Following eligibility assessment and removal of duplicate articles, 288 full text articles were selected for further assessment. Of the articles reviewed, 78 fulfilled our inclusion and exclusion criteria. We extracted two additional articles by reviewing references of studies identified through our database search. Results included 30 phase III randomized clinical trials, which were used to calculate the incidence of thyroid irAEs and 50 observational, cohort and case studies that were used to describe the clinical features of thyroid irAEs in greater detail.

Incidence

Thyroid dysfunction has been observed following treatment with both CTLA-4 and PD-1 inhibitors in multiple cancer subtypes. The mean incidence of thyroid irAEs in phase III clinical trials is 10.8%, although rates vary widely between different ICIs (Figure 1.7).

In phase III trials of ipilimumab, a CTLA-4 inhibitor, 3863 patients with melanoma or lung cancer were treated and thyroid dysfunction developed in 4.7% (range 2.0-10.4%) of exposed patients (33, 34, 83-89). Both hyperthyroidism and hypothyroidism were reported, possibly manifestations of the same disorder detected at different phases of

evolution. The incidence of thyroid irAEs did not differ between 3mg and 10mg doses of ipilimumab (84). Similarly, rates of thyroid irAEs were not significantly increased when ipilimumab was combined with dacarbazine, gp100, or platinum-based chemotherapy (33, 34, 85, 86).

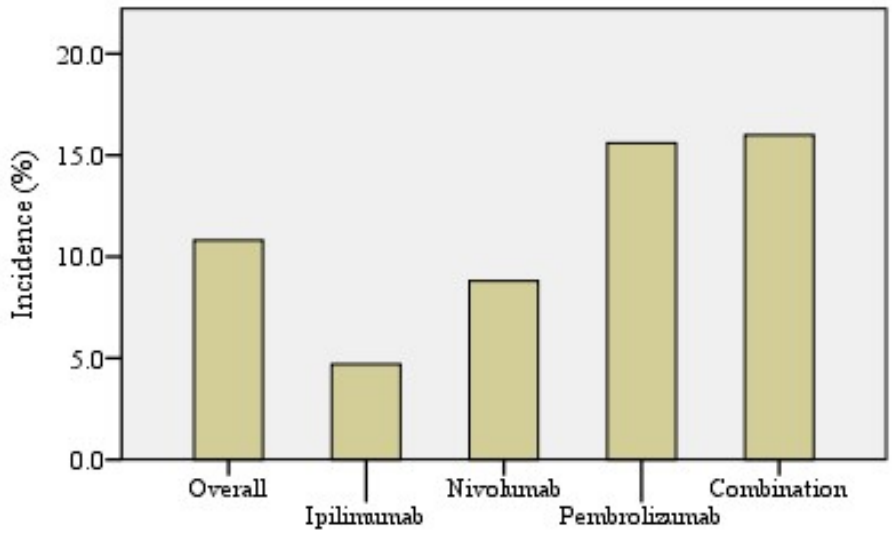

Figure 1.7: Mean incidence of thyroid irAEs in phase III randomized clinical trials of immune checkpoint inhibitors for treatment of advanced cancer

Nivolumab, a PD-1 inhibitor, has been tested for treatment of multiple cancer types, including melanoma, lung, head and neck, gastric and renal cancers. In phase III trials, 227 of 2566 (8.8%) patients treated with nivolumab developed thyroid irAEs (87, 89-96). As with ipilimumab, both hyperthyroidism and hypothyroidism were reported, and most cases were of mild severity. The prevalence of thyroid irAEs ranged from

3.7 – 15.1%, with seemingly higher rates in melanoma (11.4%) compared with other solid organ cancers (5.5%). Interestingly, in a study of melanoma patients treated with nivolumab after disease progression on ipilimumab, the incidence of thyroid irAEs was 15.1%, suggesting sequential treatment may increase risk to a similar degree as concomitant CTLA-4 and PD-1 checkpoint blockade (91).

Pembrolizumab, a second anti-PD-1 checkpoint inhibitor, has also been used successfully to treat multiple cancer subtypes. Of 4445 patients treated in phase III clinical trials, 694 (15.6%) developed a thyroid irAE (88, 97-106). This is double the incidence reported with nivolumab, despite working via inhibition of a common pathway and almost four times as frequent as with ipilimumab. The incidence of thyroid irAEs ranged from 10.6 – 27.4% and like other immune checkpoint inhibitors, the risk was not dose dependent and occurred at similar rates irrespective of the dose or dosing interval (88, 101, 102). Pembrolizumab has been used in combination with dexamethasone for the treatment of multiple myeloma in two studies (107, 108). Interestingly, the incidence of pembrolizumab-induced thyroid irAEs were lower in multiple myeloma (9.1%) then in other cancer subtypes (15.6%) (107, 108). At present it is unclear whether co-administration of dexamethasone with pembrolizumab may prevent thyroid irAEs, or if another factor may be responsible for the lower rate of irAEs observed in multiple myeloma patients.

Combination treatment with ipilimumab plus nivolumab has been trialed in melanoma, renal cell cancer and lung cancer with and without high mutational burden (89, 95, 109). Thyroid toxicity was similar to that observed with pembrolizumab treatment, but greater than with ipilimumab or nivolumab. In 1436 patients treated with combined

ipilimumab plus nivolumab, 230 (16.0%) developed a thyroid irAE. Similar to ipilimumab monotherapy, the incidence of thyroid irAEs was more common during treatment of melanoma (24.9%), then during treatment of lung cancer (11.6%) or renal cell cancer (15.5%).

PD-L1 inhibitors are another class of ICI in routine use for treatment of cancer. In phase III trials of the PD-L1 inhibitor atezolizumab, 332 of 1493 (22.2%) of patients experienced a thyroid irAE (110-113). Hypothyroidism was the reported thyroid irAE in 75% of cases, with the remainder recorded as hyperthyroidism. Durvalumab, a second PD-L1 inhibitor, was also associated with a high prevalence of thyroid irAEs, affecting 100 of 740 (13.5%) treated patients in phase III randomized clinical trials (114, 115). The ratio of hypothyroidism to hyperthyroidism with durvalumab was similar to that observed with atezolizumab.

Etiology:

The etiology of thyroid irAEs after immune checkpoint blockade remains unknown. Two case reports have been published describing histological changes observed in thyroid irAEs. The first was a cytology specimen in a patient who developed thyroiditis during combination immunotherapy with ipilimumab and nivolumab (116). Key cytologic features were lymphocytic infiltrates admixed with necrotic cells with a distinct absence of follicular cells (116). A second case underwent thyroidectomy for control of thyrotoxicosis during combined treatment with ipilimumab plus nivolumab (117). Similar to the aforementioned case, CD8+ lymphocyte predominant infiltrates were observed in association with colloid granulomas and damaged thyroid follicles

(117). Taken together, these cases suggest a destructive inflammatory thyroiditis and were histologically distinct from changes normally associated with Graves' disease.

Imaging of the thyroid in patients with ICI-associated thyroid irAEs have also suggested thyroiditis as the cause of thyroid dysfunction. Uptake scans, when performed, demonstrated diffusely reduced uptake (118-120). Thyroid ultrasound was less sensitive and specific but demonstrated heterogeneous echogenicity and hypervascularity suggestive of thyroiditis in the majority of patients scanned (121, 122). Thyroid avidity on FDG-PET was increased at the time of abnormal thyroid function in 64-100% of cases, supporting an acute hypermetabolic process (119-121). Computed tomography (CT) is uncommonly used for assessment of the thyroid, but is the main modality used to monitor ICI treatment response. Acute inflammatory thyromegaly on CT has been described in 2 cases with a doubling-tripling of thyroid volume occurring within 3 days of combined ipilimumab plus nivolumab treatment and associated with localized neck swelling (123). Thyromegaly in these cases resolved spontaneously without treatment and returned to premorbid size on serial CT examination, also in keeping with a transient inflammatory process (123).

Clinical manifestations:

Although the incidence of thyroid irAEs can be estimated from clinical trials, descriptions of the presentation and course of thyroid irAEs have been more difficult to elucidate. Historically, clinical trials reported hypothyroidism, hyperthyroidism, and thyroiditis as discrete entities. However, observational data suggests that each may represent a manifestation of the same process – a destructive thyroiditis originating with a thyrotoxic phase that progresses to hypothyroidism which may or may not

recover. Thyrotoxicosis usually develops early, within 3 to 6 weeks after drug exposure (Figure 1.8) (118, 124-132). The earliest recorded onset of thyrotoxicosis was 9 days after first dose, but subclinical hyperthyroidism may be detected as early as 24 hours after treatment initiation (130). Although most cases occur within weeks of the first dose, onset of thyrotoxicosis greater than 1-year after treatment commencement has been reported (128).

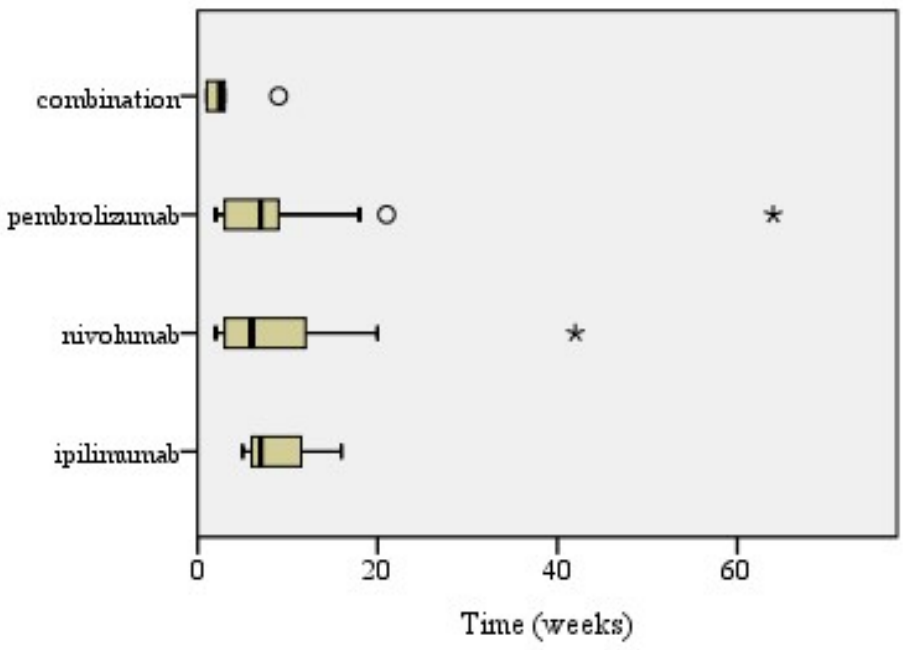

Figure 1.8: Median time to development of hyperthyroidism following first dose of immune checkpoint inhibitor.

The left, middle and right lines of the boxes correspond to the 25th, 50th and 75th percentiles respectively. The whiskers extend from minimum to maximum with ○ and * representing outlier and extreme outlier values respectively.

As with other irAEs, combination treatment with ipilimumab plus nivolumab appeared to provoke thyrotoxicosis earlier than with anti-PD-1 monotherapy (127). The thyrotoxic phase was usually painless and self-limited, lasting on average between 4 to 6 weeks (125-127, 129, 133). In the majority of cases thyrotoxicosis was asymptomatic and specific treatment with glucocorticoids or anti-thyroid medication was not required. As such, diagnosis often occurred incidentally during routine monitoring of thyroid function tests. However, severe cases have been described (134, 135). Multiple endocrine toxicities in the same patient can occur, particularly with combination therapy where up to one-third of patients who experience a thyroid irAE can develop a synchronous hypophysitis or checkpoint inhibitor associated diabetes mellitus (128, 136-140).

Following the thyrotoxic phase, most of the cases progress to hypothyroidism, although some may recover euthyroidism without an intervening hypothyroid period (125-130, 141, 142). Early onset of thyrotoxicosis (within 4 weeks) may predict progression to hypothyroidism, whereas delayed onset may be less likely to be followed by an overt hypothyroid phase (132). Subclinical hyperthyroidism also portended a good prognosis, as when present, likelihood for progression to overt hypothyroidism was significantly reduced (118, 130, 142, 143). Similar to the thyrotoxic phase, most cases of hypothyroidism were asymptomatic, although episodes of myxedema crisis have been reported (144, 145). Overt hypothyroidism without a preceding hyperthyroid phase may be the first manifestation of thyroid irAEs and the incidence of primary hypothyroidism as the initial event varies between 10-60% (124,

128, 131, 133, 143). Time to initial onset of hypothyroidism is typically longer than that seen with thyrotoxicosis predominant presentations (Figure 1.9).

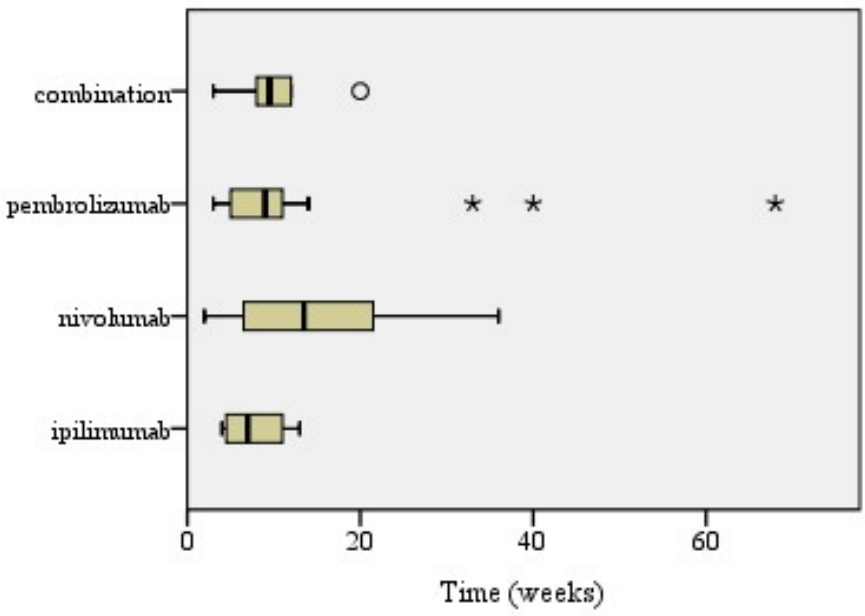

Figure 1.9: Median time to development of hypothyroidism following first dose of immune checkpoint inhibitor.

The left, middle and right lines of the boxes correspond to the 25th, 50th and 75th percentiles respectively. The whiskers extend from minimum to maximum with ○ and * representing outlier and extreme outlier values respectively.

It is unclear whether this represents a separate form of thyroid irAE, or if the transient thyrotoxic phase was asymptomatic and not clinically or biochemically detected. If overt hypothyroidism developed, it was irreversible in a majority of cases and long-term thyroid hormone replacement was required irrespective of whether

hypothyroidism was preceded by a thyrotoxic phase or not (127, 131, 133, 143, 146, 147). Subclinical hypothyroidism exhibited greater potential for recovery and only a minority required long term levothyroxine replacement (143). It is possible that milder cases may represent a non-thyroidal illness effect secondary to generalized immune activation rather than a true ICI-associated thyroiditis, although this remains to be proven. The incidence and kinetics of thyroid dysfunction after ICI treatment are summarized in Table 1.3.

Table 1.3: Incidence and kinetics of thyroid dysfunction after immune checkpoint inhibitor treatment in published observational studies reporting thyroid irAEs

	Ipilimumab (N = 195)	**Nivolumab (N = 612)**	**Pembrolizumab (N = 362)**	**Ipilimumab + PD-1 inhibitor (N = 290)**
Thyroid abnormality; n (%)				
Overt hyperthyroidism	---	9 (1.5)	3 (0.8)	---
Overt hypothyroidism	5 (2.6)	49 (8.0)	34 (9.4)	29 (10.0)
Subclinical hyperthyroidism	20 (10.3)	7 (1.1)	---	7 (2.4)
Subclinical hypothyroidism	8 (4.1)	30 (4.9)	3 (0.8)	---
Thyrotoxic phase preceding hypothyroidism; n (%)	Not reported	31/49 (63.3)	25/34 (73.5)	27/29 (93.1)
Permanent hypothyroidism; n (%)	Not reported	40/49 (81.6)	31/34 (91.2)	29/29 (100.0)
Duration of thyrotoxic phase; weeks median (range)	Not reported	7 (2-20)	5 (4-6)	6 (2-11)
N – number of patients exposed to immune checkpoint inhibitor; **n** – number of patients affected				

Thyroid function monitoring:

Routine monitoring of thyroid function at 4-6 weekly intervals is recommended for all patients on treatment with CTLA-4 and PD-1 inhibitors. Additional testing can be offered to patients who develop symptoms of potential thyroid dysfunction (fatigue, palpitations, etc.) outside of the recommended intervals (148).

Overt hypothyroidism is diagnosed in the setting of an elevated TSH and low free thyroxine (FT4) concentration. Central hypothyroidism must be excluded in all patients with biochemical hypothyroidism, especially in the setting of ipilimumab or combination immunotherapy. If present, appropriate replacement of glucocorticoids in addition to thyroid hormone is essential to avoid precipitation of adrenal crises. Subclinical hypothyroidism will usually recover without treatment, whereas most cases of overt hypothyroidism will require commencement and long-term continuation of levothyroxine. Hypothyroidism does not mandate ICI treatment interruption and can be continued during the investigation and management of thyroid dysfunction (148).

Overt hyperthyroidism is characterized by suppressed TSH and elevated FT4 levels. Most episodes of thyrotoxicosis are asymptomatic and dedicated treatment is not required. In symptomatic cases, temporary treatment with beta-blockers can help to control symptoms. Thyrotoxic patients should be monitored closely (2 weekly intervals) to detect early transition to hypothyroidism. When thyrotoxicosis is prolonged (>6 weeks) or if high clinical suspicion, patients should be investigated for Graves' disease (GD) with measurement of thyroid stimulating antibody levels (148). In most cases, ICI treatment can be continued throughout the period of thyrotoxicosis,

although patients with grade 3 or higher toxicity should have further ICI treatment withheld until the resolution of symptoms (148).

Pre-existing hypothyroidism:

Although immune checkpoint inhibition is best recognized as a cause of de novo thyroid dysfunction, patients with pre-existing hypothyroidism are also susceptible to thyroid irAEs. In one study of 63 patients with abnormal thyroid function prior to treatment with CTLA-4 or PD-1 blockade, 43 (70%) experienced an exacerbation of their thyroid dysfunction (149). The majority developed an exacerbation of hypothyroidism requiring an increase or commencement of levothyroxine. However, seven patients with established hypothyroidism on levothyroxine became thyrotoxic, mandating temporary suspension of thyroid hormone replacement (149). Similar findings have been reported in other studies of patients with pre-existing autoimmune thyroid disease, highlighting the need for surveillance of thyroid function in all patients receiving ICIs, even in those with long established hypothyroidism (60, 150-153).

Risk factors:

The etiology of irAEs is unknown, and it remains unclear why immune toxicity develops in some patients and not in others. Thyroid irAEs are among the most common and often result in permanent hypothyroidism requiring long term thyroid hormone replacement. Onset is difficult to predict and reliable predictors of thyroid irAEs have yet to be identified. The role of anti-thyroid antibodies has received the most interest, but results have been inconsistent and do not reliably predict the development or onset of thyroid irAEs.

In a Japanese study of 200 consecutive patients treated with nivolumab, age, sex and baseline TSH values were not predictive of overt thyroid dysfunction (132). However, in one study thyroid avidity on FDG-PET (prior to nivolumab treatment) significantly increased risk for development of thyroid irAEs. Positive thyroid uptake increased risk by greater than 14-times relative to those without thyroid uptake on a baseline FDG-PET (132). A second study of 99 patients treated with anti-PD-1 or combined CTLA-4 and PD-1 inhibitors also found no relationship between age or sex and risk of thyroid irAEs (142). However, unlike the Japanese study, they did find a significant relationship between baseline TSH concentration and risk of overt thyroid dysfunction (142). The mean TSH in subjects who developed overt thyroid dysfunction measured 2.72 mIU/L, compared to 1.97 mIU/L in those who did not. Based on these data, a baseline TSH >2.19 mIU/L was 76% specific and 53% sensitive for detection of overt thyroid irAEs (142).

Anti-thyroid antibodies:

The significance of thyroid peroxidase (TPOAb) and thyroglobulin (TgAb) antibodies in the pathogenesis of thyroid irAEs are unknown and their significance in the risk of thyroid irAEs remains controversial. Several studies have reported a low frequency (<30%) of positive TPOAb and TgAb in association with thyroid irAEs (122, 124, 133, 154). Others have reported increased prevalence of anti-thyroid antibodies in cases of thyroid irAEs relative to controls unaffected by thyroid dysfunction (64, 129, 155, 156). This begs the question of whether immune checkpoint blockade is simply unmasking pre-existing subclinical Hashimoto's thyroiditis (HT) which then progresses in an accelerated fashion versus a de novo process directly related to

treatment with anti-CTLA-4 and/or anti-PD-1 medicines. One study supports the latter and describes the onset of new TPOAb and TgAb positivity during the thyrotoxic phase of thyroid irAEs in patients who were antibody negative at baseline (129). In another, the presence of a TgAb at baseline provoked thyroid irAEs earlier than in TgAb negative patients and higher titers of TgAb further decreased the time to onset of thyroid dysfunction (156). Multiple other studies have not found a relationship between antithyroid antibody status and the evolution timeline of thyroiditis (127, 133).

During assessment of hyperthyroidism related to anti-CTLA-4 and/or anti-PD-1 treatment, thyroid receptor antibody levels are typically not elevated (157). However, there have been several case reports of GD occurring in patients undergoing immune checkpoint blockade and two cases of euthyroid ophthalmopathy (120, 157-159).

Prognostic significance:

Drug toxicity following anti-cancer treatment with IL-2 and tyrosine kinase inhibitors directed against the epidermal growth factor receptor is well known to correlate with tumor response and patient survival. There has therefore been great interest in whether the same relationship between toxicity and response exists in the setting of irAEs following ICI treatment. Several studies suggest this may be the case and have reported benefits in progression free survival (PFS) and overall survival (OS) following anti-PD-1 treatment with development of irAEs (71, 73). However, such results are inherently confounded by immortal time bias and no strong evidence yet supports a strong relationship between toxicity and cancer prognosis. The presence of multiple irAEs has also been shown to improve PFS and OS relative to patients with one or no

irAEs (73). Organ specific autoimmunity has also been studied, particularly dermatologic irAEs in melanoma patients treated with nivolumab and pembrolizumab, where there is an association with improved PFS and OS (73, 160, 161).

Given that thyroid irAEs are among the most common adverse effects following anti-PD-1 treatment, several studies have examined the relationship between thyroid dysfunction and clinical outcomes. Although confined to observational studies, PFS and OS were improved following the occurrence of a thyroid irAE. (71, 72, 129, 132, 162, 163). In two studies, where thyroid irAEs were subdivided into overt and subclinical, overt thyroid dysfunction showed a trend toward improved outcomes compared to when only subclinical disease was present, suggesting that not only the presence of a thyroid irAE, but also the severity may be important in the relationship between irAEs and clinical response (132, 162). Thyroiditis following blockade of PD-L1 with atezolizumab or avelumab has also been reported to improve overall survival in cancer patients in one study (164). No studies have yet reported on the prognostic significance of thyroid irAEs following anti-CTLA-4 or combined CTLA-4 and PD-1 inhibitor treatment.

Outstanding questions

Continued research is necessary to improve our understanding of autoimmune thyroid disease following immune checkpoint inhibition. The etiology of thyroid irAEs remain unclear. Clinical manifestations are heterogenous, and it is not possible to predict who will develop thyroid irAEs and whether they will progress to permanent hypothyroidism requiring long term thyroid hormone replacement. Our view is that heterogeneity of thyroid irAEs probably reflects that thyroid dysfunction arises due to

multiple mechanisms including de-novo thyroiditis, activation of underlying subclinical HT, and others. The role of thyroid autoantibodies is particularly intriguing, as they occur at higher frequency in thyroid irAEs then in euthyroid cancer patients. However, most studies have not found a causative association and antibody positivity does not fully explain susceptibility. Whether as yet unidentified antibodies directed against the thyroid exist has not been studied. Subclinical thyroid dysfunction is probably underreported, making the true incidence of thyroid irAEs unknown. Prospective collection of thyroid function tests at frequent and regular intervals could help to clarify the true incidence of thyroid irAEs and risk of progression to permanent hypothyroidism. Finally, trials combining ICIs with other immuno-modulatory therapies are ongoing. The effect of multimodal immune treatment on the risk and pattern of thyroid irAEs, and irAEs generally, is yet to be determined.

In conclusion, inhibition of CTLA-4 and PD-1 immune checkpoints have dramatically enhanced the treatment of advanced cancer. However, treatment is associated with irAEs of which thyroid dysfunction is the most common. Combined treatment with ipilimumab and nivolumab generally results in earlier onset and potentially more severe dysfunction. Most cases are mild and self-limited. Progression to permanent hypothyroidism can occur and awareness amongst medical professionals is important to limit morbidity associated with untreated thyroid dysfunction.

1.5.4 Second narrative review of thyroid irAEs encompassing major developments in understanding published after the original systematic review. Published in *Frontiers in Endocrinology*® (see Appendix 2 for print version)

ICIs are a rapidly expanding class of medications. At present, over 2000 clinical trials have been completed or are in progress to evaluate more than 30 different ICIs (165). The main immune checkpoint targets are CTLA-4 and PD-1 or its ligand PD-L1. The introduction of ICIs into cancer treatment algorithms has been transformative and durable responses are now possible in patients with previously limited therapeutic options. However, immune activation following ICI-treatment is associated with off target effects known as irAEs (43). Any organ system may be affected and thyroid dysfunction is one of the most common ICI-related toxicities (45).

Immune checkpoint physiology

Immune checkpoints are small molecules present on the cell surface of T-lymphocytes (37). Normally, T-cell activation induces immune checkpoint expression to limit the amplitude and duration of immune responses (37). Immune checkpoints are pivotal to regulate the immune response and prevent inappropriate immune activation, such as occurs in autoimmune disease (37). However, in some situations, immune checkpoints can be counterproductive, such as malignant tumors which activate immune checkpoints to evade immune mediated tumor lysis (45). Monoclonal antibodies antagonizing CTLA-4, PD-1, and other immune checkpoints have been developed to counteract such tumor evasion strategies and stimulate anti-tumor immune responses (37). These agents convey significant therapeutic benefit but are associated with wide ranging irAEs (37, 43, 45).

Immune related adverse events

The etiology of irAEs is yet to be fully elucidated. Potential mechanisms include activation of polyclonal T-cell populations, loss of regulatory T-cell function, and expansion/activation of self-reactive, antigen-specific T-cells (29). Irrespective of mechanism, ICI-associated irAEs are common, affecting up to 76% of treated patients (45). Any organ system can be affected, although there are distinct pharmacological class-specific differences between ICIs targeting CTLA-4 and those targeting PD-1 (45). Endocrine organs are commonly affected, with thyroid dysfunction, hypophysitis, insulin deficient diabetes, primary adrenal insufficiency, and hypoparathyroidism all reported following ICI-treatment (43, 78). In this review we will highlight the key features of thyroid irAEs and how our understanding of thyroid irAEs has informed our understanding of autoimmune thyroid disease more generally.

Thyroid immune related adverse events

Epidemiology

Thyroid irAEs are the most common endocrine toxicity related to ICI-treatment (78). From clinical trial data, over 10% of patients treated with ICIs develop a thyroid irAE (166). Higher rates are observed following treatment with PD-1 inhibitors relative to CTLA-4 inhibitors, with the highest rates following combined anti-PD-1 and anti-CTLA-4 treatment (166). Observational studies typically report higher rates of thyroid irAEs, usually due to inclusion of subclinical thyroid dysfunction that may be overlooked in clinical trials adverse events reporting (118, 133, 142, 167). In the two largest observational studies of thyroid irAEs to date, 42-53% experienced an ICI-associated thyroid irAE (167, 168). Thyroid dysfunction was most common following

combined anti-PD-1 and anti-CTLA-4 treatment (56%) and less frequent following anti-PD-1 (38%) and anti-CTLA-4 (25%) monotherapy (167).

Clinical manifestations

Thyroid irAEs are typically identified incidentally during routine monitoring of thyroid function as part of ICI-treatment protocols (166). Most thyroid irAEs present as a painless thyroiditis with transient thyrotoxicosis (78, 127, 166, 167). In patients with more severe (ie. biochemically overt) thyrotoxicosis, a hypothyroid phase often follows the initial thyrotoxicosis and over 40% of these patients will develop permanent hypothyroidism requiring thyroid hormone replacement (167). A smaller number of patients can present with primary hypothyroidism without a preceding thyrotoxic phase.

Onset of thyrotoxicosis usually occurs within 3 months of first ICI-exposure (78, 166). Overt and subclinical thyrotoxicosis have phenotypic differences in their presentation and disease associations. Onset of overt thyrotoxicosis typically occurs earlier and persists longer than subclinical cases (167). Overt thyrotoxicosis has also been associated with higher baseline levels of anti-thyroid antibodies which increase during ICI-inhibitor treatment (169). Treatment related changes in antibody titer have not been observed in other subtypes of thyroid irAE (169). Patients who develop overt thyrotoxicosis are more likely to develop severe irAEs and to experience two or more irAEs in extra-thyroidal organ systems (167).

Isolated hypothyroidism without a preceding thyrotoxic phase can occur. Most cases of isolated hypothyroidism are biochemically subclinical and may be related to non-ICI factors such as non-thyroidal illness syndrome. Subclinical hypothyroidism

without a preceding thyrotoxic phase typically normalizes spontaneously without need for thyroid hormone replacement (143, 167). Overt hypothyroidism can have a late-onset months to years after starting ICI-treatment (166). In contrast with subclinical hypothyroidism, overt hypothyroidism is usually permanent and lifelong treatment with thyroxine is required in most cases (78, 166, 167).

Risk factors and disease associations

Identification of reliable thyroid irAE risk factors has been elusive. To date, no reliable risk factors of thyroid irAEs have been identified, although clinical and biochemical associations have been documented in retrospective cohort studies (166). Female sex was associated with thyroid irAEs in the two largest studies to date (Table 1.4), although to a lesser degree than the roughly 8:1 female preponderance observed in autoimmune thyroid disease developing outside of the ICI-treatment setting (167, 168). Prevalence of baseline TPOAb and TgAb positivity is higher in patients who develop a thyroid irAE compared to those who do not (167, 170). Anti-thyroid antibody positivity is particularly associated with overt thyroid dysfunction (167, 170) and titers of TPOAb and TgAb can increase significantly during ICI-treatment in patients that develop overt thyrotoxicosis, which is not observed in patients with other subtypes of thyroid irAE (169). A complete list of demographic and biochemical factors that have been associated with development of thyroid irAEs are summarized in Table 1.4.

Table 1.4: Demographic and biochemical factors associated with development of thyroid irAEs

Female sex (167, 168)
Younger age (167)
Caucasian ethnicity (168)
Combined CTLA-4 + PD-1 treatment (167, 168)
Longer duration of anti-PD-1 treatment (170)
Higher body mass index (171)
Renal cell carcinoma* (168)
Baseline TSH (118, 156, 170, 171)
Baseline TPOAb positivity; treatment related increase in TPOAb titer (169, 170, 172)
Baseline TgAb positivity; treatment related increase in TPOAb titer (156, 169, 170, 172)
*Likely confounded by concomitant use of tyrosine kinase inhibitors combined with ICIs.

Pathophysiology

The etiology and biological drivers of irAEs remain elusive. It is possible that patients who experience irAEs may share a premorbid immunological state prior to ICI-initiation. A recent study using mass cytometry time of flight analysis identified higher levels of activated CD4+ memory T-cells and increased diversity in the T-cell receptor as pretreatment predictors of severe irAEs irrespective of the organ involved (173). These same factors also occurred with higher frequency in non-ICI treated patients with autoimmune disease relative to healthy controls, suggesting that irAEs may result from unmasking a latent or subclinical autoimmune process which predates ICI-initiation (173).

Traditionally, thyroid irAEs were thought to be a single entity spanning a spectrum of disease including isolated mild (asymptomatic or minimally symptomatic) thyrotoxicosis to biphasic thyroiditis to isolated overt and severe hypothyroidism. This

indeed may be the case, although more likely thyroid irAEs arise via multiple mechanisms as evidenced by phenotypic differences in presentation, antithyroid antibody positivity rates, and differential association with cancer survival outcomes (167).

Genetic factors associated with lifetime risk of autoimmune thyroid disease have been implicated in the risk of developing thyroid irAEs during ICI-treatment (174, 175). Key loci have been implicated from genes regulating the immune response such as CD69 (T-cell activation), LPP (B-cell maturation), CTLA-4 (T-cell priming) and PTPN22 (T-cell and B-cell receptor signaling). When these genes and others are combined into a polygenic risk score they can identify subgroups of patients at >6-fold increased risk of thyroid irAEs and identify patients at a lower risk of cancer death (174).

The PD-1/PD-L1 axis is significantly implicated in autoimmune thyroid disease (176). Patients with HT and GD experience a moderate increase in circulating PD-1 positive T-cells and marked increase in intrathyroidal PD-1 positive T-cells (176). Thyroid follicular cells also show high levels of PD-L1 expression, whereas multinodular goiter (non-autoimmune thyroid disease) has negligible basal expression of PD-L1 (176). The presence of high PD-L1 expression in autoimmune thyroid disease may explain why most cases are slowly progressive, as the immune stimulating effects of intrathyroidal PD-1 positive T-cells are kept in check. PD-1 positive lymphocytes and PD-L1 expression are similarly increased following ICI-treatment. However, different to autoimmune thyroid disease, blockade of the PD-1/PD-L1 axis blunts the protection against progressive immune mediated destruction and therefore thyroid dysfunction is

more rapidly progressive following ICI-treatment than in other autoimmune thyroid conditions (176, 177).

Management

Screening:

Thyroid function should be measured at baseline and repeated at 6-weekly intervals following commencement of ICI-treatment in asymptomatic patients. Additional measures of TSH and FT4 can be undertaken for case detection in patients that develop signs or symptoms of thyroid dysfunction outside this window (178). Most cases of clinically significant thyroid dysfunction will occur within 6-months of ICI-commencement (166). Therefore, monitoring frequency can be relaxed after the 6-month mark although continued periodic monitoring (3-6 monthly) to detect late onset cases is recommended.

Diagnosis:

As most cases of thyroid irAE are asymptomatic, diagnosis is most commonly via thyroid function monitoring in asymptomatic patients (166). Thyrotoxicosis is diagnosed in the setting of a low TSH with a normal or elevated FT4. Thyrotoxicosis often occurs as the initial phase of a biphasic thyroiditis and is followed by progression to hypothyroidism (elevated TSH, low FT4). More rarely, isolated hypothyroidism without a preceding thyrotoxic phase can occur. Unlike thyrotoxicosis, isolated hypothyroidism may present months to years after initiation of ICI-treatment. Special attention should be paid to the finding of an inappropriately low TSH in combination with a low or low-normal FT4. This pattern of results should alert the clinician to the possibility of hypophysitis, particularly in the setting of a CTLA-4 inhibitor. When

hypophysitis (central hypothyroidism) is suspected, the remaining pituitary hormone axes should be interrogated urgently to exclude cortisol and other hormone deficiencies prior to initiation of thyroid hormone replacement.

Treatment:

For asymptomatic or minimally symptomatic thyrotoxicosis, treatment is usually not required. Temporary use of beta blockers such as propranolol may be considered in symptomatic patients. ICI-treatment can be continued, and most cases will resolve spontaneously within days to weeks. Thyrotoxicosis lasting longer than 6-weeks or with additional features (goiter, thyroid bruit, exophthalmos) should have TSH-receptor antibody level (TRAB) measured to exclude GD (178). In severe or atypical presentations, imaging with a thyroid uptake scan can also be performed to differentiate between ICI-mediated thyroiditis and other etiologies of thyrotoxicosis. Thyrotoxicosis with marked symptoms or life-threatening complications is rare and necessitates suspension of ICI-treatment to allow for prompt investigation and specialist led treatment (178). Following identification of thyrotoxicosis, TSH and FT4 should be monitored every 2-3 weeks until recovery of normal thyroid function, as many patients will experience a biphasic illness which progresses to hypothyroidism. Regular monitoring allows early detection of hypothyroidism, which does not recover in the majority of cases (167). In the setting of overt hypothyroidism, treatment with thyroxine can be initiated, particularly when the TSH is >10 mIU/L, as recovery is highly unlikely in this setting (167). Alternatively, TSH and FT4 measurement can be repeated after 4-6 weeks and if hypothyroidism persists, treatment can be initiated at that point (178). Thyroxine doses are typically higher for patients with ICI-mediated

hypothyroidism than for HT, suggesting that gland destruction is complete in most cases of ICI-mediated thyroiditis whereas it is slowly progressive with some residual production of endogenous thyroid hormone in patients with HT (179). A simplified algorithm for the classification and management of thyroid irAEs is presented in Figure 1.10.

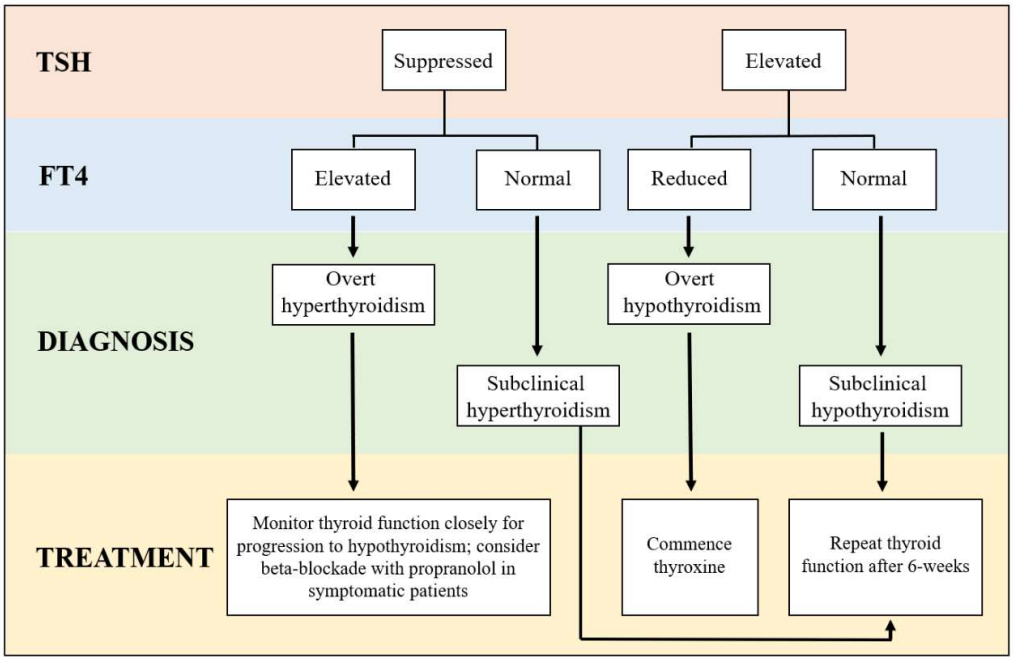

Figure 1.10: Algorithm for classification and management of thyroid irAE subtypes.

*When overt thyrotoxicosis is prolonged or severe, additional investigation with TRAB and a thyroid uptake scan should be considered to exclude other etiologies of thyrotoxicosis (Graves' disease, toxic adenoma, etc).

Special situations

Pre-existing hypothyroidism:

Patients with established hypothyroidism may experience transient effects on thyroid function following ICI-treatment (166). Compensated hypothyroidism can be exacerbated requiring initiation or increased doses of thyroxine. More rarely, patients with hypothyroidism may paradoxically experience a transient period of thyrotoxicosis requiring temporary suspension of thyroid hormone (149).

Graves' disease and thyroid eye disease:

GD has been reported following ICI-treatment (78). Whether it occurs de novo related to ICI-treatment, is unmasked by ICI-treatment, or is a coincidental occurrence remains unknown. Similarly, thyroid eye disease (TED) has been reported (120). Reactivation or progression of GD and TED is possible following initiation of ICI-treatment and in patients with known GD or TED, close consultation with an experienced endocrinologist and ophthalmologist is important for early identification and treatment.

Prognostic implications

Observational studies repeatedly document an association between irAEs and improvement in cancer outcomes (71, 76). Owing to the frequency of thyroid irAEs, they are among the most well studied and strongly associated with improvement in PFS and OS (180-182). Reported benefits are large with robust hazard ratios that are maintained following correction for immortal time bias (164, 180). However, emerging data suggest thyroid irAE subtypes are not equivalent in their association with cancer outcomes. When thyroid irAEs are classified by severity, biochemically overt thyroid

dysfunction is more strongly associated with improved PFS and OS than subclinical thyroid dysfunction (132, 167, 183). Our previous work suggests this may be limited to overt thyrotoxicosis, as no survival benefit was observed in patients with overt hypothyroidism without a preceding thyrotoxic phase (167). Permanent thyroid dysfunction requiring initiation of thyroid hormone replacement has also been associated with improved cancer outcomes (168, 184). However, an ultimate requirement for thyroxine may have been a surrogate marker for a preceding phase of overt thyrotoxicosis rather than a beneficial prognostic factor in of itself. The significance of antithyroid antibodies on cancer outcomes following ICI-treatment is unknown and current data has conflicting results (183, 185). Given the high prevalence of anti-thyroid antibodies in patients who develop overt thyrotoxicosis, an association is likely and prospective study into potential utility as a predictive biomarker is warranted (169). Interestingly, abnormal TSH level (elevated or low) prior to initiation of ICI-treatment has been associated with inferior cancer outcomes irrespective of thyroid function changes post-ICI initiation in one study (168). However, these results may have been confounded as significantly more patients with abnormal baseline TSH were receiving tyrosine kinase inhibitor treatment for metastatic renal cell cancer, which is known to significantly affect thyroid function (168). Abnormal TSH prior to ICI-treatment may also be a function of sicker patients displaying the non-thyroidal illness syndrome. Therefore, at present it is not known whether baseline thyroid function is truly associated with worse outcomes or if it simply reflects patients with a more aggressive cancer or a physiologic response to chronic illness in a less well patient at commencement of ICI-treatment.

Future directions

Further research is required to better inform our understanding of thyroid irAEs and their relationship to thyroid autoimmune conditions occurring outside the cancer immunotherapy setting. Enhanced characterization of the immune phenotype and T-cell subsets driving thyroid irAEs may allow for identification of patients at risk of thyroid (and other) irAEs and inform future research to uncouple the efficacy of ICIs from the risk of developing treatment related irAEs. Understanding the effects of immune checkpoint inhibition on the thyroid gland could also inform use of these agents for treatment of primary thyroid malignancies and prevention of autoimmune thyroid disease.

Conclusions

Thyroid irAEs are a frequent complication of anti-CTLA-4 and anti-PD-1 based ICI-treatment. Recent work has begun to unravel the molecular mechanisms underpinning development and their relationship to other autoimmune thyroid conditions. Regular monitoring of thyroid function during ICI-treatment is required, as although most cases of thyroid irAEs are asymptomatic and manageable, severe presentations with thyroid storm or myxedema can occur. Development of thyroid irAEs is associated with improved PFS and OS, especially overt thyrotoxicosis which may be a surrogate marker of patients experiencing a more robust immune response to ICI-treatment. When hypothyroidism occurs, it is usually permanent and treatment with thyroid hormone replacement will be required. Familiarity with thyroid irAEs among health professionals is important to facilitate efficient diagnosis and appropriate treatment of this increasingly common thyroid disease.

1.6 Summary and thesis aims

Thyroid irAEs are a frequent and clinically significant complication of ICI-treatment in patients with cancer. Thyroid irAEs are thought to be autoimmune in aetiology and the mechanism by which hyperthyroidism and hypothyroidism develop are unknown. Immune checkpoint inhibitor associated thyroid irAEs are a novel model of thyroid (and other organ) autoimmunity and may shed light on the pathogenesis of these conditions. The aims of this thesis were to characterize the clinical, biochemical, immune, and genetic factors involved in development of thyroid irAEs following ICI treatment. Specifically, we aimed to:

1. Determine the incidence and phenotypically describe clinical and biochemical associations of thyroid irAEs in a large cohort of patients treated with immune checkpoint inhibitors. These data are presented in Chapter 2.
2. Characterize the prevalence and possible association of thyroid autoantibodies with the development of thyroid irAEs. These data are presented in Chapter 3.
3. Study the immune phenotype of thyroid irAEs and determine whether specific immune cell populations are preferentially upregulated in association with thyroid irAEs. These data are presented in Chapter 4.
4. Test whether polymorphisms in genes associated with autoimmune thyroid disease are also overrepresented in patients with thyroid irAEs. These data are presented in Chapter 5.
5. Investigate whether novel autoantibodies against other thyroid antigens (non-TPOAb, non-TgAb) are associated with the development of thyroid irAEs. These data are presented in Chapter 6.

Chapter 2

Characterization of thyroid irAEs in patients with melanoma undergoing immune checkpoint inhibitor treatment

2.1 Introduction

Prior to this study, checkpoint inhibitors were already in use for treatment of multiple malignancies, including advanced and metastatic melanoma. The indication list has continued to expand since that time, with some estimates indicating over 30% of patients with cancer may be eligible for ICI-treatment (186). From the earliest trials, it was apparent that thyroid dysfunction was, and would continue to be, a frequently occurring drug-related toxicity. However, at that time, thyroid irAEs were poorly characterized by randomized clinical trials and only catalogued as hyperthyroidism, hypothyroidism, or thyroiditis without further clinical or biochemical description. Apart from the limited reporting in large randomized controlled trials, only small retrospective cohort studies had been reported, again with marked heterogeneity and insufficient detail to effectively phenotype the spectrum, clinical presentation, and prognostic significance of thyroid irAEs occurring because of ICI-treatment. As such, we set out to comprehensively describe and precisely define thyroid irAEs in a cohort of cancer patients with sufficient size to produce reliable and informative data. We partnered with Melanoma Institute Australia (MIA), who provided us with demographic and clinical data for over 1200 ICI-treated patients. Clinical data was then linked to thyroid function test results for each patient to enable us to address our research question. The results of this study were published in the *Journal of Clinical Endocrinology and Metabolism* in 2021 and are presented in Section 2.2 (for print version, see Appendix 3).

2.2 Retrospective cohort study of thyroid irAEs in patients with melanoma receiving ICI treatment.

Published in the *Journal of Clinical Endocrinology and Metabolism*® (see Appendix 3 for print version)

ICIs are standard of care in the management of melanoma and many other cancer types (80). Through antagonism of the CTLA-4 and PD-1 pathways, ICIs increase T-cell activation and anti-cancer immune responses (37). Enhancements in anti-tumor immunity related to ICI treatment have improved patient survival, but are associated with wide ranging irAEs (43, 187). Thyroid dysfunction is the most commonly occurring endocrine irAE, affecting more than 10% of exposed patients (166). PD-1 inhibitors are associated with higher rates of thyroid dysfunction relative to CTLA-4 inhibitors, with the highest rates observed in patients undergoing combination treatment with both anti-CTLA-4 and anti-PD-1 ICIs (166).

The etiology of thyroid irAEs has generally been assumed to be secondary to destructive, immune-mediated thyroiditis. However, onset of thyroid dysfunction is highly variable and not all patients develop the classic thyroiditis-like presentation of transient thyrotoxicosis followed by a hypothyroid phase (166). Isolated thyrotoxicosis and hypothyroidism have both been reported, as well as subclinical disease that is often short-lived and transient (166). Some studies have reported thyroid irAEs to be associated with improvements in PFS and OS (71, 129, 132, 164). Thyroid autoantibody positivity rates vary widely between studies with prevalence rates of 17-100% reported (119, 124, 126, 127, 156). Low level thyroid antibody positivity has been associated with improvement in PFS and OS, however the role of anti-thyroid antibodies in the pathogenesis and susceptibility to thyroid irAEs remains unknown (166, 183).

Given the heterogeneity and lack of large studies examining thyroid dysfunction following ICI treatment, the aim of the present study was to comprehensively characterize thyroid irAEs in a large cohort of patients with advanced or metastatic melanoma.

Methods:

Study design and participants:

We reviewed outcomes in a cohort of adult patients undergoing ICI treatment for advanced or metastatic melanoma between 01-Nov-2009 and 31-Dec-2019. Demographic, treatment, and cancer outcome information was extracted from a prospectively maintained central database. Measurement of serum TSH was performed in all subjects prior to commencement of immunotherapy and thyroid function was repeated at subsequent ICI doses q3-4 weekly during the first 6-months of follow-up. In patients with documented thyroid dysfunction or symptoms of thyroid dysfunction, testing was performed at increased frequency on an individualized basis. After the first 6-months of ICI-treatment, the testing interval was extended to q6-8 weekly and then to q12 weekly after 1-year. Patients with normal thyroid function at baseline and at least one further measurement of thyroid function following the first dose of ICI were included. Patients with pre-existing thyroid disease (defined as abnormal thyroid function at baseline or treatment with thyroxine at baseline and patients who developed hypophysitis with central hypothyroidism were excluded.

Definitions:

Thyroid irAEs were defined by detection of new thyroid dysfunction developing after treatment with an ICI and were sub-categorized by the biochemical pattern of thyroid dysfunction. Overt thyrotoxicosis was defined by a TSH level below the lower reference

interval with concomitant elevation of FT4 above the upper reference interval. Subclinical thyrotoxicosis was defined by a TSH level below the lower reference interval in the presence of normal serum concentrations of FT4. Overt hypothyroidism was defined by a TSH level above the upper reference limit with concomitant FT4 levels below the lower reference interval or by TSH >10.0 mIU/L irrespective of FT4. Subclinical hypothyroidism was defined as normal FT4 level in the presence of TSH elevation above the upper limit of the normal reference range and <10.0 mIU/L. Euthyroid hyperthyroxinemia and hypothyroxinemia were defined as a normal TSH with FT4 concentrations above or below the normal reference interval, respectively.

Time to thyroid dysfunction was measured as the time to first documented thyroid function test abnormality following receipt of the initial dose of ICI. Duration of thyrotoxicosis or hypothyroidism was defined as the time to restoration of normal TSH following the first laboratory documented episode of abnormal thyroid function.

Assay characteristics:

Serum concentration of thyroid hormones and antibodies were made using commercially available kits from Abbott Diagnostics. TSH had a measuring range of 0.01-127 mIU/L with precision (CV) of 1.9-5.3% across a wide range of TSH values. FT4 had a measuring range of 0.0-77.2 pmol/L with precision (CV) of 3.6-7.8% across a wide range of FT4 values. Full assay characteristics are available online via Abbott Diagnostics package insert instructions. Local reference intervals for TSH and FT4 were set at 0.40-4.20 mIU/L and 9.0-19.0 pmol/L respectively. TPOAb and TgAb titers were measured using the Abbott Architect platform with values of >5.61 IU/mL and >4.11 IU/mL considered positive, respectively.

Procedures and outcomes:

The primary outcome was to describe the prevalence, clinical phenotypes and factors associated with thyroid irAEs in a large cohort of melanoma patients undergoing ICI-treatment. Biobank serum specimens were retrieved to measure TPOAb and TgAb titers in a subset of patients using samples collected and stored at baseline (prior to receipt of first ICI dose). Additional analyses were performed to assess the association of thyroid irAEs with cancer survival.

Statistical analysis:

Continuous variables were summarized as median and interquartile range (IQR) or frequencies and percentages reported for categorical variables. Baseline characteristics of patients with thyroid irAEs were compared to those of patients without thyroid irAEs in Table 2.1 using Mann-Whitney U-test for continuous outcome variables and Pearson's chi-square test for categorical outcome variables. Associations between demographic factors and thyroid irAEs were assessed using multivariable logistic regression that included all factors with p-value <0.20 from a univariate analysis. Results of multivariable logistic regression are presented as OR and associated 95% confidence interval (CI). Survival differences between patients with thyroid irAEs and patients without thyroid irAEs were compared using Kaplan-Meier log-rank analysis. The effect of thyroid irAEs on OS and PFS was estimated using Cox regression adjusted with age, sex, brain metastases and ICI-type. All statistical tests were 2-tailed with two-sided significance level set at p=0.05. Pre-specified subgroup analyses were performed in patients with overt thyrotoxicosis and overt hypothyroidism compared to patients with euthyroidism. Statistical analyses were conducted using Statistical Package for Social Sciences (SPSS)

version 26 (SPSS Inc, IL, USA) and figures were created using GraphPad Prism, version 8. All patients consented for their data to be recorded in the MIA central database, and for that information to be used for research purposes. Ethical approval was provided by the Sydney Local Health District human research and ethics committee.

Results:

Study population:

One thousand two hundred forty-six patients met inclusion criteria. The median age was 65 years (IQR 54-74) and 824 (66%) participants were male, consistent with a typical melanoma population. Immune checkpoint inhibitor treatment consisted of the anti-CTLA-4 antibody ipilimumab (13%), anti-PD-1 antibodies nivolumab (19%) or pembrolizumab (36%), combination ipilimumab and nivolumab (23%), combination ipilimumab and pembrolizumab (7%) or the anti-PD-L1 antibodies atezolizumab/avelumab (2%). Baseline characteristics are presented in Table 2.1.

Incidence of thyroid dysfunction:

The entire cohort (n=1246) had a median follow-up of 11.3 (IQR 5-24) months. During follow-up, ICI-associated thyroid irAEs occurred in 518 (42%) patients. The highest incidence of thyroid irAEs occurred with combination CTLA-4 and PD-1 inhibitor treatment (56%), followed by anti-PD-1 (38%) and anti-CTLA-4 (25%) monotherapies (Figure 2.1a). Multiple patterns of thyroid irAEs were observed. Primary thyrotoxicosis was the most common thyroid irAE, affecting 388 (31%) patients. Of primary hyperthyroid irAEs, thyrotoxicosis was subclinical in 234 patients and overt in 154 patients (Figure 2.1b). In patients with overt thyrotoxicosis, the median maximum FT4 concentration was 25.6 pmol/L (IQR 21.9-35.1). Primary hypothyroidism at onset (i.e.

without a preceding hyperthyroid phase) occurred in 100 (8%) patients and, again, most cases were subclinical (Figure 2.1b). Euthyroid hyperthyroxinemia and hypothyroxinemia developed in 10 (<1%) and 6 (<1%) patients, respectively. Non-thyroidal illness (isolated low triiodothyronine) occurred in 14 (1%) patients (Figure 2.1b). As a proportion of total thyroid irAEs, overt thyroid dysfunction occurred more commonly following combination CTLA-4 and PD-1 inhibitor treatment (47%) than after anti-PD-1 (37%) or anti-CTLA-4 (19%) based monotherapy.

Kinetics of thyroid dysfunction:

There were kinetic differences in the temporal course of TSH and FT4 in patients with thyrotoxicosis compared to those who developed hypothyroidism from onset (Figure 2.2). Time to thyroid dysfunction was significantly shorter for patients with thyrotoxicosis compared to those with hypothyroidism (median 41 vs. 90 days, $p<0.001$). Of patients with thyrotoxicosis, a classical thyroiditis-like pattern of thyrotoxicosis immediately followed by a hypothyroid phase occurred in 111/388 (29%) patients. Progression to hypothyroidism was more common following overt thyrotoxicosis, with 91/154 (59%) patients progressing compared to 20/234 (9%) patients following an initial presentation with subclinical thyrotoxicosis (Table 2.2). Permanent hypothyroidism requiring thyroid hormone replacement was highest in patients with overt hypothyroidism at onset (74%). In patients with subclinical thyrotoxicosis 227 (97%) returned to a euthyroid state during follow-up, whereas in patients with overt thyrotoxicosis, only 88 (57%) recovered normal thyroid function during follow-up and 66 patients (43%) progressed to permanent hypothyroidism requiring thyroid hormone replacement. (Table 2.2).

Table 2.1: Baseline characteristics

	All patients	Thyroid irAE	No thyroid irAE	p-value
Patients; n (%)	1246 (100)	518 (42)	728 (58)	
Age (years); median (IQR)	65 (54-74)	63 (51-71)	67 (56-75)	<0.001
Male; n (%)	824 (66)	306 (59)	518 (71)	<0.001
Checkpoint inhibitor; n (%) - CTLA-4 - PD-1/PD-L1 - Combination CTLA-4 + PD-1	 165 (13) 705 (57) 376 (30)	 41 (8) 266 (51) 211 (41)	 124 (17) 439 (60) 165 (23)	<0.001
AJCC stage (188); n (%) - III a - III b - III c - IV M1a - IV M1b - IV M1c	 17 (1) 63 (5) 150 (12) 83 (7) 167 (13) 726 (58)	 6 (1) 29 (6) 55 (11) 35 (7) 69 (13) 314 (61)	 11 (1) 34 (5) 95 (13) 48 (7) 98 (14) 412 (57)	0.70
ECOG status; n (%) - 0 - 1 - 2 - 3	 744 (60) 365 (29) 37 (3) 8 (1)	 330 (64) 138 (27) 14 (3) 2 (<1)	 414 (57) 227 (31) 23 (3) 6 (1)	0.14
Brain metastases; n (%)	258 (21)	131 (25)	127 (17)	0.001
Elevated LDH; n (%)	336 (27)	133 (26)	203 (28)	0.29
TSH (mIU/L); median (IQR)	1.32 (0.93-1.94)	1.24 (0.81-1.90)	1.38 (0.98-1.99)	0.003
FT4 (pmol/L); median (IQR)	13.1 (12.0-14.3)	13.1 (12.0-14.7)	13.0 (12.0-14.1)	0.32
TPOAb positive; n (%)	27/163 (17)	27/128 (21)	0/35 (0)	<0.001
TgAb positive; n (%)	42/163 (26)	41/128 (32)	1/35 (3)	<0.001

Immune-related adverse events in extra-thyroidal organ systems were common, affecting 865 (69%) patients. Rates of extra-thyroidal irAEs were significantly increased in patients with overt thyrotoxicosis relative to hypothyroid and euthyroid patients (87 vs. 67 vs. 65%,

p<0.001). Similarly, overt thyrotoxicosis was associated with higher rates of multi-system irAEs and CTCAE grade 3 or higher (more severe) irAEs compared to hypothyroid and euthyroid patients (Table 2.2).

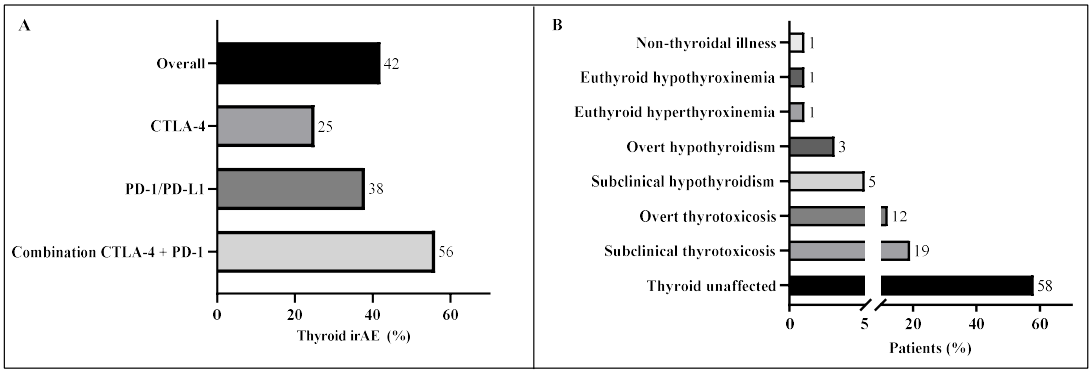

Figure 2.1: Incidence and biochemical subtypes of thyroid irAEs

(A) Proportion of patients experiencing a thyroid irAE by ICI exposure. (B) Patterns and incidence rates of thyroid dysfunction developing after ICI treatment.

Clinical and biochemical associations:

Combination immunotherapy with anti-CTLA-4 and anti-PD-1 ICIs was associated with higher rates of thyroid irAE relative to either CTLA-4 (OR 3.76; 95% CI 2.49-5.75; p<0.001) or PD-1 (OR 1.90; 95% CI 1.45-2.49; p<0.001) based monotherapy. Thyroid irAEs were significantly more frequent in women (OR 1.62; 95% CI 1.27-2.08; p<0.001) and were negatively associated with age (OR 0.88 per 10-years; 95% CI 0.81-0.96; p=0.005) and brain metastases (OR 0.71; 95% CI 0.53-0.94; p<0.02). The incidence of overt thyrotoxicosis was higher following combination ICI treatment compared with anti-CTLA-4 (OR 10.8; 95% CI

4.51-25.6; p<0.001) or anti-PD-1 (OR 3.30; 95% CI 2.23-4.90; p<0.001) monotherapy (Table 2.3).

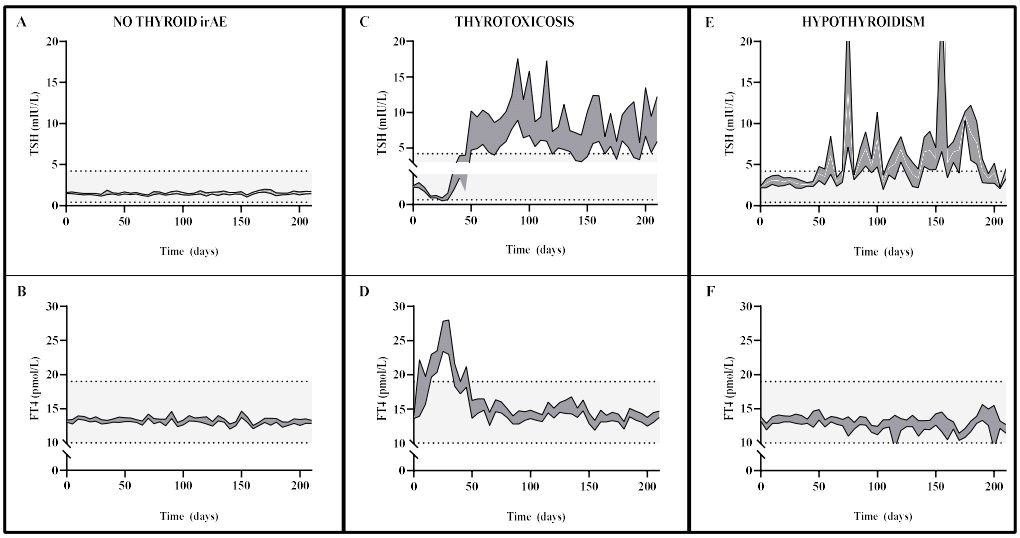

Figure 2.2: Kinetics of TSH and FT4 following ICI treatment

(A) Change in TSH over time (mean ±SEM) in patients who remained euthyroid during follow-up. (B) Change in FT4 over time in patients who remained euthyroid during follow-up. (C) Change in TSH over time in patients who developed primary thyrotoxicosis (subclinical and overt) during follow-up. (D) Change in FT4 over time in patients who developed primary thyrotoxicosis during follow-up. (E) Change in TSH over time in patients who developed primary hypothyroidism (subclinical and overt) during follow-up. (F) Change in FT4 over time in patients who developed primary hypothyroidism during follow-up. TSH=thyroid stimulating hormone. SEM=standard error of the mean. FT4=free thyroxine.

Table 2.2: Clinical phenotypes of thyroid dysfunction in patients with thyroid irAEs

	Thyrotoxicosis		Hypothyroidism		p-value
	Subclinical	Overt	Subclinical	Overt	
Patients; n	234	154	61	39	
Age (years); median (IQR)	63 (49-71)	60 (49-69)	68 (55-74)	62 (51-73)	<0.001
Male; n (%)	147 (63)	83 (54)	42 (69)	16 (41)	<0.001
Checkpoint inhibitor; n (%) - CTLA-4 - PD-1/PD-L1 - Combined CTLA-4 + PD-1	 23 (10) 110 (47) 101 (43)	 6 (4) 62 (40) 86 (56)	 6 (10) 48 (79) 7 (11)	 1 (3) 29 (74) 9 (23)	<0.001
Baseline TSH (mIU/L); median (IQR)	0.95 (0.63-1.41)	1.29 (0.89-1.88)	2.32 (1.43-3.03)	1.94 (1.20-3.07)	<0.001
Baseline FT4 (pmol/L); median (IQR)	12.8 (11.9-13.7)	13.6 (12.2-15.1)	12.5 (11.7-14.1)	14.1 (12.5-15.6)	0.23
TPOAb positive; n (%)	3/58 (5)	18/53 (34)	2/4 (50)	4/13 (31)	
TgAb positive; n (%)	8/58 (14)	26/53 (49)	1/4 (25)	6/13 (46)	
Time to onset of thyroid dysfunction (weeks); median (IQR)	8 (4-14)	5 (2-8)	10 (3-26)	14 (8-25)	<0.001
Time to normalization of TSH after onset of thyroid dysfunction (weeks); median (IQR)	4 (1-8)	12 (7-24)	3 (1-8)	10 (1-24)	0.37
Progression to hypothyroidism; n (%)	20 (9)	91 (59)	n/a	n/a	n/a
Permanent hypothyroidism requiring thyroxine; n (%)	7 (3)	66 (43)	n/a	29 (74)	0.13
Extra-thyroidal irAEs; n (%)	173 (74)	134 (87)	38 (62)	29 (74)	0.01
Multi-system extra-thyroidal irAEs; n (%)	13 (6)	24 (16)	0 (0)	2 (5)	0.01
Severe (CTCAE grade 3-4) extra-thyroidal irAEs; n (%)	56 (24)	37 (24)	9 (15)	5 (15)	0.03

Overt thyrotoxicosis was more common in women (OR 2.02; 95% CI 1.37-2.95; p<0.001) and in younger patients (OR 0.83 per 10-years; 95% CI 0.72-0.95; p=0.007). Combination ICI-treatment did not significantly influence odds of overt hypothyroidism compared to CTLA-4 (OR 7.28; 95% CI 0.89-59.4; p=0.06) and PD-1 (OR 0.70; 95% CI 0.31-1.59; p=0.40) based monotherapy. Baseline TSH did not predict thyroid irAEs overall. However, baseline TSH levels were significantly higher in patients with overt hypothyroidism from onset (OR 2.33 per mIU/L; 95% CI 1.61-3.33; p<0.001). Using ROC curve analysis, baseline TSH values greater than 2.47 mIU/L were 90% specific and 40% sensitive for prediction of subsequent primary overt hypothyroidism.

Thyroid autoantibodies:

TPOAb and TgAb were measured in a subset of 163 patients with available biobank serum specimens collected prior to ICI-treatment initiation. Overall, TPOAb, TgAb or both were elevated in 46 patients (28%). Anti-thyroid antibody positivity rates were low in patients who did not develop a thyroid irAE, with only 1 patient (3%) affected by a positive TPOAb (Table 2.1). TPOAb and TgAb positivity rates were higher in patients who ultimately developed a thyroid irAE, affecting 21% and 32% of patients, respectively. In patients with thyroid irAEs and positive antibodies, 22/45 (48%) were positive for both TPOAb and TgAb, whereas no euthyroid patients were positive for both antibodies. The prevalence of anti-thyroid antibody positivity, including dual antibody positivity, was higher in patients with overt thyroid irAEs (thyrotoxicosis or hypothyroidism) compared to those with subclinical thyroid irAEs (Table 2.2).

Table 2.3: Demographic associations with ICI-associated thyroid irAEs (including subgroup analyses of overt thyroid irAEs) compared with patients that remained euthyroid during follow-up

Factor	Any thyroid irAE OR (95% CI)	*p*-value	Overt thyrotoxicosis OR (95% CI)	*p*-value	Overt hypothyroidism OR (95% CI)	*p*-value
Age (per 10-years)	0.88 (0.81-0.96)	0.005	0.83 (0.72-0.95)	0.007	0.87 (0.68-1.10)	0.23
Sex (female vs male)	1.62 (1.27-2.08)	<0.001	2.02 (1.37-2.95)	<0.001	3.31 (1.67-6.56)	0.001
Immune checkpoint inhibitor						
- CTLA-4+PD-1 combination vs CTLA-4	3.76 (2.49-5.75)	<0.001	10.8 (4.51-25.6)	<0.001	7.28 (0.89-59.4)	0.06
- CTLA-4+PD-1 combination vs PD-1	1.90 (1.45-2.49)	<0.001	3.30 (2.23-4.90)	<0.001	0.70 (0.31-1.59)	0.40
Baseline TSH (per 1.0 mIU/L)	1.05 (0.91-1.22)	0.48	1.08 (0.84-1.38)	0.55	2.33 (1.61-3.33)	<0.001
Brain metastases (absent vs present)	0.71 (0.53-0.94)	0.02	0.78 (0.50-1.22)	0.27	0.70 (0.31-1.59)	0.39

Prognostic significance:

Median follow-up was not different between patients with thyroid irAEs (11.5 months; IQR 5.0-23.9) and patients without thyroid irAEs (11.1 months; IQR 4.8-25.3). No difference in treatment response (iRECIST criteria (189)) was observed in patients with ICI-associated thyroid irAEs relative to patients that remained euthyroid (58% vs. 56%; p=0.55). Similarly, mortality did not differ between patients with and patients without thyroid irAEs (33% vs 37%; p = 0.14). After adjustment for age, sex, brain metastases, and ICI-type, OS and PFS did not differ between patients with thyroid irAEs and patients that remained euthyroid. In thyroid irAE patients, hazard ratios (HR) for OS and PFS were 0.95 (95% CI 0.77-1.16; p=0.59) and 0.97 (95% CI 0.81-1.16; p=0.74) respectively.

Survival analyses were performed separately for patients with overt thyrotoxicosis or overt hypothyroidism. Patients who developed overt thyrotoxicosis had longer OS (log-rank test; p=0.001) and PFS (log-rank test; p=0.001) compared to patients who did not experience a thyroid irAE. These results were confirmed by multivariable Cox regression analysis (adjusted for age, sex, brain metastases and ICI-type) with a HR of 0.57 (95% CI 0.39-0.84; p=0.005) for OS and a HR of 0.68 (95% CI 0.49-0.94; p=0.02) for PFS (Figure 2.3). No improvement in PFS or OS was observed in association with overt hypothyroidism.

Discussion:

We report clinical and biochemical characteristics of ICI-associated thyroid irAEs in the largest cohort of patients studied to date. The incidence of thyroid dysfunction in our study was substantially higher than reported in other cohorts with sufficient patient numbers to examine this condition (127, 133, 183). We confirmed that combined ICI treatment resulted in more frequent and severe thyroid irAEs (166, 190). Notably, with the power of our large

dataset we were able to clearly delineate subsets of thyroid dysfunction and describe new clinical and biochemical associations. Our findings suggest that ICI-associated overt thyrotoxicosis (with or without subsequent hypothyroidism) and hypothyroidism (without preceding thyrotoxicosis) may be etiologically distinct, rather than different presentations of the same disease process.

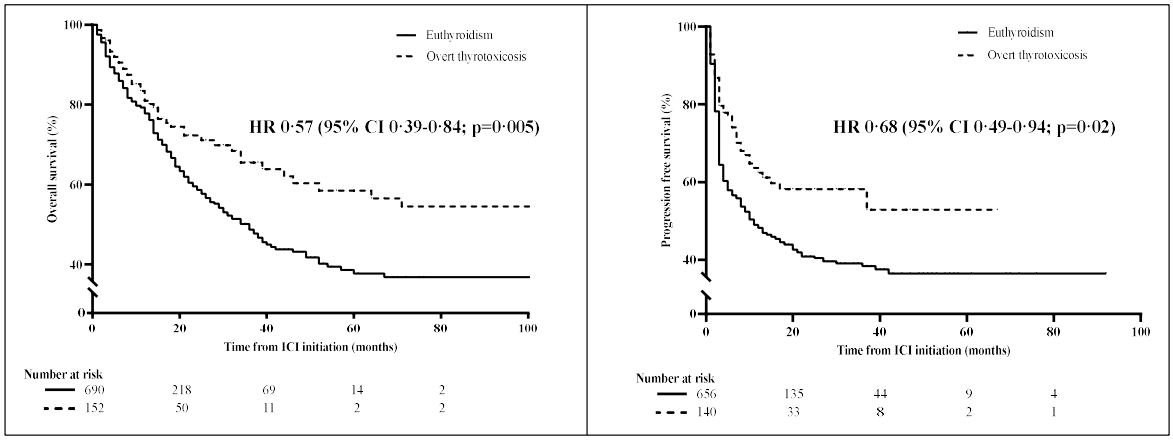

Figure 2.3: Overall survival and progression free survival in patients with thyroid irAEs

(A) Overall survival in patients with overt primary thyrotoxicosis relative the patients who remained euthyroid. (B) Progression free survival in patients with overt primary thyrotoxicosis relative the patients who remained euthyroid.

Whereas previous studies have failed to identify clinical factors related to thyroid irAEs, our study identified multiple new demographic characteristics that were associated with incident thyroid irAEs. In our cohort, female sex was a strong predictor of thyroid irAEs. An association between sex and thyroid irAE risk had not previously been reported, possibly due

to small sample sizes and lack of statistical power (142, 156). A predilection of thyroid irAEs toward women is similar to the female preponderance of autoimmune thyroid disease (AITD) observed in the general population (191). Age also acted as an independent risk factor for thyroid irAEs, with younger patients experiencing higher rates of thyrotoxicosis, but not hypothyroidism. These findings are consistent with other forms of thyroiditis, such as subacute thyroiditis, which typically develop between the third and fifth decades (192, 193).

Kinetic changes of TSH and FT4 that occurred following ICI treatment demonstrated several distinct patterns. In patients with overt thyrotoxicosis, 93% presented with abnormal thyroid function within three months of ICI treatment initiation compared to only 38% of patients presenting with overt hypothyroidism as the primary manifestation of ICI-associated thyroid irAE. Whereas the time to onset of thyrotoxicosis was uniform, there was marked heterogeneity in the time to onset of hypothyroidism, ranging from one week to 70 weeks after the start of ICI treatment. The mechanisms underpinning late onset hypothyroidism are poorly characterized. However, anti-thyroid antibody positivity rates in hypothyroid patients approached 50%, suggesting that late onset hypothyroidism may be secondary to ICI-mediated acceleration of lymphocytic infiltration of the thyroid in the context of a subclinical HT that predated ICI-treatment.

The role of anti-thyroid antibodies in the pathogenesis of ICI-associated thyroid irAEs remains controversial and poorly characterized (166). We measured baseline TPOAb and TgAb status using stored serum samples in 163 ICI-treated patients using a single assay and within a single run. Our results clearly demonstrated an association between TPOAb and/or TgAb positivity at baseline and the incidence of ICI-associated thyroid irAEs. Interestingly, these findings share similarity to other causes of thyroiditis (ie. postpartum), where

antithyroid antibodies are present in up to 50% of affected patients but may not be pathognomonic (194). TPOAb and TgAb positivity was particularly prominent in patients who developed overt thyroid dysfunction, suggesting that pre-existing thyroid autoimmunity may be a potent risk factor for future development of ICI-associated thyroid toxicity. This hypothesis is strengthened by our additional findings that TPOAb and TgAb titres and dual antibody positivity rates were higher in patients with overt thyroid irAEs compared to patients with subclinical or no thyroid irAE.

As expected, thyrotoxicosis was significantly associated with the type of ICI treatment received. Combination therapy resulted in significantly higher rates of both overt and subclinical thyrotoxicosis relative to CTLA-4 and PD-1 inhibitors used alone. However, no such association was observed with respect to overt hypothyroidism, where the incidence of thyroid irAEs was similar across all ICI types. Baseline TSH concentrations were significantly higher in patients who developed overt hypothyroidism as their initial thyroid irAE. The association of hypothyroidism and baseline TSH had been observed previously and again may suggest progression of a pre-existing, subclinical HT accelerated by ICI-treatment, rather than a de novo ICI-mediated thyroiditis (118, 142, 171). Our data are consistent with this hypothesis, although our finding of differential thyroid irAE sensitivity to ICI-type and high prevalence of antithyroid antibodies needs to be validated in other large ICI-patient cohorts.

Overall, thyroid irAEs were associated with increased rates of extra-thyroidal irAEs (hepatitis, colitis, etc.) including multi-system and CTCAE grade 3 or higher (more severe) irAEs relative to euthyroid patients. Increased rates of non-thyroidal irAEs were driven mainly by patients with overt thyrotoxicosis. Primary hypothyroidism alone was not

associated with extra-thyroidal, multi-system or severe irAEs, which in this group of patients occurred at similar rates to that observed in patients who remained euthyroid. Taken together, our data suggest that overt thyrotoxicosis could be a surrogate marker of more robust immune response to ICI-treatment. Overt thyrotoxicosis was associated with high rates of extra-thyroidal immune toxicity, but also improved cancer survival outcomes. Similar autoinflammatory sequalae and survival improvements were not observed in association with other thyroid irAE subtypes. Previous studies have inconsistently reported benefit in PFS and OS associated with thyroid irAEs (71, 73, 162, 164). Improvements in PFS and OS have been reported with overt relative to subclinical thyroid irAEs, but no studies had examined the differential effect of thyrotoxicosis and hypothyroidism with respect to cancer survival (132, 183). Differences in clinical and biochemical presentation of ICI-associated thyroid irAEs in our study support the hypothesis that ICI-associated thyrotoxicosis and hypothyroidism represent distinct clinical entities with differing underlying pathogenesis – thyrotoxicosis as an acute destructive thyroiditis and hypothyroidism as a progression of pre-existing autoimmune lymphocytic thyroiditis.

Our study had limitations inherent to the retrospective study design. Although patients were followed prospectively, and thyroid function tests were usually performed on a 3-4 weekly schedule, not all patients had tests performed at uniform time points, and it is possible that some cases of thyroid dysfunction may have been missed. We did not routinely test for TSH-receptor antibodies in hyperthyroid patients and therefore GD cannot be excluded. However, all patients in our study developed transient thyrotoxicosis which was self-limited and resolved without treatment. We believe this makes GD highly unlikely. Stored serum at baseline was not available for all patients. As such, anti-thyroid antibodies were measured in

a subset of patients only. However, we believe these results can be generalized to the larger cohort, as patients were sampled from all subgroups of thyroid dysfunction and demographically were not significantly different from untested patients of their respective thyroid irAE subtypes.

In summary, this study comprehensively details the clinical and biochemical features of ICI-associated thyroid irAEs. As the use of ICIs continue to increase, irAEs (thyroid and non-thyroid) will become an increasingly key component of endocrine and oncology practice. Our study reports new associations of thyroid irAEs with age, sex, TPOAb/TgAb, extra-thyroidal irAEs, and other factors, which in totality suggest there are multiple mechanisms underpinning development of thyroid dysfunction following ICI treatment.

Conclusion

This work represents the most comprehensive description of thyroid irAEs to date in a pure cohort of 1246 patients with melanoma, free from pre-existing thyroid disease, undergoing ICI-treatment. We reported multiple novel aspects of thyroid irAEs, including a prevalence of >50% in patients receiving combined CTLA-4 and PD-1 checkpoint inhibition. We produced a detailed description of the kinetic changes in thyroid function that occur following ICI-treatment, with most affected patients experiencing a rapid onset of transient thyrotoxicosis followed by spontaneous restoration of euthyroidism or progression to hypothyroidism. However, we also showed that a small number of patients have delayed presentations, sometimes years after the commencement of ICI-treatment. We identified age, sex, and baseline TSH level as being associated with the development of a thyroid irAE and were the first to show that the most severe presentations of thyroiditis following ICI-treatment were associated with major improvements in cancer related PFS and OS.

Chapter 3

Association of anti-thyroid antibodies in checkpoint inhibitor-associated thyroid irAEs

3.1 Introduction

Apart from the relationship to ICI-treatment, our previous work in chapter 2 demonstrated that thyroid irAEs have a similar clinical phenotype to painless thyroiditis that occurs sporadically due to thyroid autoimmunity (167). In patients with painless autoimmune thyroiditis, thyroid autoantibodies are present in up to 90% of cases (82). Based on these observations, we hypothesized that autoimmune thyroiditis and thyroid irAEs may share mechanistic similarities with a similarly high prevalence of anti-thyroid antibodies in patients that experience a thyroid irAE. Prior to our study, reports in the literature were sparse, and mainly consisted of small retrospective studies at high risk for bias, where anti-thyroid antibodies had been measured unsystematically and without a control population. To address this knowledge gap, and to test our hypothesis that anti-thyroid antibodies were likely to be implicated in thyroid irAE pathogenesis, we systematically measured anti-thyroid antibodies via radioimmunoassay in ICI-treated cancer patients with and without thyroid irAEs. Additionally, we measured TRAB antibodies to definitively show they are not routinely involved in thyroid irAE pathogenesis and looked at IL-6 levels, a pro-inflammatory cytokine that has been implicated in irAEs affecting other organs such as the colon. The results of this study were published in the *Journal of Clinical Endocrinology and Metabolism* in 2022 and are presented in Section 3.2 (for in print version, see Appendix 4).

3.2 Retrospective cohort study of TPOAb, TgAb, TRAB, and IL-6 levels in patients with melanoma receiving ICI treatment.

Published in the *Journal of Clinical Endocrinology and Metabolism*® (see Appendix 4 for print version)

ICIs are associated with wide ranging irAEs (43). The thyroid is particularly susceptible, and thyroid dysfunction is one of the most common irAEs (78, 166). The etiology of thyroid irAEs is not fully understood and multiple mechanisms may underly disease onset (167, 195). The significance of TPOAb and TgAb positivity on risk for thyroid irAEs remains unknown.

We recently described thyroid irAEs in a large cohort of ICI-treated patients with melanoma (167). We showed that TPOAb and TgAb were mainly found in association with biochemically overt thyroid irAEs, particularly overt thyrotoxicosis where we also found an association with longer PFS and OS (167). Based on these findings, the current study aimed to further characterize the association of TPOAb/TgAb and changes in TPOAb/TgAb during treatment with thyroid irAEs, with a particular interest in patients with overt thyrotoxicosis. TRAB and IL-6, a proinflammatory cytokine associated with irAEs in other non-endocrine organ systems, were also studied.

Materials and methods:

Study design and participants:

We included adult patients undergoing ICI treatment for melanoma with biobank serum samples to study the prevalence and kinetics of TPOAb, TgAb and IL-6. Demographic, treatment, and cancer outcome information was extracted from a prospectively maintained central database. As part of routine care, measurement of serum TSH was performed in all patients prior to commencement of immunotherapy and thyroid

function was repeated at subsequent ICI doses q3-4 weekly. Biobank samples collected and stored at baseline (prior to receipt of first ICI dose) and at onset of thyroid irAE (or 30-60 days after treatment initiation in patients that remained euthyroid) were retrieved and used to measure TPOAb, TgAb, TRAB and IL-6 levels.

Definitions:

Thyroid irAEs were defined by new thyroid dysfunction developing after treatment with an ICI and were sub-categorized by the biochemical pattern of thyroid dysfunction. Overt thyrotoxicosis was defined by a TSH level below the lower reference interval with concomitant elevation of FT4 above the upper reference interval. Subclinical thyrotoxicosis was defined by a TSH level below the lower reference interval in the presence of normal serum concentrations of FT4. Overt hypothyroidism was defined by a TSH level above the upper reference limit with concomitant FT4 levels below the lower reference interval and no preceding thyrotoxic phase.

Assay characteristics:

Serum concentration of thyroid hormones were measured using the Abbott Architect I-2000 immunoassay (TSH catalog # 7K62, RRID: AB_2883972; FT4 catalog # 7K65, RRID: AB_2801665). Local reference intervals for TSH and FT4 were set at 0.40-4.20 mIU/L and 9.0-19.0 pmol/L respectively. TPOAb and TgAb titers were measured using Abbott Architect with values of >5.61 IU/mL and >4.11 IU/mL considered positive, respectively (TPOAb catalog # 2K47, RRID: AB_2904180; TgAb catalog # 2K46, RRID: AB_2904179). TRAB levels were measured using Roche Diagnostics cobas e601 analyzer (catalog # 04388780190, RRID: AB_2801453), with a measuring range of 0.8-40.0 IU/L (CV 7.6, 1.9 and 0.9% for TRAB levels of 1.7, 5.9 and 24.6 IU/L

respectively). TRAB titers >1.8 IU/L were considered positive. IL-6 was measured using Siemens IMMULITE 2000-XPI enzyme-labelled, chemiluminescent immunometric assay (ELISA; catalog # L2K6P2, RRID: AB_2904178) with the reference range set at 0.0-3.4 pg/mL.

Procedures and outcomes:

The primary outcome was change in TPOAb and TgAb from baseline during ICI-treatment and whether increases in TPOAb/TgAb were associated with the development of a thyroid irAE. Change in TPOAb or TgAb from baseline were considered present if there was new antibody positivity or a >50% increase in antibody titer for patients already positive at baseline. Other outcomes of interest included IL-6 levels at baseline and change in IL-6 level during ICI-treatment. Additionally, TRAB levels were measured in patients at time of development of thyroid irAE to exclude GD.

Statistical analysis:

Baseline characteristics are presented as median and IQR or frequency and percentages. Changes in TPOAb, TgAb and IL-6 from baseline were tested using Wilcoxon signed rank test. All statistical tests were 2-tailed with two-sided significance level set at p=0.05. Statistical analyses were conducted using SPSS version 26 (SPSS Inc, IL, USA) and figures were created using GraphPad Prism, version 8. All patients consented for their data to be recorded in the MIA central database, and for that information to be used for research purposes. Ethical approval was provided by the Sydney Local Health District human research and ethics committee.

Results:

Patient characteristics:

Paired serum samples at baseline and on treatment were obtained for 122 patients. Samples came from patients with subclinical thyrotoxicosis (n=47), overt thyrotoxicosis (n=37), overt hypothyroidism (n=7) and persistent euthyroidism (n=31). The median follow-up of the study cohort was 13.8 months (IQR 5.6-25.2). At baseline, 19 patients (16%) had TPOAb elevated, and 28 patients (23%) had TgAb elevated above the upper reference interval. Sixteen patients (13%) were positive for both TPOAb and TgAb. Baseline characteristics of all patients are presented in Table 3.1 and stratified by thyroid irAE subtype in Table 3.2.

Table 3.1: Baseline characteristics

	All patients (n=122)
Sex; n (%) - Male - Female	 73 (60) 49 (40)
Age (years); median (IQR)	65 (53-75)
Checkpoint inhibitor; n (%) - CTLA-4 - PD-1/PD-L1 - CTLA-4 + PD-1	 3 (2) 75 (62) 44 (36)
TSH (mIU/L); median (IQR)	1.27 (0.91-1.82)
FT4 (pmol/L); median (IQR)	13.1 (12.1-14.4)
TPOAb positive; n (%)	19 (16)
TPOAb titre (IU/mL); median (IQR)	0.33 (0.10-0.75)
TgAb positive; n (%)	28 (23)
TgAb titre (IU/mL); median (IQR)	0.77 (0.00-3.32)
Dual antibody positive; n (%)	16 (13)
Interleukin-6 elevated; n (%)	65 (53)
Interleukin-6 (pg/mL); median (IQR)	3.7 (1.3-6.0)

Table 3.2: Baseline characteristics by thyroid irAE subtype

	Euthyroid	Subclinical thyrotoxicosis	Overt thyrotoxicosis	Overt hypothyroidism
N	31	47	37	7
Sex; n (%) - Male - Female	 23 (74) 8 (26)	 28 (60) 19 (40)	 20 (54) 17 (46)	 2 (29) 5 (71)
Age (years); median (IQR)	68 (58-77)	69 (57-75)	55 (44-64)	74 (56-81)
Checkpoint inhibitor; n (%) - CTLA-4 - PD-1/PD-L1 - CTLA4 + PD1	 2 (6) 22 (71) 7 (23)	 1 (2) 27 (57) 19 (41)	 0 (0) 20 (54) 17 (46)	 0 (0) 6 (86) 1 (14)
TSH (mIU/L); median (IQR)	1.56 (1.16-1.90)	0.96 (0.58-1.40)	1.32 (0.92-1.77)	3.29 (1.94-3.33)
FT4 (pmol/L); median (IQR)	12.5 (11.1-14.0)	13.1 (12.5-14.2)	13.7 (12.1-15.3)	13.6 (12.5-14.3)
TPOAb positive; n (%)	1 (3)	2 (4)	13 (35)	3 (43)
TPOAb titre (IU/mL); median (IQR)	0.17 (0.09-0.39)	0.24 (0.07-0.43)	0.57 (0.34-78.9)	0.38 (0.18-278)
TgAb positive; n (%)	0 (0)	5 (11)	20 (54)	3 (43)
TgAb titre (IU/mL); median (IQR)	0.80 (0.00-1.45)	0.41 (0.00-0.92)	6.55 (0.91-43.6)	0.40 (0.0-427)
Dual antibody positive; n (%)	0 (0)	2 (4)	11 (30)	3 (43)
Interleukin-6 elevated; n (%)	17 (55)	21 (45)	23 (62)	4 (57)
Interleukin-6 (pg/mL); median (IQR)	3.6 (1.0-6.3)	3.1 (1.0-5.7)	4.1 (2.1-6.2)	4.7 (3.0-7.0)

Thyroglobulin antibody:

TgAb positivity at baseline was 100% specific (31% sensitive) for development of a thyroid irAE. Five patients (11%) with subclinical thyrotoxicosis had a positive TgAb at baseline. By comparison, 20 patients (54%) with overt thyrotoxicosis and 3 patients (43%) with overt hypothyroidism had a positive TgAb at baseline (Figure 3.1a). No patients that remained euthyroid had a positive TgAb at baseline. In patients with a positive baseline TgAb, median TgAb titre was significantly higher in patients with overt thyroid irAEs compared to patients with subclinical irAEs (Table 2, $p<0.001$). In patients with overt thyrotoxicosis, progression to hypothyroidism following the initial thyrotoxic phase was significantly higher in TgAb positive patients compared to TgAb negative patients (85.0% vs. 41.2%; $p=0.01$). No patients remained thyrotoxic and the remainder spontaneously returned to euthyroidism.

During ICI treatment, 34 patients experienced a >50% increase in TgAb titre or became positive from a negative baseline level. In 24/34 (71%) patients, increases or new positivity in TgAb were observed in association with overt thyrotoxicosis. TgAb positivity at baseline had a positive predictive value of 71.4% and a positive likelihood ratio of 6.0 for future development of overt thyrotoxicosis. Conversely, absence of TgAb had a negative predictive value of 81.9% and a negative likelihood ratio of 0.5 for future development of overt thyrotoxicosis. In patients with overt thyrotoxicosis, TgAb titer at the time of thyroid dysfunction was significantly increased compared to baseline (37.6 vs 6.55 IU/mL; $p<0.001$). Treatment-related increases in TgAb were not observed in patients who remained euthyroid or in patients with subclinical thyrotoxicosis and overt hypothyroidism (Figure 3.2a).

Thyroid peroxidase antibody:

TPOAb positivity at baseline was 97% specific (20% sensitive) for identification of patients destined to develop a thyroid irAE. Two patients (4%) with subclinical thyrotoxicosis had a positive TPOAb at baseline. By comparison, 13 patients (35%) with overt thyrotoxicosis and 3 patients (43%) with overt hypothyroidism had a positive TPOAb at baseline (Figure 3.1b). Only 1 patient (3%) who remained euthyroid had a positive TPOAb at baseline. In patients with a positive baseline TPOAb, median TPOAb titer was significantly higher in patients with overt thyroid irAEs compared to patients with subclinical irAEs (Table 2, $p<0.001$). In patients with overt thyrotoxicosis, progression to hypothyroidism following the initial thyrotoxic phase was not significantly different in TPOAb positive patients compared to TPOAb negative patients (76.9% vs. 58.3%; $p=0.26$). No patients remained thyrotoxic and the remainder spontaneously returned to euthyroidism.

During ICI-treatment, 16 patients experienced a >50% increase in TPOAb titer or became positive from a negative baseline level. In 13/16 (81%) patients, increases or new positivity in TPOAb were observed in association with overt thyrotoxicosis. TPOAb positivity at baseline had a positive predictive value of 68.4% and a positive likelihood ratio of 5.0 for future development of overt thyrotoxicosis. Conversely, absence of TPOAb had a negative predictive value of 76.6% and a negative likelihood ratio of 0.7 for overt thyrotoxicosis. In patients with overt thyrotoxicosis, TPOAb titer at the time of thyroid dysfunction was significantly increased compared to baseline ($p<0.001$). Significant treatment-related increases in TPOAb were not observed in

patients who remained euthyroid or in patients with subclinical thyrotoxicosis and overt hypothyroidism (Figure 3.2b).

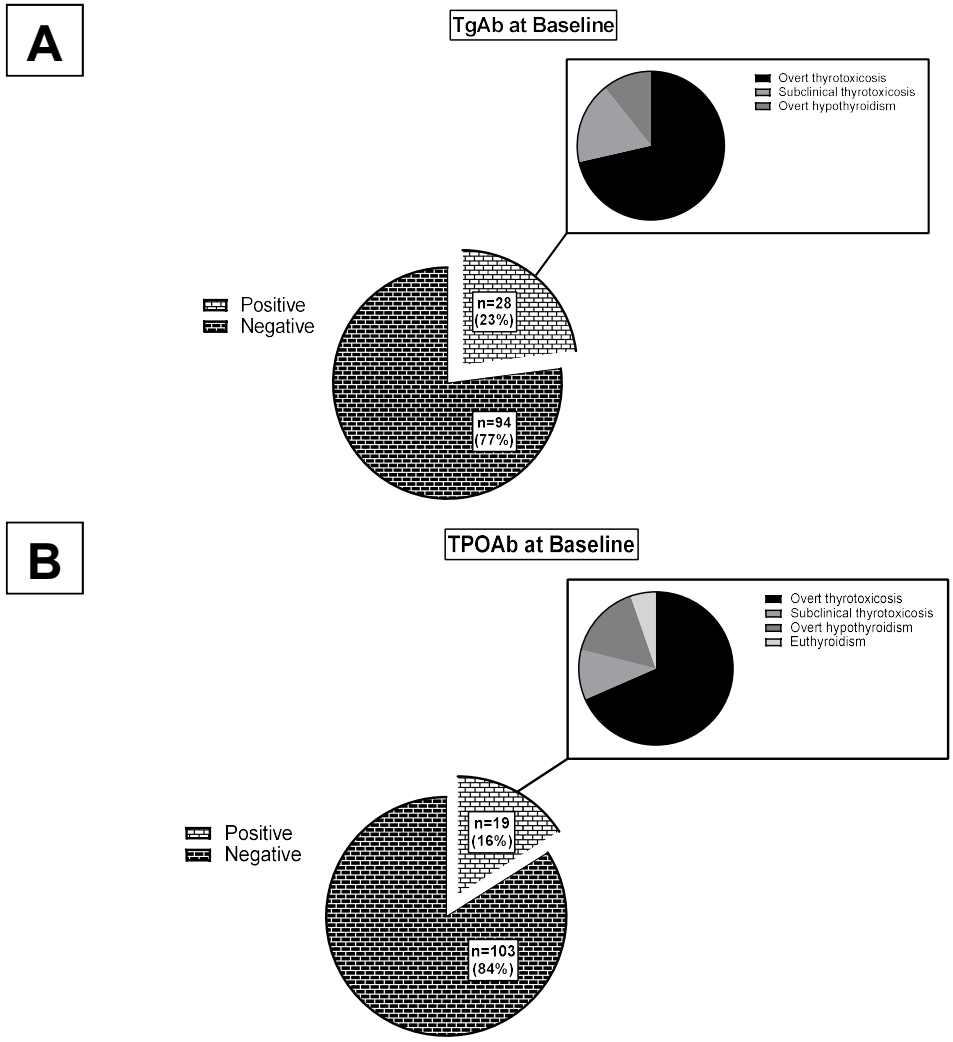

Figure 3.1: Prevalence of TPOAb and TgAb at baseline.

(A) Proportion of patients with positive TgAb at baseline by thyroid irAE subtype. (B) Proportion of patients with positive TPOAb at baseline by thyroid irAE subtype.

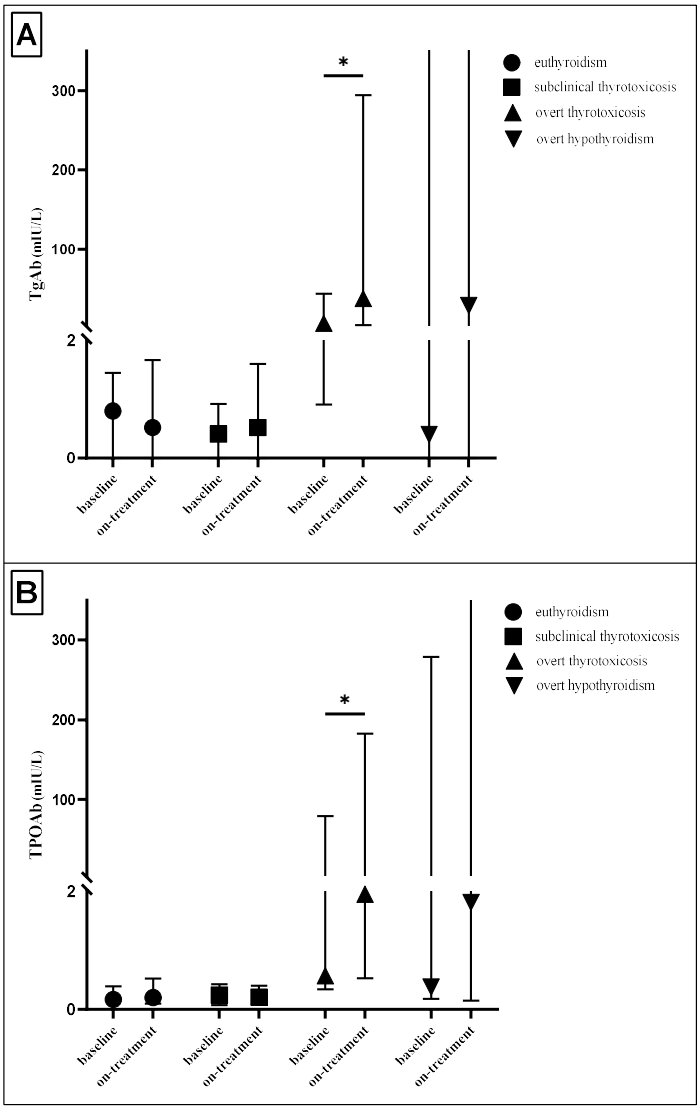

Figure 3.2: Change in TPOAb and TgAb titer following exposure to ICI-treatment.

(A) Median and interquartile range of TgAb at baseline and during ICI-treatment by thyroid irAE subtype (* p<0.001). (B) Median and interquartile range of TPOAb at baseline and during ICI-treatment by thyroid irAE subtype (* p<0.001).

TSH-receptor antibody:

TRAB was measured at the time of thyroid irAE development in all patients. TRAB positivity was not documented in any patient.

Interleukin-6:

Elevation of IL-6 at baseline was present in 65 (53%) patients. Median baseline IL-6 measured 3.70 pg/mL (IQR 1.3-6.0). Rates of IL-6 elevation and absolute IL-6 levels were not different in patients with positive TPOAb or TgAb and those without. Similarly, IL-6 levels were not different across different subtypes of thyroid irAE (Table 3.2). During ICI-treatment there was a significant increase in IL-6 levels from baseline in patients with overt hypothyroidism (p=0.03). Treatment-related increases in IL-6 were not observed in patients who remained euthyroid or in patients with subclinical or overt thyrotoxicosis (Figure 3.3).

Discussion:

This study examined the prevalence and change in TPOAb and TgAb during ICI-treatment in patients with melanoma. We found the prevalence of TPOAb and TgAb positivity to be higher in patients who developed overt thyroid dysfunction (thyrotoxicosis or hypothyroidism) compared to patients who remained euthyroid or those with subclinical thyrotoxicosis. However, only overt thyrotoxicosis was associated with large increases or new positivity in TPOAb and TgAb during ICI-treatment. In patients with overt thyrotoxicosis, TgAb positivity was associated with progression to permanent hypothyroidism following an initial thyrotoxic phase. TPOAb positivity was also higher in patients who developed hypothyroidism following an

initial thyrotoxic phase, although the association did not attain statistical significance in our cohort.

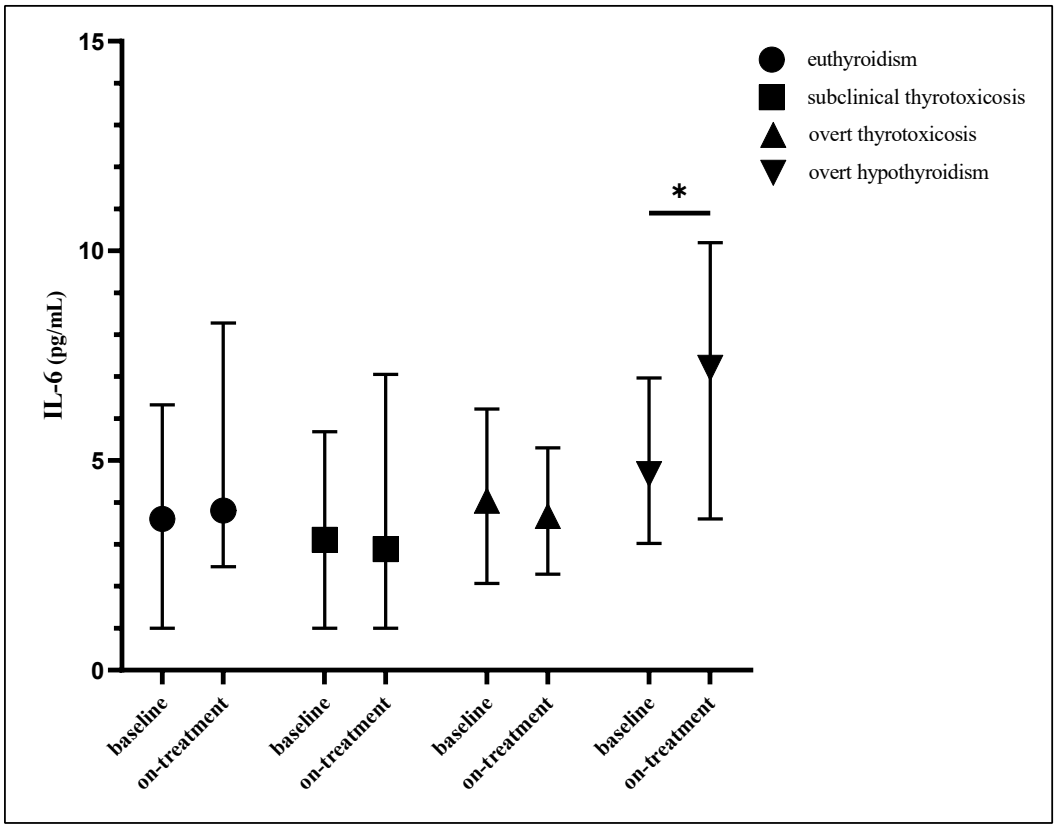

Figure 3.3: Change in IL-6 level following exposure to ICI-treatment

Median and interquartile range of IL-6 at baseline and during ICI-treatment by thyroid irAE subtype (* p=0.03)

Thyroid irAE onset is variable and can be missed as symptoms may be minimal or conflated with those related to the underlying malignancy (166). Pre-treatment selection of at-risk patients would be clinically useful, as overt thyrotoxicosis commonly

progresses to permanent hypothyroidism and treatment with thyroid hormone replacement may be delayed (167). Predictive biomarkers to identify patients at risk of thyroid irAEs could promote timely diagnosis and reduce risk of thyroid irAE related morbidity.

TPOAb or TgAb are positive in >90% of patients with autoimmune thyroiditis, but their relevance to thyroid irAE pathogenesis is poorly characterized (82). The clinical course of thyroid irAEs often mimics painless sporadic thyroiditis, a subtype of AITD. Thyroid irAEs are associated with higher TPOAb and TgAb positivity rates relative to the general population, but do not approach the 90% prevalence observed in AITD and therefore irAE pathogenesis cannot be solely antibody related (126, 127, 156, 196). In our study, all but one patient with positive TPOAb or TgAb went on to develop thyroid dysfunction, although all patients with thyroid irAEs did not have positive anti-thyroid antibodies. It may be that a subgroup of thyroid irAE patients with positive TPOAb or TgAb are predisposed to AITD, which is unmasked following inhibition of immune checkpoints important for the maintenance of self-tolerance in the thyroid (176). This is supported by other studies showing early onset of thyroid irAE in antibody positive patients (156, 172).

Immune-related adverse events correlate with improvements in PFS and OS in immunotherapy treated cancer patients (45). Thyroid irAEs are often less severe than irAEs in other organ systems but have also been shown to associate with improved cancer survival, particularly when thyroid dysfunction is overt (132, 162, 164, 167, 183). Limited data has been published on the significance of anti-thyroid antibodies with respect to PFS and OS. A retrospective analysis of 137 patients with advanced

non-small cell lung cancer treated with PD-1 inhibitors did not find improved OS or PFS in patients with positive anti-thyroid antibodies (185). However, TPOAb and TgAb were associated with improved PFS and OS in patients with levels above compared to below the median value in a second cohort of 168 anti-PD-1 treated patients (183). Our previous work in a large cohort of over 1200 patients demonstrated that overt thyrotoxicosis (relative to no thyroid irAE or subclinical thyroid irAEs) were associated with increased immune toxicity in other organ systems as well as improved cancer survival and is hypothesized to be a surrogate marker of patients who demonstrate more robust immune activation following ICI-treatment (167). In our current study, we found higher incidence and titers of TPOAb and TgAb in patients with overt thyrotoxicosis but were not powered to examine the effect of antibody positivity on cancer survival.

Dermatologic irAEs and colitis have been associated with low baseline IL-6 levels or increases in IL-6 during ICI treatment (197, 198). We did not find an association between baseline IL-6 and thyroid irAEs in our study. This is consistent with one other study examining the role of IL-6 in thyroid irAEs (172). However, we did observe a significant increase in IL-6 at time of diagnosis in patients who developed overt hypothyroidism which was not observed with other thyroid irAE subtypes, potentially supporting our hypothesis that thyrotoxicosis and hypothyroidism arise via distinct mechanisms in the setting of ICI-associated thyroid dysfunction.

Strengths of our study include the use of uniformly stored samples, which were processed together in a single run to limit analytical variation. Limitations of our study are that it may have been underpowered to measure associations of TPOAb and TgAb with overt hypothyroidism. Although numerically TPOAb and TgAb values did

increase in patients with overt hypothyroidism during ICI-treatment, samples were available for only 7 patients. Although highly specific, TPOAb and TgAb lacked sensitivity, which reduces their utility as a clinically useful biomarker. Anti-thyroid antibodies other than TPOAb and TgAb may be involved in pathogenesis of thyroid irAEs (199). We did not test non-classical anti-thyroid antibody levels and cannot exclude that they may have contributed to development of thyroid irAEs in antibody negative patients.

In conclusion, ICI-treatment indications are rapidly expanding and thyroid toxicity is a common side effect of anti-CTLA-4 and anti-PD-1 based treatment (43). Predictive biomarkers will be important to identify patients at risk of irAEs, and our data suggest that baseline TPOAb and TgAb are highly specific and may be useful to identify patients at risk of overt thyrotoxicosis. Prospective studies are warranted to further evaluate the utility of TPOAb and TgAb as predictive biomarkers.

Conclusion

The results of this study prospectively examined the importance of TPOAb and TgAb to the development of thyroid irAEs in 122 patients. It represents the first study to address this issue in a standardized fashion, and in so doing clarifies the clinical relevance of these biomarkers and their association with thyroid irAE susceptibility. Furthermore, we showed that TRAB is not a key driver of thyrotoxicosis in the ICI-setting, and that use of the inflammatory cytokine IL-6 has no value in identifying patients at risk of thyroid irAEs, whether measured at baseline or during the initial period of thyroid dysfunction.

Chapter 4

Immune cell representations following immune checkpoint blockade in patients experiencing thyroid irAEs

4.1 Background

Thyroid irAEs are a frequent side effect of ICI-treatment and often result in permanent hypothyroidism requiring lifelong thyroid hormone replacement (166, 200). However, thyroid irAEs have also been associated with significant improvements in cancer-related PFS and OS, which offsets morbidity related to thyroid dysfunction for many affected patients (167). Despite the frequency of thyroid irAEs and their prognostic importance, the pathogenic mechanisms driving onset remain incompletely characterized. Enhanced knowledge of these mechanisms is important, as they could improve identification of at risk patients, prevent unnecessary treatment interruption and hospitalization, and enable development of next generation ICIs with potent anti-tumor efficacy, but improved safety profiles (201).

Phenotypically, thyroid irAEs demonstrate similarities, but also distinct differences, to autoimmune thyroiditis occurring in the non-ICI-treatment setting (167). Therefore, overlapping mechanistic explanations that could account for both conditions were initially sought. In AITD, the role of immune checkpoints (particularly PD-1) appears critical. Multiple SNPs in genes encoding CTLA-4, PD-1 and PD-L1 have been associated with susceptibility to AITD (202-205). Furthermore, relative to patients with multinodular goiter (a non-autoimmune thyroid disease), patients with AITD have increased circulating PD-1^+ T-lymphocytes in peripheral blood and markedly increased PD-1^+ T-cell infiltrates within the thyroid gland itself (176). PD-L1 expression is also

increased in thyroid follicular cells of patients with AITD (176). However, despite intense activation of the PD-1/PD-L1 axis locally in the thyroid, PD-1 mediated dampening of the immune response is insufficient to prevent progression of AITD (176). In thyroid irAEs, the PD-1 axis is also intimately involved due to inhibition of the immune-tolerant effects of PD-1/PD-L1 using targeted monoclonal antibodies. This may explain why there is usually a rapid evolution of thyroiditis in the ICI-setting, whereas HT is often indolent and slowly progressive. Similar histologic findings to AITD have been identified in thyroid irAE patients, with markedly increased numbers of PD-1^+ $CD4^+$ and $CD8^+$ T-lymphocytes infiltrating the thyroid gland compared to healthy controls (164). Concurrently, PD-L1 expression is also increased within the thyroid of patients who develop a thyroid irAE (206). Differences in circulating immune cell populations have been observed in patients with thyroid irAEs relative to healthy controls but overlap with immunophenotyping in HT patients (119, 177). More recent animal studies further elucidate potential mechanisms driving thyroiditis occurring in response to ICI-treatment, and suggest $CD4^+$ Th17 cells and gamma-delta T_{17}-cells could mediate development of ICI-associated thyroid irAEs (207). Th17-cells and IL-17 have been previously implicated in HT, although the role of gamma-delta T-cells in AITD is unknown (208, 209).

No study has yet compared immune cell subpopulations in patients with thyroid irAEs to ICI-treated patients who did not develop a thyroid irAE. Therefore, previous findings of specific immune cell changes occurring in association with thyroid irAEs may not be specific to thyroid toxicity, but to melanoma or an ICI-treatment effect more generally. To bridge this knowledge gap, we employed high dimensional time of flight mass

cytometry to compare immune cells at baseline and on treatment in ICI-treated patients with and without thyroid irAEs.

4.2 Materials and methods

We studied immune cell populations in peripheral blood of patients with melanoma receiving ICI-treatment. Patients were identified from our previous work and categorized into a thyroid irAE group and a euthyroid control group (167). Patients were allocated to the thyroid irAE group if they developed overt thyrotoxicosis within 12 weeks of commencing ICI-treatment. Patients were allocated to the control group if their TSH and FT4 levels remained within the normal reference interval over 12 weeks following initiation of ICI-treatment.

Peripheral blood mononuclear cells (PBMC) were retrieved from the MIA Biospecimen Bank at two timepoints for each patient with available samples. The first PBMC sample was collected at baseline, prior to ICI-treatment. The second PBMC sample was collected at the time of first abnormal TSH (thyroid irAE group) or at 8-12 weeks following the start of ICI-treatment (control group). As such, patient samples were allocated to one of four groups: (1) thyroid irAE group – baseline samples; (2) thyroid irAE group – on treatment samples; (3) no thyroid irAE (control) group – baseline samples; (4) no thyroid irAE (control) group – on treatment samples.

4.2.1 Mass cytometry sample preparation and treatment

Prior to mass cytometric analysis, cryopreserved PBMC samples underwent rapid thawing and resuscitation through slow dilution in warm Roswell Park Memorial Institute (RPMI)-1640 medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) and 1:10,000 universal nuclease (Thermo Fisher Scientific). Cell

counts were verified using Trypan blue exclusion variability dye, then washed in fluorescence-activated cell sorting (FACS) buffer containing Dulbecco's PBS supplemented with 1% FBS and 0.05% ethylenediaminetetraacetic acid (EDTA).

Immunophenotyping of PBMCs was performed using a selection of monoclonal antibodies covering a wide range of T-cell, B-cell, myeloid cell, and natural killer (NK) cell subpopulations. Specific antibodies used are shown in Table 4.1 and Table 4.2.

Table 4.1: Metal-conjugated antibodies used for mass cytometry analysis

Label	Antibody target	Clone	Manufacturer
89	CD8	RPA-T8	BioLegend
104	CD45	HI30	BioLegend
108	CD45	HI30	BioLegend
113	CD56	NCAM16.2	BD
115	Ki67	B56	BD
141	CD196 (CCR6)	11A9	BD
142	CD19	HIB19	BioLegend
143	CD117	104D2	BioLegend
144	TCRγδ	B1	BioLegend
145	PE	PE001	BioLegend
146	CD1c	L161	BioLegend
147	CD45RO	UCHL1	BioLegend
148	CD16	3G8	BD
149	CD45RA	HI100	BD
150	FITC	FIT-22	BioLegend
151	CD278 (ICOS)	DX29	BD

152	CD66b	G10F5	BD
153	CD68	KP1	BioLegend
154	CD3	UCHT1	BD
155	EOMES	WD1928	eBioscience
156	CD10	HI10a	BioLegend
158	CD33	WM53	BD
159	CD197 (CCR7)	150503	R&D Systems
160	CD14	M5E2	BD
161	CD274 (PD-L1)	29E.2A3	BioLegend
162	Foxp3	PCH101	eBioscience
163	CD183 (CXCR3)	G025H7	BioLegend
164	GATA3	L50-823	BD
165	Biotin	1D4-C5	BioLegend
166	CD185 (CXCR5)	RF8B2	BD
167	CD11c	Bu15	BioLegend
168	CD4	SK3	BioLegend
169	CD25	M-A251	BioLegend
170	CD152 (CTLA-4)	14D3	eBioscience
171	CD24	ML5	BioLegend
172	Cy5	CY5-15	Sigma
173	CD141	AD5-14H12	Miltenyi Biotech
174	HLA DR	L243	BioLegend
175	CD279 (PD-1)	EH12.2H7	BioLegend
176	APC	APC003	BioLegend
209	T-bet	4B10	BD

Table 4.2: Fluorophore-conjugated antibodies used for mass cytometry analysis

Antibody target	Clone	Fluorophore	Manufacturer
CCR3 (extracellular)	5B3	BIOTIN	BioLegend
CD161 (extracellular)	HP-3G10	FITC	BioLegend
GrZB (intracellular)	GB11	APC	BioLegend
RORɣT (intracellular)	Q21-559	Cy5	BD
CD11b (extracellular)	ICRF44	PE	BioLegend

Samples were plated in 96-well U-bottom plates for staining. Once plated, samples were centrifuged at room temperature for 5 minutes at 500-times gravity. To identify viable cells, resultant cell pellets were added to 100µl cisplatin (1:4000 dilution in RPMI; Fluidigm®) at room temperature. After three minutes, 100µl RPMI was added to quench the reaction. Cells were barcoded using metal-conjugated lymphocyte common antigen (CD45) antibodies in incubation for 30-minutes. Following incubation, samples were washed with FACS buffer and combined. The combined sample was suspended and incubated in an antibody cocktail containing fluorophore-conjugated surface antibodies for 30-minutes at 4°C. Following this step, FACS buffer was used to wash cells prior to resuspension and incubation in an antibody cocktail containing metal-conjugated surface antibodies for 30-minutes at 4°C. On completion of the two incubation steps, cells were again washed with FACS buffer, then permeabilized using BD Pharmagen transcription factor buffer set (BD Biosciences®) for 40-minutes at 4°C. Permeabilized cells were treated with fluorophore-conjugated intracellular antibodies for 40-minutes at 4°C, washed in Perm/Wash buffer (1:20 dilution; eBioscience™), followed by treatment with

metal-conjugated intracellular antibodies for a further 40-minutes at 4°C. Prior to fixation with 4% paraformaldehyde solution containing DNA intercalator (0.125µM iridium-191/193; Fluidigm®), cells were washed twice with Perm/Wash buffer and once with FACS buffer. Fixed cells were incubated overnight at 4°C. Following overnight incubation, cells were washed and diluted to 800,000 cells/mL in Milli-Q® water and 1:10 diluted EQ™ four element calibration beads (Fluidigm®) prior to filtering through a 35µm nylon mesh. Filtered cells were acquired at a rate of 200-400 cells/second using a CyTOF 2 Helios upgraded mass cytometer (Fluidigm®). Samples were run in 1 batch. Data from the Helios analysis were normalized using CyTOF software (Fluidigm®) analysis of concurrently run EQ beads.

4.2.2 Statistical analysis

Mass cytometry data was analyzed using FlowJo™ software version 10.6.1 (BD Biosciences®). To identify viable cells for analysis, pre-gating based on DNA intercalator and cisplatin staining, as well as CD45 expression was performed prior to data exportation. R-statistics was used to perform computational analyses with FlowSOM clustering performed via the R package, *Spectre* (210, 211). Principal component analysis (PCA) was performed through *Spectre*, with between group differences assessed by multivariate permutational analysis of variance (PERMANOVA) using the R package *vegan* (212). Prior to analysis, data were scaled (to promote balanced comparison between parameters) using the Euclidean distance calculated between points. Pairwise comparisons were made using the R package *pairwiseAdonis* with Holm's correction for multiple comparisons. To visualize immune cell populations, uniform manifold approximation and projection plots (UMAP) were

generated in combination with FlowSOM clustering data via *Spectre* (213). Unbiased cell sorting was initially employed to visualize data and identify cell populations of interest. Manual gating was performed as a sensitivity analysis to confirm changes identified in the initial unsupervised analysis and classify major immune cell populations within the sample using the following labels.

- The total cell population was identified by: CCR3, CCR6, CCR7, CD1c, CD3, CD4, CD8a, CD10, CD11b, CD11c, CD14, CD16, CD19, CD24, CD25, CD33, CD45RA, CD45RO, CD56, CD66b, CD68, CD117, CD141, CD161, CTLA-4, CXCR3, CXCR5, EOMES, Foxp3, GATA3, granzyme B, HLA-DR, ICOS, Ki67, PD-1, PD-L1, ROR$_\gamma$t, T-bet, and TCR$\gamma\delta$.
- The T-lymphocyte population was identified by: CCR7, CD3, CD4, CD8a, CD16, CD25, CD45RA, CD45RO, CD56, CXCR3, CXCR5, Foxp3, GATA3, granzyme B, Ki67, ROR$_\gamma$t, T-bet, and TCR$\gamma\delta$.
- The myeloid cell population was identified by: CCR3, CCR7, CD11b, CD11c, CD14, CD16, CD45RA, CD45RO, CD56, CD66b, CD68, CD117, CTLA-4, granzyme B, HLA-DR, ICOS, Ki67, and T-bet.

Statistical analyses performed outside R were conducted using SPSS, version 26 and additional figures were created using GraphPad Prism, version 8. Parametric and non-parametric tests were used to compare groups as appropriate. All statistical tests were two-tailed with two-sided significance level set at p=0.05. To compare samples between groups (i.e. thyroid irAE baseline vs. no thyroid irAE baseline), a Browne-Forsyth and Welch one way ANOVA with Dunnett T3 multiple comparison test was performed. To compare paired samples (i.e. thyroid irAE baseline vs. thyroid irAE toxicity), mixed

effects analysis with Šidák's multiple comparison test was used. Written consent was provided for each included patient to the MIA Biospecimen Bank, and institutional ethics approval for this study was obtained through the Sydney Local Health District human research ethics committee.

4.3 Results

Ten patients receiving ICI-treatment for melanoma and with available PBMC samples were selected for the study. Seven 'case' patients were selected on the basis that they developed a typical thyroid irAE (acute onset overt thyrotoxicosis within three months of commencing ICI-treatment). Three 'control' patients were selected who did not develop a thyroid irAE and additionally had no (or very mild) irAEs affecting other organ systems. Cohort characteristics are presented in Table 4.3. Unfortunately, due to sample availability issues related to COVID-19, there was an imbalance in ICI-treatment between groups. Whereas most thyroid irAE group patients received combination ICI-treatment, control group patients were all treated with PD-1 monotherapy. Future studies are planned to extend the cohort and eliminate the between group differences present in the current study.

4.3.1 Global circulating immune cell population profile

Mass cytometry analysis was performed on PBMC samples from all patients at baseline and repeated on samples collected at the time of a thyroid irAE (or at a matched timepoint for control group patients) to define the composition of circulating immune cells and associated ICI-treatment induced changes. Initial unsupervised FlowSOM clustering was performed on all samples (baseline and on-treatment) in both groups and identified 40 meta-clusters representing cell populations within the innate and adaptive immune

compartments. Meta-clusters were further consolidated manually into 14 broad categories encompassing major innate and adaptive immune cell populations (Figure 4.1a). Major populations were further divided into subpopulations that were used to perform additional within and between group comparisons (Figure 4.1b).

Table 4.3 – Cohort characteristics of thyroid irAE and control patients

	Thyroid irAE (n=7)	**Control** (n=3)
Age (years); median (range)	58 (49-69)	81 (57-85)
Sex (male); n (%)	2 (29)	2 (67)
Checkpoint inhibitor; n (%)		
- PD-1/PD-L1 alone	1 (14)	3 (100)
- Combined CTLA-4 + PD-1	6 (86)	0 (0)
AJCC stage; n (%)		
- Stage IIIc	2 (29)	0 (0)
- Stage IV M1a – M1c	5 (71)	3 (100)
Brain metastases; n (%)	0 (0)	2 (67)
LDH elevated; n (%)	3 (43)	0 (0)
Baseline TSH (mIU/L); median (range)	1.35 (0.85-3.40)	1.60 (1.17-2.49)
Baseline FT4 (pmol/L); median (range)	13.2 (10.9-14.9)	12.6 (11.1-16.3)
Time to onset of thyroid irAE (days); median (range)	28 (15-61)	n/a
Maximum FT4 level measured during follow-up (pmol/L); median (range)	28.3 (23.2-53.8)	14.2 (12.2-17.7)
Additional non-thyroid irAEs; n		
- Colitis	4	0
- Arthritis	2	0
- Hepatitis	1	1
- Pneumonitis	0	1
- Nephritis	1	0
- Hypophysitis	1	0
- Uveitis	1	0
- Vitiligo	1	0

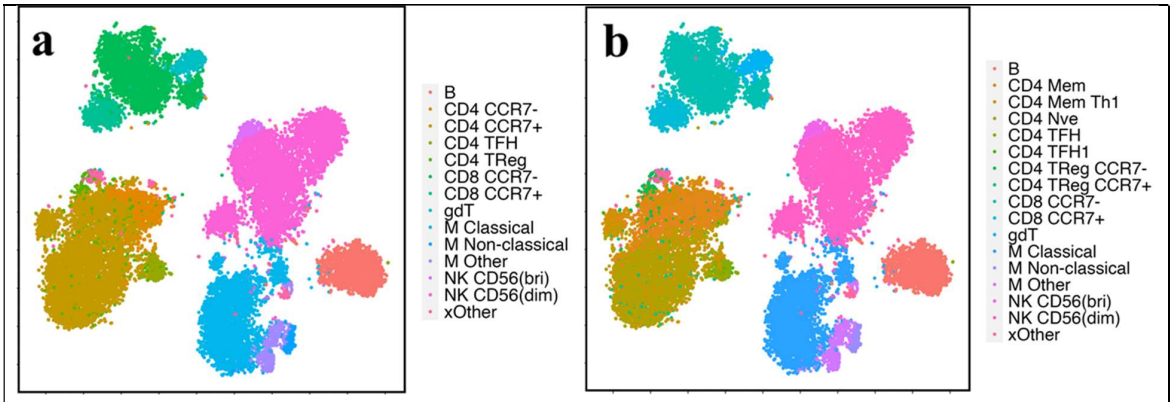

Figure 4.1: FlowSOM clustering of immune cells in patients receiving ICI-treatment.

FlowSOM clustering of PBMC samples visualized as UMAP plots of (a) major immune cell populations and (b) lineage subpopulations from all samples collected at both time points.

Abbreviations: B, $CD19^{+}$ B-lymphocytes; CD4 $CCR7^{-}$, total effector/memory $CD4^{+}$ T-cells; CD4 $CCR7^{+}$, total naive memory $CD4^{+}$ T-cells; CD4 TFH, total T-follicular helper cells; CD4 Treg, total $CD4^{+}$ regulatory T-cells; CD8 $CCR7^{-}$, effector/memory $CD8^{+}$ T-lymphocytes; CD8 $CCR7^{+}$, naive $CD8^{+}$ T-lymphocytes; gdT, gamma-delta T-lymphocytes; M Classical, $CD14^{++}CD16^{-}$ classical monocytes; M Non-classical, $CD14^{+}CD16^{++}$ non-classical monocytes; M Other, $CD14^{+}CD16^{+}$ intermediate monocytes; NK CD56(bri), $CD56^{(bright)+}$ natural killer cells (predominant in secondary lymphoid tissues); NK CD56(dim), $CD56^{(dim)+}$ natural killer cells (predominant in peripheral blood); xOther, all cells not meeting criteria for inclusion in other populations; CD4 Mem, total $CD4^{+}$ memory T-cells; CD4 Mem Th1, committed effector memory $CD4^{+}$ T-cells; CD4 Nve, naïve $CD4^{+}$ T-cells; CD4 TFH1, committed T-follicular helper cells; CD4 Treg $CCR7^{-}$, effector memory $CD4^{+}$ regulatory T-cells; CD4 Treg $CCR7^{+}$, naive memory $CD4^{+}$ regulatory T-cells; CD4 Treg, total regulatory T-cells.

4.3.2 Circulating immune cell profile at baseline

Differences in circulating immune cells were present at baseline between the two groups (Figure 4.2). Baseline changes were independent of ICI-treatment, as samples were collected prior to ICI-initiation. As a percentage of total cells, B-lymphocytes were higher in thyroid irAE group patients (median 9%, range 7-13) compared with control patients (median 4%, range 4-6; p=0.02). Conversely, NK-cells were lower in thyroid irAE group patients (median 22%, range 8-24) compared with control group patients (median 38%, range 27-40; p=0.02). As a percentage of total cells at baseline, $CD4^+$ T-cells, $CD8^+$ T-cells, monocytes, and gdT-cells were not different between groups.

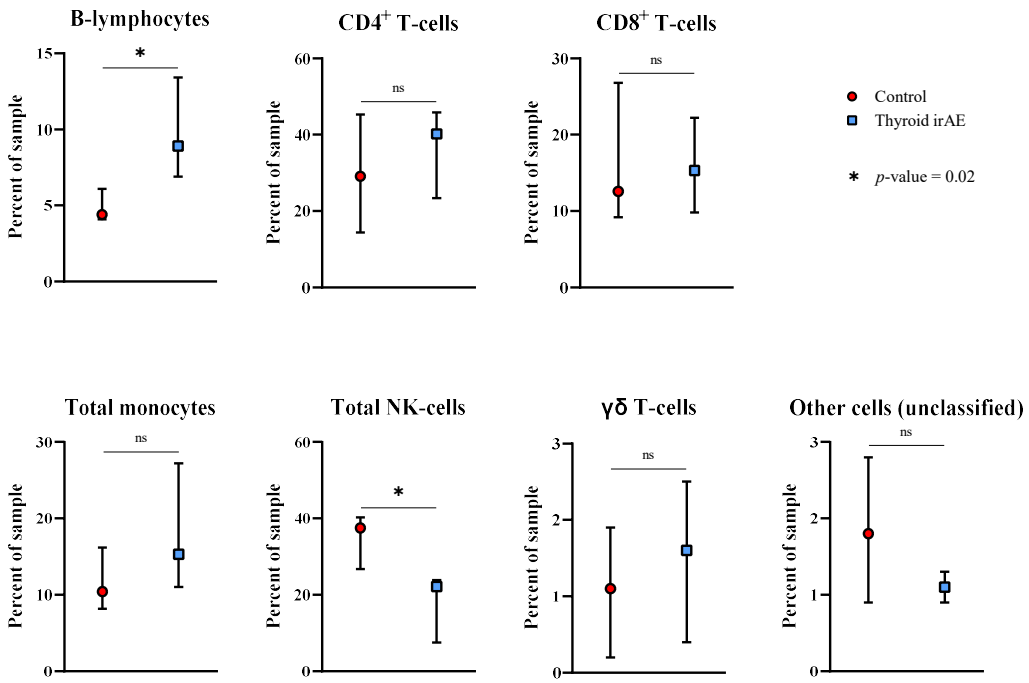

Figure 4.2: Circulating immune cell populations at baseline

Comparison of baseline immune cell populations as a percentage of total sample in patients with and without thyroid irAEs. Data presented as median and range; *p = 0.02; *ns* = not significant.

Circulating baseline immune cell subpopulations were recalculated as a percentage of their respective lineage to amplify differences that could otherwise be obscured when assessed as a percentage of the total sample. No baseline between group differences were observed in any monocyte, NK-cell, $CD4^+$ T-cell, and $CD8^+$ T-cell lineage subpopulations (Figure 4.3).

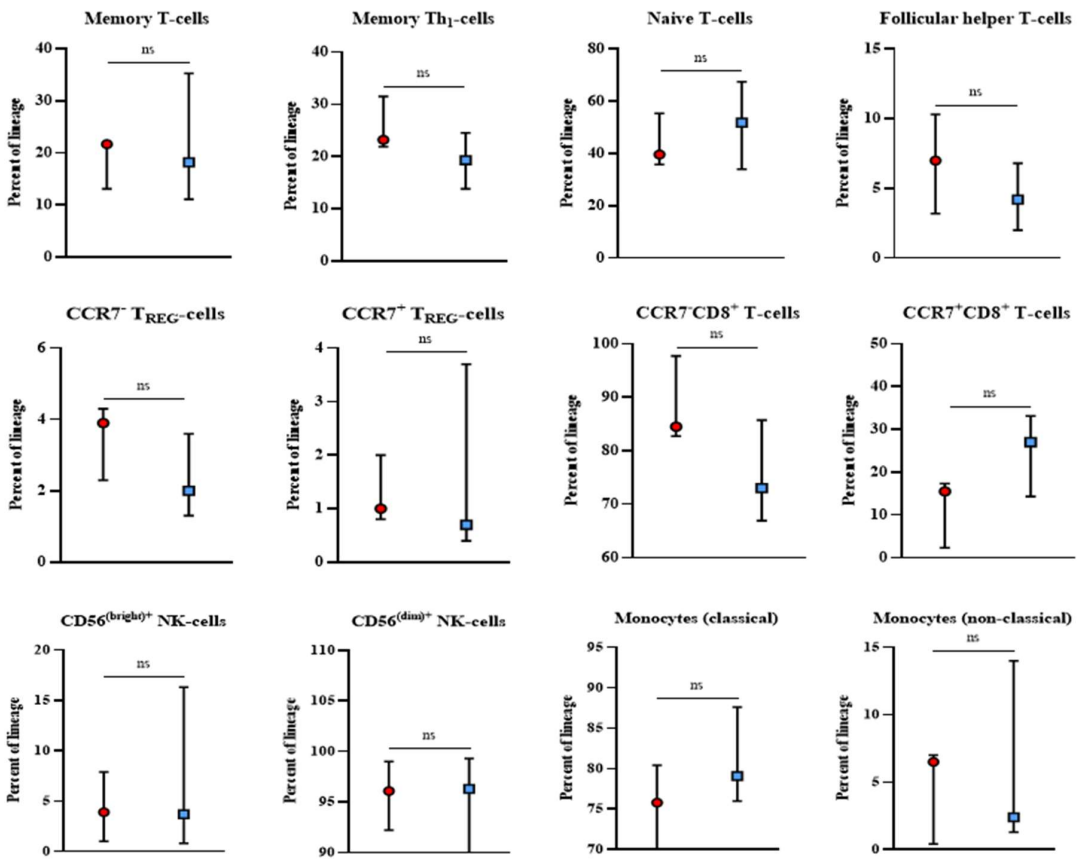

Figure 4.3: Circulating immune cell subpopulations at baseline

Comparison of baseline immune cell populations as a percentage of cell lineage in patients with and without thyroid irAEs. Data presented as median and range; *ns* = not significant.

4.3.3 ICI-treatment related changes in circulating immune cell profile

Thyroid irAE patients experienced significant alterations in the composition of circulating cells related to ICI-treatment, whereas no ICI-treatment related changes were observed in any cell lineage in control group (no thyroid irAE) patients.

As a percentage of the total sample, B-cells declined significantly in thyroid irAE group patients during ICI-treatment (median 6%, range 2-10) compared to baseline (median 9%, range 7-13; p=0.03). Total $CD4^+$ T-cells also decreased during ICI-treatment (median 30%, range 14-35) compared to baseline (median 40%, range 23-46; p=0.02). No significant changes in total $CD8^+$ T-cells, NK-cells or monocytes were observed in relation to ICI-treatment ($p \geq 0.05$). Results are displayed graphically in Figure 4.4.

As with baseline analyses, circulating immune cell subpopulations were recalculated as a percentage of their respective lineage to amplify differences that could be obscured when assessed as a percentage of the total sample. Although total $CD4^+$ T-cells decreased during ICI-treatment in thyroid irAE group patients, $CCR7^-$effector memory T_{REG} cells increased relative to baseline as a percentage of total $CD4^+$ cells (median 4%, range 3-6 vs. median 2%, range 1-3; $p<0.01$). Other subpopulations in the $CD4^+$ compartment did not change significantly in response to ICI treatment.

Despite no overall change in $CD8^+$ T-cells as a proportion of the total sample in thyroid irAE group patients, the proportion of $CCR7^-CD8^+$ effector T-cells increased during ICI-treatment relative to baseline (median 89%, range 76-91 vs. median 73%, range 67-86; $p<0.01$). Conversely, the proportion of naïve $CCR7^+CD8^+$ T-cells decreased during ICI-treatment relative to baseline (median 11%, range 9-24 vs. median 27%, range 14-33;

p<0.01). A significant decrease in intermediate monocytes was also associated with ICI-treatment in thyroid irAE group patients (median 7%, range 3-15 vs. median 11%, range 7-22; p=0.03).

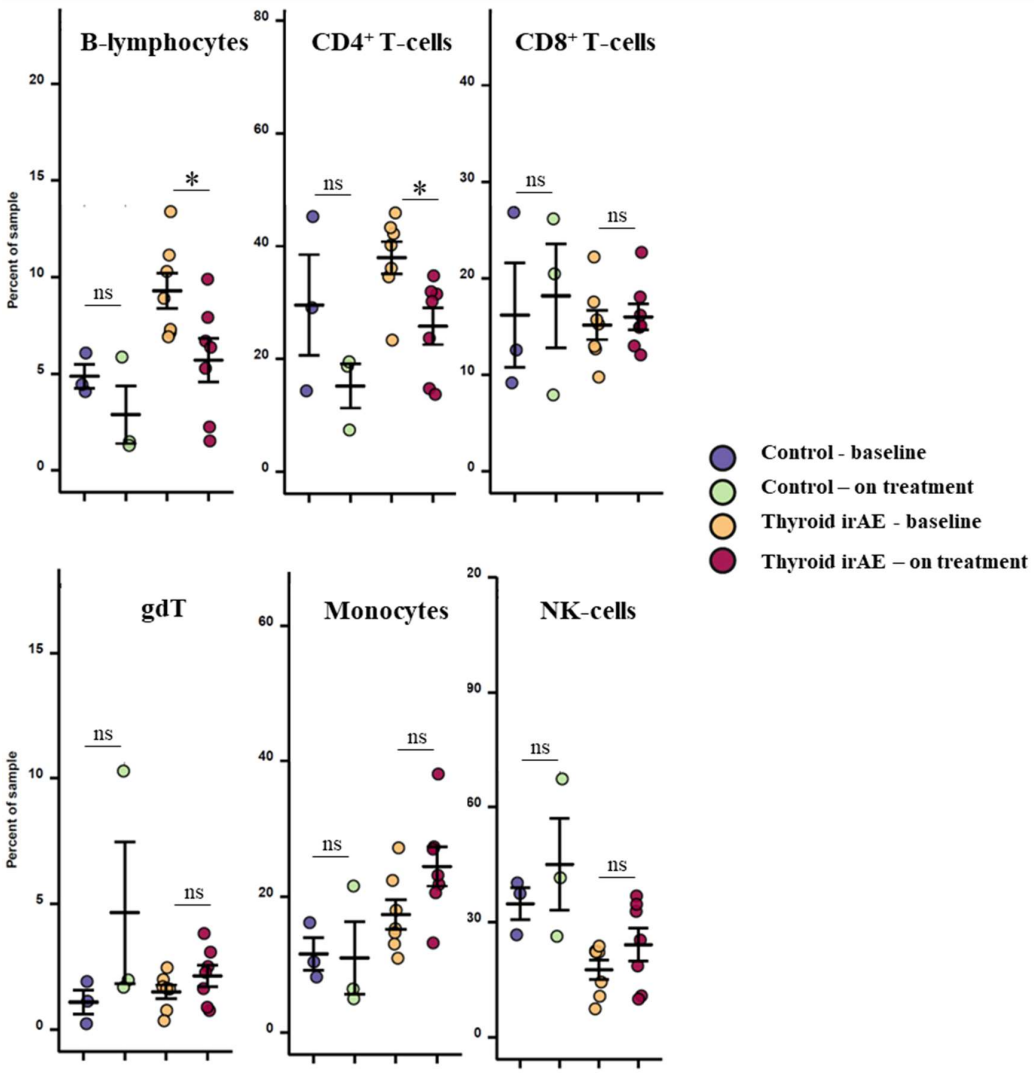

Figure 4.4: Circulating immune cell populations before and after ICI-treatment

Comparison of baseline and on-treatment immune cell populations as a percentage of total cells in patients with and without thyroid irAEs. Data presented as median and range; *$p < 0.05$; *ns* = not significant.

We examined cellular expression of Ki67 to identify proliferating immune cell populations potentially mediating the effects of ICI-inhibitors on thyroid toxicity. Interestingly, following ICI-treatment in the thyroid irAE group, Ki67 expression significantly increased in $CD4^+$ T-cells despite an overall decrease in total $CD4^+$ cells as a proportion of the total sample. Conversely, in control group patients Ki67 expression did not change in $CD4^+$ T-cells (Figure 4.5). Examination of Ki67 expression in $CD4^+$ subpopulations demonstrated that increases were concentrated in Th1 effector memory T-cells, whereas other T-cells in the $CD4^+$ compartment did not increase Ki67 expression. Thyroid irAE group patients also had a significant increase in Ki67 expression in $CD8^+$ T-cells following ICI-treatment, whereas control patients did not (Figure 4.5). Examining $CD8^+$ subpopulations demonstrated that increased Ki67 was due to expansion of effector $CCR7^-CD8^+$ T-cells rather than naive $CCR7^+CD8^+$ T-cells. Granzyme B (GZMB) expression, a marker of T-cell activation and proliferation also increased significantly in effector $CCR7^-CD8^+$ T-cells, but not in naïve $CCR7^+CD8^+$ T-cells (Figure 4.6). Ki67 and GZMB expression did not significantly change from baseline in monocytes, NK-cells or gdT-cells following ICI-treatment in both control and thyroid irAE groups.

4.4 Discussion

The immunology of AITD is complex and incompletely understood. In general terms, a combination of genetic predisposition and environmental exposure promotes loss of immune tolerance and progressive autoimmunity directed against the thyroid (214). In GD there is predominately a humoral response characterized by TRAB mediated activation of the TSH receptor (214). In HT, a cellular T-cell mediated response

predominates, although autoantibodies are also present as a secondary phenomenon and promote progressive lymphocytic infiltration and destruction of the thyroid gland (214). Thyroid irAEs have phenotypic similarity to AITDs which occur outside the cancer immunotherapy setting. However, the etiology of thyroid irAEs is less well understood, and whether a T-cell, B-cell, combination, or other immune response drives pathogenesis is not known.

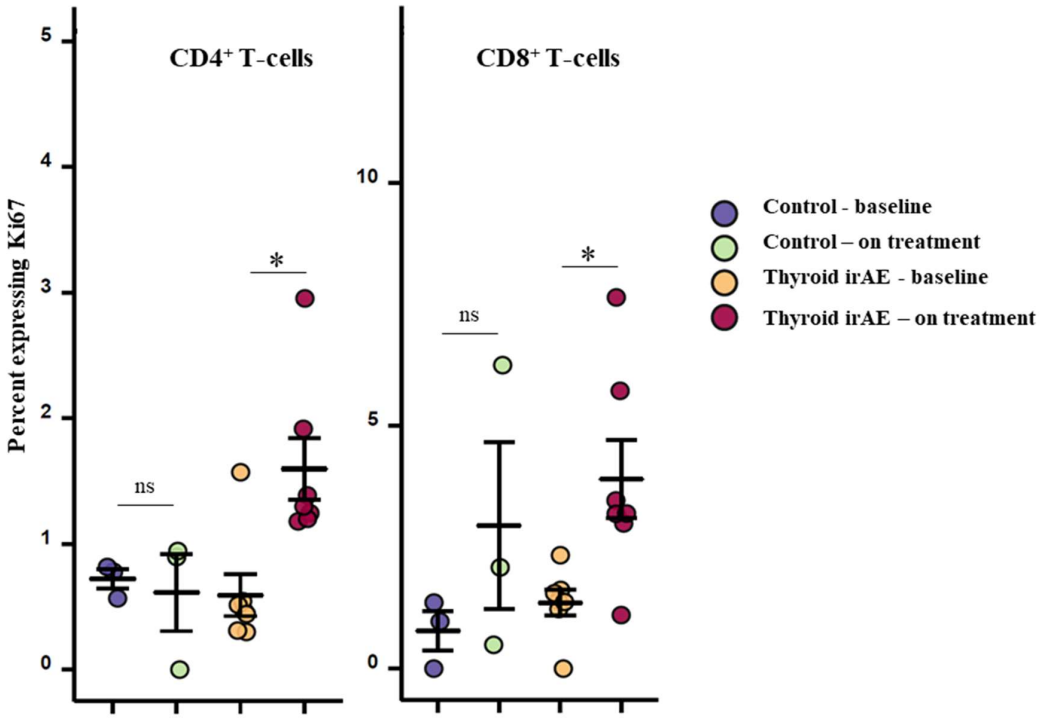

Figure 4.5: Ki67 expression in $CD4^+$ and $CD8^+$ T-cells at baseline and during checkpoint inhibitor treatment

Comparison of baseline and on-treatment Ki67 expression in $CD4^+$ and $CD8^+$ T-cells as a percentage of total cells in patients with and without thyroid irAEs. Data presented as median and range; *p = 0.01; *ns* = not significant.

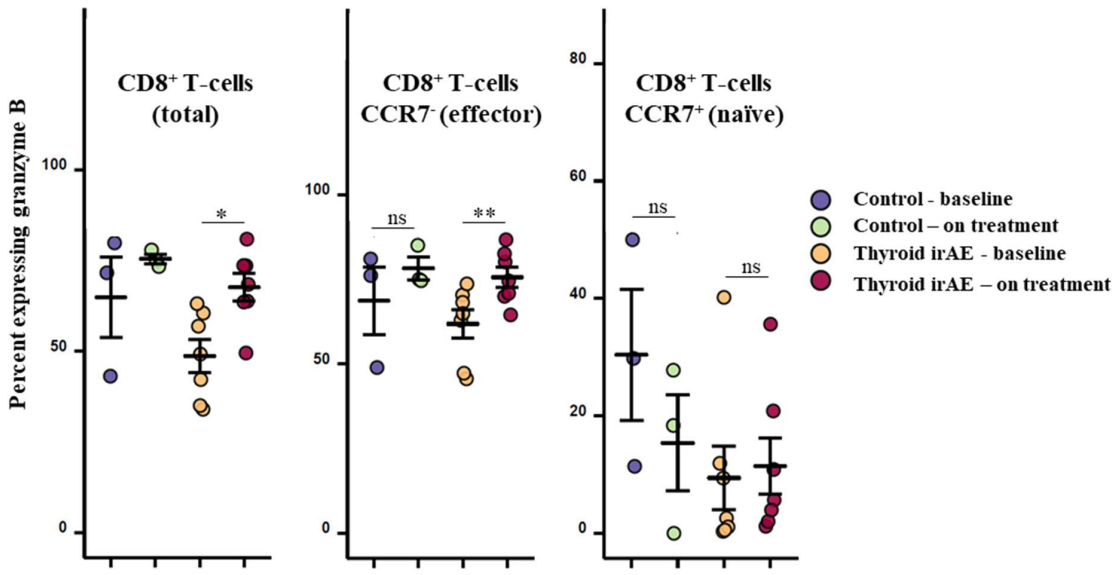

Figure 4.6: CD8$^+$ T-cells - granzyme B expression phenotype

Granzyme B expression (a marker of T-cell activation and proliferation) was measured at baseline and on treatment in patients with and without thyroid irAEs as a percentage of total CD8$^+$ T-cells (left panel), CCR7$^-$ 'effector' CD8$^+$ T-cells (center panel), and CCR7$^+$ 'naïve' CD8$^+$ T-cells (right panel). Data presented as median and range; *p<0.01; **p=0.03; *ns* = not significant.

In this study, we analyzed the circulating immune profile of ten melanoma patients receiving ICI-treatment to identify immune cells involved in the pathogenesis of thyroid irAEs. Important pre-ICI treatment differences were noted between subjects who developed thyroid irAEs and those who did not and may have relevance as biomarkers of thyroid irAE susceptibility; our ability to interpret post-treatment changes was limited

by an imbalance in ICI treatments between groups and are therefore reported as pilot data.

At baseline (before ICI-treatment), B-lymphocytes were significantly higher as a proportion of total cells in patients who experienced a thyroid irAE. Our results suggest that baseline B-cell concentrations >7% have potential use as a biomarker of thyroid irAE susceptibility. B-lymphocytic involvement in thyroid irAE pathogenesis has biological plausibility. Our previous work clearly demonstrated an association of B-cell derived anti-thyroid antibodies with thyroid irAE development (169). Unfortunately, we did not know the TPOAb and TgAb status of patients included in this study. It could be postulated that higher B-lymphocyte populations at baseline suggest an increased likelihood of TPOAb and TgAb positivity in these patients. Baseline antithyroid antibodies would suggest the immune system is primed against the thyroid prior to ICI-treatment and progresses rapidly following breakdown in immune tolerance related to checkpoint inhibitor blockade. As such, B-cell responses prior to ICI-treatment could be important mediators of thyroid irAE susceptibility.

Treatment related changes associated with thyroid irAEs included proliferation of $CD4^+$ and $CD8^+$ T-cell subpopulations (which were static in those who remained euthyroid), but as noted there is possible confounding by the dual-ICI treatments these patients received, compared to single-agent anti-PD1 treatments in the control group. Nevertheless, many changes identified in our results are consistent with phenotypic changes in the immune profile reported in other published studies of immunotherapy-related toxicity. Also, B-lymphocytes decreased as a proportion of the total sample during ICI-treatment in thyroid irAE group patients. This replicates findings from

another study, which also showed decreased circulating B-cell levels in ICI-associated thyroiditis patients compared to healthy controls (177). Decreases in B-lymphocyte concentration during treatment are interesting when considered in conjunction with the concomitant increase in $CD4^+$ and $CD8^+$ effector T-cells we observed in our study. Such changes suggest that B-cell responses may prime the immune system against the thyroid, but under influence of checkpoint inhibitors there is a shift toward a predominate T-cell phenotype which further mediates immune infiltration of thyrocytes and promotes the rapidly progressive thyroiditis that is characteristic of thyroid irAEs.

Previous studies have demonstrated increased PD-1 expression in tumor infiltrating lymphocytes and increased PD-L1 expression within the thyroid gland in patients with AITD (176). Extension of this line of reasoning presupposes that thyroid irAEs may arise in patients with pre-existing, not clinically apparent HT, whereby ICI-treatment accelerates the immune response against thyroid tissue already primed by the presence of antithyroid antibodies and tissue specific effector T-lymphocytes. We observed proliferation and expansion of $CD8^+$ effector T-cells related to ICI-treatment in patients with thyroid irAEs. Infiltration of the thyroid gland by T-lymphocytes has been previously described following ICI-treatment (177, 206, 215). In fact, fine needle thyroid aspirates in patients with ICI-associated thyroiditis have demonstrated high levels of $CD8^+$ T-cells relative to healthy controls (177, 216). Additionally, the phenotype of $CD8^+$ T-cells in thyroid irAE patients was different relative to HT and healthy volunteers (216). Thyroid irAE cases were more likely to have GZMB, CXCR6, and interferon gamma-positive $CD8^+$ T-cells as opposed to EOMES-positive 'autoimmune-mediator' T-cells and $TIGIT^+PD1^+$ 'exhausted' T-cells which predominated in AITD patients and

healthy controls respectively (216). We also found increased GZMB expression in a subpopulation of effector $CD8^+$ T-cells, lending support to the importance of this cell population in the etiology of thyroid irAEs.

In our study, baseline NK-cells were significantly reduced as a proportion of the total sample in thyroid irAE group patients compared to control group patients. This mimics results from a study which also found lower proportions of NK-cells in patients with ICI-induced colitis, supporting a key role of innate immune responses in the pathogenesis of thyroid irAEs (217). Reduction in NK-cell activity has been demonstrated in patients with AITD outside the ICI-setting, lending further support for a potential shared mechanism between these distinct disease entities (218). Increased levels of $CD16^+CD56^+$ NK-cells have been reported in both thyroid irAEs and AITD compared to healthy volunteers (119, 177). We did not observe an increase in NK-cell levels during treatment, however our control group was ICI-treated euthyroid controls and therefore does not allow for direct comparison of our results with previously published data.

Limited other studies have examined the role of specific immune cells in ICI-associated thyroiditis. A mouse model of ICI-thyroiditis identified intrathyroidal IL-$17A^+$ T-cells and gdT_{17} cells to be associated with thyroiditis following ICI-treatment (207). The same study also showed that blockade of IL-17 using a monoclonal antibody antagonist prevented lymphocytic infiltration of the thyroid gland without impairing anti-tumor efficacy of ICI-treatment (207). We did not identify significant changes in gdT-cells at baseline or during ICI-treatment in our cohort. Therefore, the significance of these findings is unknown, and confirmation in cancer patients is required, as there may be differences in irAE pathogenesis between human subjects and mice.

Our study has limitations. The study population was limited to ten patients and there were baseline differences between patients in the thyroid irAE and control groups. We cannot exclude that between group differences in age, sex and ICI-treatment may have influenced immune cell composition at baseline and during ICI-treatment. Future studies are planned to mitigate these differences. Finally, we did not have thyroid tissue, and only measured circulating immune cell populations. As such, we could not assess the immune phenotype within the target organ of interest, and it is possible that circulating immune cell changes may not directly mimic immune cell populations within the thyroid microenvironment.

4.5 Conclusion

This study demonstrates a unique immune signature associated with thyroid irAEs in patients undergoing ICI-treatment for melanoma. Our results support a role for multiple immune cell types, including B-lymphocytes, T-cells, and NK-cells in the pathogenesis of ICI-induced thyroiditis with similarities to the distinct immune changes long known to be associated with AITD more generally. As thyroid irAEs are common and well-established biomarkers of ICI response, elucidating their mechanistic underpinnings may improve understanding of irAE susceptibility more generally and promote development of next generation ICIs which uncouple the anti-tumor benefits from irAE toxicity.

Chapter 5

FLT3 polymorphisms and FLT3 ligand levels in thyroid irAEs

5.1 Introduction

Thyroid irAEs are a frequent complication of ICI-treatment (166, 200). In many aspects, the clinical presentation and course of thyroid irAEs are similar to sporadic thyroiditis in patients with AITD not receiving ICI-treatment (167). Additionally, anti-thyroid antibodies are highly prevalent in patients with thyroid irAEs, although to a lesser degree than in GD, HT and other forms of AITD (169). AITD is highly heritable, with more than one-third of patients with HT having one or more affected siblings and higher concordance of HT and GD in monozygotic, compared to dizygotic, twins (219, 220). Multiple susceptibility genes have been identified which predispose an individual to future thyroid autoimmunity (221). Emerging data suggest that genetics also influence risk of thyroid irAEs, involving polymorphisms in many genes like those implicated in AITD (174, 175). This chapter will review the genetic susceptibilities to AITD and thyroid irAEs, followed by new experimental data we produced examining the association of fms-related tyrosine kinase 3 (FLT3) variants to risk of thyroid irAEs.

5.1.1 Genetics of autoimmune thyroid disease

Autoimmune thyroid disease encompasses a spectrum of thyroid conditions affecting up to 5% of the general population (222). A much greater number, perhaps up to 20%, of adult women, have measurable anti-thyroid antibodies without active manifestations of thyroid disease (222). Despite varying clinical presentations, GD, HT, and other AITDs share multiple genetic susceptibility loci (223). Affected loci are mainly

clustered within genes that regulate immune responses and genes related to thyroid structure and function (224).

Thyroglobulin (Tg) and thyroid stimulating hormone receptor (TSHR) are frequent antigen targets of the immune response in AITD. Perhaps expectedly, genetic and epigenetic polymorphisms in Tg gene and TSHR gene are two of the main thyroid-specific genes that have been associated with increased susceptibility to AITD (224). Relative to alterations in thyroid-specific genes, many more immune-specific genes have been associated with susceptibility to AITD. Aberrations have been identified in genes involved with maintenance of peripheral tolerance (forkhead box P3 and CD25), T-cell co-stimulation (CD40 and CTLA-4), and antigen presentation (HLA-DR), reflecting the critical importance of immune factors in the development of thyroid autoimmunity (221, 223).

5.1.2 FLT3 polymorphisms and risk of AITD

A recent genome wide association study (GWAS) of 30,000 AITD patients and 700,000 controls identified 99 sequence variants associated with increased susceptibility to AITD (225). Among them was a previously unknown, low frequency (1.4%) SNP in FLT3 (rs76428106-C) (225). FLT3 is a tyrosine kinase receptor that regulates development of monocytes and dendritic cells from haematopoietic progenitors (226). FLT3 minor allele rs76428106-C conferred a larger effect on risk of AITD (OR 1.46) than all other identified variants, including previously well-established susceptibility genes such as CTLA-4 and HLA-DR (225). Functional studies demonstrate that patients expressing the rs76428106-C variant produce a truncated FLT3 protein without a functional tyrosine kinase domain (225). As such, there is accumulation of FLT3 ligand

(FLT3-L), with a doubling of plasma FLT3-L levels for each copy of rs76428106-C (225).

5.1.3 Genetics of thyroid irAEs

Thyroid irAEs are associated with improved treatment response and better PFS and OS in cancer patients receiving ICI-treatment (164, 167, 180). Therefore, understanding which patients will develop thyroid irAEs, and the mechanisms underlying development are prognostically useful and important. Potential demographic predictors of thyroid irAEs have been identified but lack generalizability with many additional studies failing to find similar associations (78, 166, 200). As such, interest in identifying genetic susceptibility loci which promote thyroid irAEs, and consequently suggest improved response and survival to ICI-treatment has intensified.

Two studies have examined the genetic underpinnings of thyroid irAEs (174, 175). In one study, a polygenic risk score (PRS) for hypothyroidism or use of thyroid medications derived from UK Biobank data was applied to a cohort of 744 patients receiving ICI-treatment (175). Interestingly, development of a thyroid irAE was significantly associated with the UK Biobank hypothyroidism PRS, with a HR of 1.34 per SD of PRS (175). These associations were validated in a second cancer cohort of 561 patients, where a similar effect size was observed (175). A second study applied a similar hypothyroidism PRS including 140 variants with highest probability of association to 2616 patients receiving atezolizumab treatment across multiple phase III clinical trials (174). They also found the PRS for hypothyroidism was associated with a greater than six-fold increased risk of thyroid irAEs in their atezolizumab-treated cohort (174). Variants identified with the highest estimated importance to irAE

susceptibility were strikingly like the variants known to increase susceptibility to AITD such as CTLA-4, PTPN22 and Tg (174).

Given the overlap in genetic variants predicting thyroid irAEs and AITD, plus the recent discovery that loss of function mutations in FLT3 are associated with increased susceptibility to AITD, we hypothesized that FLT3 may also be associated with susceptibility to thyroid irAEs following ICI-treatment. To test this hypothesis, we assessed for FLT3 rs76428106-C SNPs and FLT3-L levels in a cohort of melanoma patients undergoing ICI-based cancer treatment.

5.2 Materials and methods

To examine the association between FLT3 rs76428106-C SNP and risk of thyroid irAEs, we performed a pilot, case-control study of adult patients undergoing ICI-treatment for melanoma. Patients who developed thyroid irAEs were considered cases and those who remained euthyroid were considered controls. Demographic, treatment, and thyroid function test data were extracted from a prospectively maintained MIA database and from laboratory records. Separately, germline DNA was isolated from patient blood samples in the MIA Biospecimen Bank and GWAS genotyped as part of an unrelated MIA study (227). From this GWAS data, FLT3 rs76428106-C SNP data was provided for 482 patients receiving ICI-treatment and with known thyroid irAE outcomes. For a subset of 128 patients, we retrieved fresh MIA Biospecimen Bank blood samples to measure FLT3-L levels. Soluble FLT3-L was duplicate measured in plasma using quantitative sandwich ELISA as per the manufacturer's instruction (R&D Systems, Human Flt-3 Ligand/FLT3L Quantikine® ELISA kit, catalog DFK00).

Our primary outcome was to test whether thyroid irAE prevalence differed by FLT3 rs7648106-C genotype. We hypothesized that thyroid irAEs would be most prevalent in patients with homozygous FLT3 rs76428106-C variants, intermediate prevalent in heterozygous individuals, and least prevalent in patients with wild type FLT3. Secondary outcomes included measurement of FLT3-L levels in a subset of patients as a surrogate marker of FLT3 genotype, and regression analysis of relevant factors (including FLT3 rs76428106-C genotype) to assess for associations with thyroid irAE.

5.2.1 Statistical analysis

As a pilot study with a small sample size, our aims were predominately hypothesis generating. Baseline characteristics are presented as frequency and percentage or mean and SD. The genotypes of patients in the thyroid irAE group and no thyroid irAE group were compared by Pearson chi-square analysis with statistical significance level set at $p<0.05$. Unpaired t-test was used to compare FLT3-L levels between groups. Logistic regression analysis was performed to correlate the risk of thyroid irAE and FLT3 genotype using age, sex, ICI-type, and the first ten principal components as covariates.

5.3 Results

A total of 536 patients were available for analysis. Thyroid irAEs occurred in 276 patients (52%), with no thyroid irAEs in the remaining 260 patients (48%). Baseline characteristics are presented in Table 5.1. GWAS data for FLT3 rs76428106-C SNPs was available for 482 patients (90%). FLT3-L levels were measured in 128 patients (24%). The combination of both FLT3 SNP data and FLT3-L levels were available for 74 patients (14%).

Table 5.1: Baseline characteristics

	Entire cohort (N=536)	**Thyroid irAE** (n=276)	**No thyroid irAE** (n=260)
Sex (male); n (%)	347 (65)	159 (58)	188 (72)
Age (years); mean (SD)	63 (14)	61 (14)	65 (14)
TSH (mIU/L); mean (SD)	1.54 (0.80)	1.50 (0.85)	1.58 (0.74)
FT4 (pmol/L); mean (SD)	13.2 (2.0)	13.4 (2.2)	13.0 (1.8)
Checkpoint inhibitor; n (%)			
• CTLA-4 only	38 (7)	14 (5)	24 (9)
• PD-1/PD-L1 only	303 (57)	139 (50)	164 (63)
• Combination ICI	195 (36)	123 (45)	72 (28)
Non-thyroidal irAEs; n (%)			
• Any	413 (77)	223 (81)	190 (73)
• Severe (CTCAE ≥3)	108 (20)	72 (26)	36 (14)
• Multisystem (≥2)	31 (6)	23 (8)	8 (3)

5.3.1 Prevalence and distribution of FLT3 variant rs76428106-C

The allelic frequency of rs76428106-C in our cohort was 1.9%, similar to the low frequency (1.4%) observed in a large European study of over 700,000 individuals (225). Only two patients in our study demonstrated rs76428106-C homozygosity for the FLT3 variant both of whom developed a thyroid irAE. Heterozygosity was present in 14 individuals, eight of whom developed a thyroid irAE, while the rest remained euthyroid. Although proportionally greater in thyroid irAE patients, the rs76428106-C variant was not statistically associated with an increased risk of thyroid irAEs in our study. Genotype distributions and analysis of FLT3 polymorphism rs76428106-C by thyroid irAE outcome are presented in Table 5.2.

Table 5.2: Genetic distribution of FLT3 variant rs76428106-C

FLT3 rs76428106 genotype	Thyroid irAE	No thyroid irAE	X^2 test	*p*-value
Homozygous (CC); n (%)	2 (1)	0 (0)		
Heterozygous (CT); n (%)	8 (3)	6 (2)	2.577	0.28
Wild type (TT); n (%)	224 (96)	242 (98)		

5.3.2 FLT3 ligand levels

FLT3-L was measured in 128 patients with a median level of 88 pg/mL (range 21-287). The distribution of FLT3-L levels in our cohort is presented in Figure 5.1. In patients who developed a thyroid irAE, median FLT3-L level measured 88 pg/mL (range 22-287).

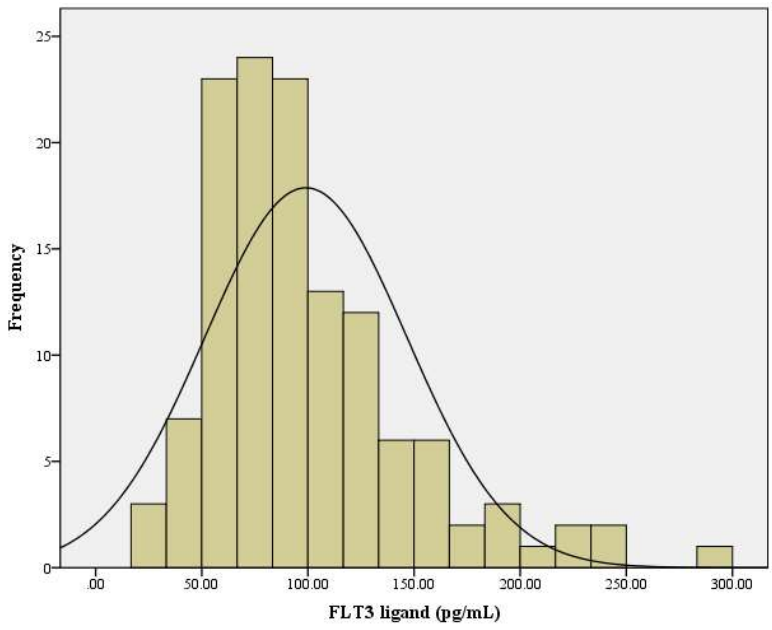

Figure 5.1: Distribution of FLT3-L levels in 128 patients receiving ICI-treatment

In patients that did not experience a thyroid irAE, median FLT3-L level measured 89 pg/mL (range 21-223). FLT3-L levels in thyroid irAE patients were not statistically different from levels in patients that did not develop a thyroid irAE (Figure 5.2; p=0.72).

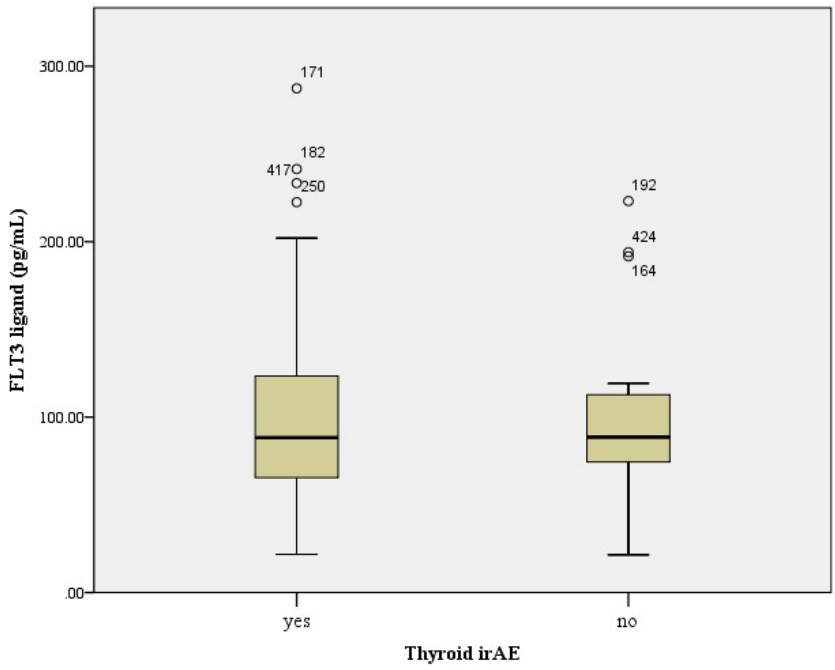

Figure 5.2: FLT3 ligand levels in patients with and without thyroid irAEs

Boxplot shows the median (horizontal lines), IQR (box), and 10th and 90th centiles (vertical lines) as well as significant outliers (numbered dots)

5.3.3 Logistic regression analysis of thyroid irAE related factors

A logistic regression analysis was performed to ascertain the effect of FLT3 rs76428106-C variant on the likelihood patients with melanoma developed a thyroid

irAE while receiving ICI-treatment. For this analysis, heterozygous and homozygous cases were combined into a single group due to the low allele frequency and small number of homozygous cases (n=2). The logistic regression model was statistically significant ($X^2(15) = 44.2$, $p<0.001$) and explained 12% of the variance in thyroid irAE development (Nagelkerke R^2). Our model classified 61% of cases correctly. In our cohort, the presence of SNP rs76428106-C in the FLT3 gene was not associated with increased susceptibility to thyroid irAE development (Table 5.3). Associations were observed for male sex, who were 42% less likely to develop an irAE than women (OR 0.58, 95% CI 0.39-0.87). Combination CTLA-4 and PD-1 ICI-treatment was associated with increased likelihood of thyroid irAE compared to both CTLA-4 and PD-1 ICI-treatment alone (Table 5.3).

Table 5.3: Logistic regression analysis of factors associated with thyroid irAEs

Independent variable	*B*	SE	*p*-value	Exp (*B*)	95% CI for Exp (*B*)
Age (per year)	-0.012	0.007	0.09	0.99	0.98-1.00
Sex • Female (ref) • Male	 -0.544	 0.204	 0.01	 0.58	 0.39-0.87
Checkpoint inhibitor • Combination (ref) • CTLA-4 only • PD-1/PD L1 only	 -1.160 -0.725	 0.403 0.211	 <0.01 <0.01	 0.31 0.48	 0.14-0.69 0.32-0.73
rs76428106-C • Hetero- or homozygous (ref) • Wild type	 0.543	 0.546	 0.32	 1.72	 0.59-5.01

5.4 Discussion

The current study tested whether a recently discovered AITD susceptibility variant in FLT3 gene (rs76428106-C) was associated with development of thyroid irAEs in 482 patients with melanoma receiving ICI-treatment. Despite biological plausibility of an association, our results did not suggest significant differences in prevalence of FLT3 rs76428106-C variant between patients with and patients without thyroid irAEs. Additionally, we measured FLT3-L levels in a subset of patients and did not find a significant difference between patients that developed a thyroid irAE and patients that remained euthyroid. Logistic regression analysis confirmed data from our previous study and showed that sex and ICI-type were significantly associated with increased likelihood of thyroid irAEs (167). However, FLT3 rs76428106-C genotype was again not significantly associated.

FLT3 (also known as CD135) is a tyrosine kinase receptor present mainly within cells of the haematopoietic system (228). FLT3 is activated via binding its ligand, FLT3-L which then regulates differentiation, proliferation, and survival of multiple haematopoietic progenitor cells both directly and via interaction with other cytokines such as type 1 interferons (228). Somatic activating mutations of FLT3 have been implicated in multiple disease states, such as acute myeloid leukemia (AML), and follicular thyroid carcinoma (228-230). More recently, an inactivating mutation in FLT3 (rs76428106-C) was associated with increased susceptibility to autoimmune disease states such as type 1 diabetes mellitus and coeliac disease (225). The rs76428106-C variant resulted in a premature stop codon and truncated FLT3 protein without functional tyrosine kinase activity (225). As such, rs76428106-C mutations are

predicted to cause a loss of function of the FLT3 protein, which has been supported by functional studies which showed a 22% decrease in functional FLT3 transcripts for each rs76428106-C allele present (225). Reduction in the number of full length (wild-type) FLT3 transcripts was associated with marked increases of FLT3-L in individuals carrying the rs76428106-C variant (225).

FLT3-L may have a pivotal function in determining susceptibility to autoimmune disease. Individuals born with rare forms of combined immunodeficiency have unique predisposition to autoimmune disease, and have FLT3-L levels 100-fold greater than an immunocompetent population (231). Animal models of autoimmune thyroiditis also have increased FLT3-L levels in proportion to disease severity, and administration of FLT3-L to normal mice induces lymphocytic destruction of the thyroid gland (232). FLT3-L levels are elevated in humans with autoinflammatory arthritis and systemic sclerosis, where it may have potential as a biomarker for more severe disease (233, 234). Hence, it is hypothesized that increased levels of FLT3-L observed in association with rs76428106-C variants of the FLT3 gene may induce autoimmunity via preferential amplification of immunogenic (rather than tolerogenic) signaling pathways (225). Interestingly, inhibitors to FLT3 have been developed and investigated for treatment of FLT3-mutated AML, and thyroid dysfunction was not observed as an adverse effect of FLT3 inhibition despite presumably high levels of FLT3-L (235, 236).

In our study, we did not observe a difference in FLT3-L levels between patients with thyroid irAEs and those without. However, our results do not preclude FLT3-L as a potential biomarker of thyroid irAE susceptibility.

Our study has limitations. As an exploratory pilot study, there were several reasons we may have failed to show a significant association between rs76428106-C SNP in FLT3 gene and susceptibility to thyroid irAEs. Firstly, as in the landmark study demonstrating the association between FLT3 (rs76428106-C) and AITD in a European population, we also documented a very low risk allele frequency of <2% in our Australian cohort (225). Therefore, our study was underpowered to detect an association of rs76428106-C with thyroid irAEs. That said, our results are interesting in that both patients homozygous for the rs76428106-C FLT3 risk allele developed a thyroid irAE, whereas no patients without a thyroid irAE had two copies of the rs76428106-C variant. Secondly, FLT3-L could only be performed in a subset of 128 patient, of which 76 were wild-type, two were rs76428106-C heterozygous, and 50 had an unknown genotype. Therefore, we had limited power to detect differences in FLT3-L levels between groups, especially given the low frequency of the risk allele in the population.

5.5 Conclusions

The FLT3 gene polymorphism rs76428106-C and FLT3-L levels have been associated with AITD but were not associated with thyroid irAEs in our study. Further examination of this SNP and FLT3-L levels in a larger population of ICI-treated patients is warranted given the strong association between other genetic susceptibility loci in AITD and thyroid irAEs as well as the prognostic benefits observed in cancer patients in association with thyroid irAEs. However, given its low allelic frequency, it seems unlikely to fully explain thyroid irAEs, which occur at a much higher rate than could be explained by FLT3 polymorphisms alone.

Chapter 6

Development of an animal model to identify novel thyroid auto-antibodies mediating thyroid irAE development

6.1 Prelude

This chapter was intended to co-opt an animal model used previously to identify novel central nervous system (CNS) autoantigens in multiple sclerosis patients. We had intended to use this same animal model to identify novel thyroid autoantigens in ICI-treated patients experiencing thyroid irAEs. The study was conceptualized and submitted to the animal ethics committee in 2019. However, due to COVID-19, related lockdowns, and repeated laboratory closures, the study was not commenced until late 2022. Within the time confines of this thesis, final testing of rodent thyroid by indirect immunofluorescence (IIF) was not possible but will be performed after thesis submission. Therefore, this chapter has evolved and in place of IIF data, will instead detail our optimization of the rodent perfusion fixation procedure, thyroid extraction, and immunohistochemical staining of resected rodent thyroid confirming adequate tissue perfusion-fixation to permit future autoantibody testing via IIF.

6.2 Background

Thyroid irAEs are a common drug-related side effect of ICI-treatment and approximately 30-40% of patients that develop overt thyroid irAEs will have detectable TPOAb or TgAb (169, 200). Although TPOAb and TgAb are more prevalent in cancer patients with thyroid irAEs compared to those without, TPOAb and TgAb positivity is still well below the >90% prevalence observed in HT and other AITDs (82, 169). TPOAb (and TgAb to a lesser extent) are likely to be pathogenic in HT as a secondary

phenomenon following an initial immune insult (82). TPOAb can fix complement in vitro, and helps direct a primed immune system against the thyroid gland leading to progressive lymphocytic infiltration and eventual gland fibrosis and failure (82, 237). Whether TPOAb or TgAb participate in the pathogenesis of thyroid irAEs, and if so, why they are less prevalent then in HT, remains unknown.

Antibodies against other thyroid structures have been identified and appear overrepresented in patients with AITD. For instance, 10-20% of patients with HT and GD have IgG antibodies against the sodium iodide symporter (NIS), the channel responsible for iodine uptake into the thyroid gland (238, 239). Similarly, antibodies against pendrin, a channel protein which mediates movement of iodide into the follicular lumen, can be detected in 9% of patients with AITD, and are not observed in healthy controls (239, 240). Thyroid hormone (T4, T3) antibodies are even more common, present in up to 40% of patients with HT and 20% of patients with GD (241). To date, the significance of non-TPO, non-Tg antibodies in the pathogenesis of AITD has not been established, and therefore they are not routinely measured in clinical practice (242). Furthermore, no studies have examined for non-traditional thyroid antibodies in association with thyroid irAEs, or whether they are present in affected patients without traditional anti-TPO and anti-Tg antibodies. Determining the mechanism by which patients develop thyroid irAEs, and how they differ from AITD occurring outside the cancer setting is important and could have practical implications that would help determine appropriate surveillance and management of thyroid function in these patients.

In chapter 3, we demonstrated that patients who develop thyroid irAEs are more likely to have positive TPOAb and TgAb than patients that remain euthyroid (169). However, we also showed that many thyroid irAE patients did not have detectable TPOAb or TgAb (169). Based on this discrepancy, we hypothesized that other (non-TPOAb, non-TgAb) antibodies directed against the thyroid may be involved in the pathogenesis of thyroid irAEs. To test our hypothesis, we developed an animal model to test for additional antibodies (i.e. NIS, pendrin, etc.) in ICI-treated patients with TPO/TgAb-negative thyroid irAEs.

6.3 Materials and methods

We developed a pilot study to assess whether autoantibodies to non-classical thyroid antigens (NIS, pendrin, T4, etc.) were present in patients with ICI-associated thyroid irAEs. MIA Biospecimen Bank serum samples were chosen from the same cohort of patients used in our previous antibody study, so that TPOAb and TgAb titers were known for all patient samples (169). The study was assessed and approved by the Animal Ethics Committee, Northern Sydney Local Health District (reference RESP/21/003). Experimentation was conducted in alignment with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines (243).

6.3.1 Experimental design - overview

A total of eight female Lewis rats were used. On arrival from Animal Resources Centre, Western Australia, rats were approximately 10 weeks of age and were housed in pairs in standard clear polycarbonate cages within Kearns animal facility (Kolling Institute of Medical Research, St. Leonard's, NSW, Australia). During a two-week acclimatization period animals were fed ad lib and maintained on a 12-hour light-dark

schedule. Animals were monitored and weighed twice weekly during acclimatization. At 12-weeks of age, rats were anaesthetized and then euthanized via thoracotomy and intra-cardiac perfusion fixation. Intra-cardiac fixation was chosen over immersion fixation, as it uses the animal's own vasculature to deliver fixative to tissues, thus preserving tissue more rapidly and uniformly, which limits hypoxic damage degrading the quality of the tissue specimen (244). Following fixation, the thyroid and other target organs were harvested, and flash frozen in liquid nitrogen. Because rat and human thyroid tissues are structurally similar, rat thyroid can act as a useful proxy to study thyroid disease in humans (245).

6.3.2 Anaesthesia protocol

Prior to surgery, animals were lightly anaesthetized using 2% isoflurane in oxygen via induction chamber. Once anaesthetized, animals were placed on a heating pad to expose the abdomen and 1% isoflurane was continued via nose cone. Animals were then administered 80 mg/kg intraperitoneal ketamine into the lower left quadrant of the abdomen via 25-gauge needle to induce a deeper plane of anaesthesia.

6.3.3 Thoracotomy protocol

Following confirmation that a surgical plane of anaesthesia had been achieved (animal unresponsive to firm toe pinch), the chest cavity was opened using a lateral incision through the chest wall just inferior to the rib cage (Figure 4.1). Blunt dissection was used to separate the diaphragm from the liver and other viscera (246). Vertical incisions lateral to each rib cage were made. Using a hemostat, the sternum and ribs were reflected cranially to expose cardiac structures and major vessels within the pleural cavity (Figure 6.1).

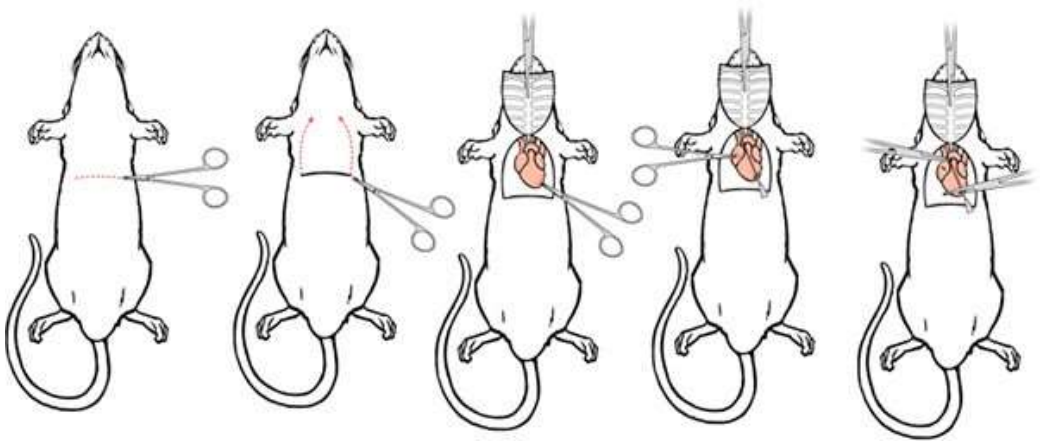

Figure 6.1: Thoracotomy procedure used to expose cardiac organs

Image taken from (246) Gage et al. Whole animal perfusion fixation for rodents. *J Vis Exp*. 2012;65:3564.

6.3.4 Perfusion-fixation protocol

Using a 5.0 nylon suture, the left ventricle was pierced to anchor the heart prior to making a small incision at the apex. A 21-gauge blunt cannula was fed through the incision and advanced gently to sit within the ascending aorta (246). A second incision was made to the right atrium to create an outflow tract. To begin, phosphate buffered saline (PBS) was administered manually via an infusion set attached to the aortic cannula until clear fluid drained from the right atrium and the liver was pale. Intra-cardiac PBS infusion was followed by perfusion with 4% paraformaldehyde via the same cannula (Figure 6.2). We monitored for vigorous tremors during perfusion and animal stiffness at the end of the procedure was used as an indicator of a successful fixation.

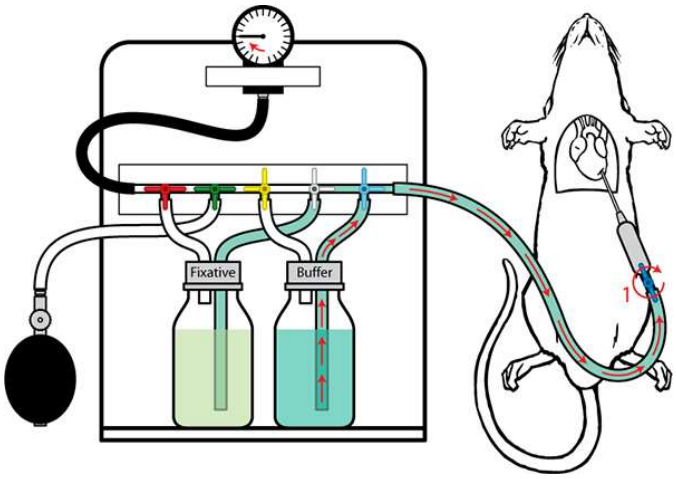

Figure 6.2: Perfusion procedure used to perform whole animal fixation

Image taken from (246) Gage et al. Whole animal perfusion fixation for rodents. *J Vis Exp*. 2012;65:3564.

6.3.5 Tissue extraction and preparation

Following completion of the perfusion-fixation process, the thyroid was harvested and flash frozen in isopentane (cooled in liquid nitrogen). Thyroid tissue blocks were stored at -80°C until sectioned (5 µm) via cryostat microtome and placed onto slides. Cryostat sections were sequentially stained using Mayer's haematoxylin and Scott's bluing solution. Slides were checked for correct ratio of staining prior to dehydration in ethanol and xylene. Dehydrated tissues were then mounted using a resinous media. Future plans are for rat thyroid to be treated with human sera from melanoma patients with ICI-associated thyroid irAEs ex vivo. Rat tissues will be assessed for the presence of autoantibodies using indirect IIF. Patients with thyroid irAEs and high titers of TPOAb

and TgAb (identified from our previous work) will be used as positive controls. Patients with thyroid irAEs without detectable TPOAb or TgAb will also be subjected to IIF to address whether non-TPO, non-Tg antibodies are present and potentially involved in the pathogenesis of ICI-induced thyroid dysfunction.

6.4 Discussion

The effects of checkpoint inhibitor treatment on the immune system are wide ranging, and multiple immune mechanisms likely contribute to development of irAEs (247). ICI-effects on T-cells are best characterized, although interactions with innate immune populations and cells of the B-lymphocyte lineage are increasingly recognized as playing important roles (247). In this chapter, we describe optimization of an animal model that will be used to test for non-traditional autoantibodies in association with thyroid irAEs. This model has previously been validated in patients with multiple sclerosis, where novel CNS antibodies have been identified (248). Unfortunately, IIF experiments could not be completed prior to thesis submission, but much work has been done to optimize the perfusion-fixation method and to extract and store fixed thyroid tissue for future testing.

Anti-thyroid antibodies (TPOAb, TgAb) are more prevalent in patients who experience a thyroid irAE relative to those that do not (169). However, over half of patients with a thyroid irAE do not have measurable TPOAb or TgAb (169). We propose that other thyroid antibodies are involved in the pathogenesis of TPOAb/TgAb-negative thyroid irAEs and that determination of non-traditional autoantibodies mediating thyroid irAE development is important, with potential for utility as a clinical biomarker and to

develop personalized management strategies for patients at the highest risk of thyroid toxicity.

The role of non-traditional antibodies has already been established in many irAEs affecting non-thyroid organ systems. For instance, pre-treatment antibodies against guanine nucleotide binding protein (GNAL) and integral membrane protein 2B (ITM2B), two proteins found within normal pituitary tissue, are associated with development of ICI-induced hypophysitis (66). Similarly, BP180 antibodies targeting desmosomes within the dermal-epidermal junction are overrepresented in patients with skin-based irAEs and antibodies to CD74, which normally has modest expression in lung tissue, are present in patients with ICI-associated pneumonitis (66, 249). Other tissue specific antibodies have been documented in association with irAEs such as dermatomyositis, Raynaud's phenomenon, and cerebritis (247). Moreover, non-specific antibodies such as antinuclear antibody (ANA), extractable nuclear antigens and anti-smooth muscle antibodies have been associated with irAE development in multiple discrete organ systems (247).

Interestingly, patients with pre-existing autoimmune disease have increased susceptibility to disease reactivation or de novo irAEs following treatment with ICIs (59-61). It has been proposed that pre-treatment antibody positivity may signal subclinical autoimmunity that is unleashed by the effects of antibodies against the PD-1 and CTLA-4 pathways. In one study, Gowen et al used an unbiased proteomic microarray to compare global antibody profiles in pre-treatment sera of melanoma patients treated with ICIs (250). Toxicity specific antibody signatures were observed

capable of discriminating between patients with and without irAEs with >90% accuracy, sensitivity and specificity (250).

Identification of patients at increased risk of ICI-toxicity is relevant and worthwhile, as the development of an irAE has been shown to associate with improved cancer outcomes, particularly thyroid irAEs (70-74, 166-168). Although antibodies are associated with irAE development, until recently their prognostic significance independent of irAE development was less clear. Emerging data suggest antibodies are independently associated with ICI-related outcomes. For instance, baseline positivity for rheumatoid factor and ANA has been associated with improved PFS, objective response rate and disease control rates in a retrospective study of 137 patients treated with PD-1 inhibitors (185). Conversely, some antibodies may protect against irAE development. Antibodies against fibroblast growth factor receptor-1 appear protective against irAE development, but were associated with lower overall survival (251).

Another possibility is that patients with TPOAb/TgAb-negative thyroid irAE cases may have a non-antibody mediated aetiology and represent a distinct class of thyroid disorder separate to patients with TPOAb/TgAb-positive thyroid irAEs.

6.5 Conclusion and future directions

The work presented in this chapter outlines an experimental approach to test for non-traditional antibodies against thyroid antigens. We optimized an intra-cardiac perfusion fixation technique and employed it to collect thyroid specimens from eight rats. These samples will be foundational for future IIF work using targeted antibodies against non-traditional thyroid antigens to establish if such antibodies are associated with development of thyroid irAEs.

Determination of the immune mechanisms underpinning thyroid irAEs is important clinically, as pre-treatment sampling could be an easy and effective method for measuring biomarkers with capacity to identify patients at highest risk of irAEs and those most likely to respond to ICI-treatment.

Chapter 7

Summary and future directions

7.1 Summary and application of thesis findings

Immune checkpoint blockade represents a huge step forward in the treatment of advanced malignancies, many having previously been considered a death sentence. However, ICI-associated irAEs remain a challenge and can lead to treatment interruption, organ-specific morbidity, and rarely death. Thyroid irAEs are among the most frequent immune toxicity in any organ system. Aims of this thesis were to characterize the clinical, biochemical, immune, and genetic factors involved in development of thyroid irAEs following ICI-treatment. Despite the challenges imposed by COVID-19, research presented within this thesis accomplished many of those aims. Our initial work produced the most comprehensive description of thyroid irAEs to date in a pure cohort of 1246 patients with melanoma, free from pre-existing thyroid disease, undergoing ICI-treatment. This work reported multiple novel aspects of thyroid irAEs, including a prevalence of >50% in patients receiving combined CTLA-4 and PD-1 checkpoint inhibition. We produced a detailed description of the kinetic changes in thyroid function that occur following ICI-treatment, with most affected patients experiencing a rapid onset of transient thyrotoxicosis followed by spontaneous restoration of euthyroidism or progression to hypothyroidism. However, we also showed that a small number of patients have delayed presentations, sometimes years after the commencement of ICI-treatment. We identified age, sex, and baseline TSH level as being associated with the development of a thyroid irAE and were the first to show that the most severe presentations of thyroiditis following ICI-treatment were

associated with major improvements in cancer related PFS and OS. As evidence of the importance of this work, it was recognized as one of the top 10% of cited articles in the *Journal of Clinical Endocrinology and Metabolism* in 2021.

A follow-up study in a subset of 122 patients prospectively examined the importance of TPOAb and TgAb to the development of thyroid irAEs. We were the first group to address this issue in a standardized fashion, and in so doing clarified the clinical relevance of these biomarkers and their association with thyroid irAE susceptibility. Furthermore, we showed that TRAB is not a key driver of thyrotoxicosis in the ICI-setting, and that use of the inflammatory cytokine IL-6 has no value in identifying patients at risk of thyroid irAEs, whether measured at baseline or during the initial period of thyroid dysfunction.

As less than 50% of patients with a thyroid irAE were positive for TPOAb or TgAb at baseline, we posited that antibodies against other thyroid antigens may be involved in the pathogenesis of thyroid irAEs. To test this hypothesis, we optimized an animal model previously validated to identify novel autoantibodies by indirect immunofluorescence in patients with multiple sclerosis (248). To date, we have successfully implemented the trans-cardiac perfusion fixation technique and harvested thyroid glands from eight animals. Although initial perfusions failed to adequately fix thyroid tissue, troubleshooting improved outcomes with immunohistochemical analysis of more recently perfused thyroids demonstrating higher quality tissue suitable for IIF testing.

During my candidature, a landmark paper was published in *Nature* identifying a polymorphism in the FLT3 gene as having the largest effect on susceptibility to

development of AITD. As we were interested in genetic determinants of thyroid irAEs and given their many similarities to AITD occurring in the non-cancer setting, we tested whether the same FLT3 SNP was associated with susceptibility to thyroid irAE development. Although low allelic frequency of the variant limited the power of our study to detect an association, we found that FLT3 rs76428106-C heterozygous patients were 50% more likely to develop a thyroid irAE and both FLT3 rs76428106-C homozygous patients developed a thyroid irAE. These findings suggest further study of this variant in thyroid irAEs is warranted.

Finally, we measured circulating immune populations in ICI-treated patients with and without thyroid irAEs. In so doing we identified a baseline immune signature that was unique to thyroid irAEs and may have future potential as a biomarker. The results also have mechanistic implications and suggest that immune cells of multiple lineages contribute to the development of thyroid irAEs.

7.2 Future directions

The body of work presented in this thesis significantly advances our understanding of thyroid irAEs as a complication of ICI-treatment. However, more work needs to be done. Improved understanding of the mechanisms contributing to irAEs in the thyroid and other organ systems is important to refine ICI-treatment and uncouple the anti-tumor effects from the detrimental autoimmune/autoinflammatory side effects which underpin irAE development. Further research has potential to improve patient outcomes. By identification of demographic, biochemical, genetic, and immune biomarkers of irAEs, it may be possible to stratify patients and tailor monitoring and treatment regimens to encompass their personalized likelihood of an adverse outcome.

In this situation, patients at highest risk of severe or permanent immune toxicity could been offered alternative treatments or have a tailored monitoring program. Finally, and perhaps most importantly, ICI-associated thyroid irAEs represent a novel model of autoimmunity and further research into this entity could help to elucidate factors promoting susceptibility to and development of thyroid autoimmunity more generally. As one of the most prevalent autoimmune disorders in any organ system with no currently available treatment apart from hormone replacement, the implications of such findings are potentially practice changing.

References

1. Bona CA, Bonilla FA. Textbook of Immunology: CRC press; 2019.

2. Callahan MK, Postow MA, Wolchok JD. Targeting T cell co-receptors for cancer therapy. Immunity. 2016;44(5):1069-78.

3. Schildberg FA, Klein SR, Freeman GJ, Sharpe AH. Coinhibitory pathways in the B7-CD28 ligand-receptor family. Immunity. 2016;44(5):955-72.

4. Brunet J-F, Denizot F, Luciani M-F, Roux-Dosseto M, Suzan M, Mattei M-G, et al. A new member of the immunoglobulin superfamily—CTLA-4. Nature. 1987;328(6127):267-70.

5. Magistrelli G, Jeannin P, Herbault N, Benoit de Coignac A, Gauchat JF, Bonnefoy JY, et al. A soluble form of CTLA-4 generated by alternative splicing is expressed by nonstimulated human T cells. Eur J Immunol. 1999;29(11):3596-602.

6. Gerold KD, Zheng P, Rainbow DB, Zernecke A, Wicker LS, Kissler S. The soluble CTLA-4 splice variant protects from type 1 diabetes and potentiates regulatory T-cell function. Diabetes. 2011;60(7):1955-63.

7. Scalapino KJ, Daikh DI. CTLA-4: a key regulatory point in the control of autoimmune disease. Immunological Rev. 2008;223(1):143-55.

8. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity. 1995;3(5):541-7.

9. Kuehn HS, Ouyang W, Lo B, Deenick EK, Niemela JE, Avery DT, et al. Immune dysregulation in human subjects with heterozygous germline mutations in CTLA4. Science. 2014;345(6204):1623-7.

10. Schubert D, Bode C, Kenefeck R, Hou TZ, Wing JB, Kennedy A, et al. Autosomal dominant immune dysregulation syndrome in humans with CTLA4 mutations. Nat Med. 2014;20(12):1410-6.

11. Schneider H, Martin M, Agarraberes FA, Yin L, Rapoport I, Kirchhausen T, et al. Cytolytic T lymphocyte-associated antigen-4 and the TCRζ/CD3 complex, but not CD28, interact with clathrin adaptor complexes AP-1 and AP-2. J Immunol. 1999;163(4):1868-79.

12. Shiratori T, Miyatake S, Ohno H, Nakaseko C, Isono K, Bonifacino JS, et al. Tyrosine phosphorylation controls internalization of CTLA-4 by regulating its interaction with clathrin-associated adaptor complex AP-2. Immunity. 1997;6(5):583-9.

13. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, et al. CTLA-4 control over Foxp3+ regulatory T cell function. Science. 2008;322(5899):271-5.

14. Rudd CE, Taylor A, Schneider H. CD28 and CTLA-4 coreceptor expression and signal transduction. Immunological Rev. 2009;229(1):12-26.

15. Fallarino F, Grohmann U, Hwang KW, Orabona C, Vacca C, Bianchi R, et al. Modulation of tryptophan catabolism by regulatory T cells. Nat Immunol. 2003;4(12):1206-12.

16. Zhang X, Schwartz J-CD, Guo X, Bhatia S, Cao E, Chen L, et al. Structural and functional analysis of the costimulatory receptor programmed death-1. Immunity. 2004;20(3):337-47.

17. Wei SC, Duffy CR, Allison JP. Fundamental mechanisms of immune checkpoint blockade therapy. Cancer Discovery. 2018;8(9):1069-86.

18. Ishida Y, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. EMBO J. 1992;11(11):3887-95.

19. Nielsen C, Ohm-Laursen L, Barington T, Husby S, Lillevang ST. Alternative splice variants of the human PD-1 gene. Cellular Immunol. 2005;235(2):109-16.

20. Wan B, Nie H, Liu A, Feng G, He D, Xu R, et al. Aberrant regulation of synovial T cell activation by soluble costimulatory molecules in rheumatoid arthritis. J Immunol. 2006;177(12):8844-50.

21. Nishimura H, Nose M, Hiai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity. 1999;11(2):141-51.

22. Nishimura H, Okazaki T, Tanaka Y, Nakatani K, Hara M, Matsumori A, et al. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. Science. 2001;291(5502):319-22.

23. Terawaki S, Chikuma S, Shibayama S, Hayashi T, Yoshida T, Okazaki T, et al. IFN-α directly promotes programmed cell death-1 transcription and limits the duration of T cell-mediated immunity. J Immunol. 2011;186(5):2772-9.

24. Loke Pn, Allison JP. PD-L1 and PD-L2 are differentially regulated by Th1 and Th2 cells. PNAS. 2003;100(9):5336-41.

25. Yokosuka T, Takamatsu M, Kobayashi-Imanishi W, Hashimoto-Tane A, Azuma M, Saito T. Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. J Experimental Med. 2012;209(6):1201-17.

26. MacCarty WC. Longevity in cancer: a study of 293 cases. Ann Surgery. 1922;76(1):9.

27. Gross L. Intradermal immunization of C3H mice against a sarcoma that originated in an animal of the same line. Cancer Res. 1943;3(5):326-33.

28. Hellström I, Hellström KE, Pierce GE, Yang JP. Cellular and humoral immunity to different types of human neoplasms. Nature. 1968;220(5174):1352-4.

29. Korman AJ, Garrett-Thomson SC, Lonberg N. The foundations of immune checkpoint blockade and the ipilimumab approval decennial. Nat Rev Drug Discovery. 2021:1-20.

30. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. Science. 1996;271(5256):1734-6.

31. Van Elsas A, Hurwitz AA, Allison JP. Combination immunotherapy of B16 melanoma using anti–cytotoxic T lymphocyte–associated antigen 4 (CTLA-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF)-producing vaccines induces rejection of subcutaneous and metastatic tumors accompanied by autoimmune depigmentation. J Experimental Med. 1999;190(3):355-66.

32. Postow MA, Callahan MK, Wolchok JD. Immune checkpoint blockade in cancer therapy. J Clin Oncol. 2015;33(17):1974.

33. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med. 2010;363(8):711-23.

34. Robert C, Thomas L, Bondarenko I, O'Day S, Weber J, Garbe C, et al. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. N Engl J Med. 2011;364(26):2517-26.

35. Brahmer JR, Drake CG, Wollner I, Powderly JD, Picus J, Sharfman WH, et al. Phase I study of single-agent anti–programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. J Clin Oncol. 2010;28(19):3167.

36. Naimi A, Mohammed RN, Raji A, Chupradit S, Yumashev AV, Suksatan W, et al. Tumor immunotherapies by immune checkpoint inhibitors (ICIs); the pros and cons. Cell Comm Signaling. 2022;20(1):1-31.

37. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer. 2012;12(4):252-64.

38. Zou W, Chen L. Inhibitory B7-family molecules in the tumour microenvironment. Nat Rev Immunol. 2008;8(6):467-77.

39. Butte MJ, Keir ME, Phamduy TB, Sharpe AH, Freeman GJ. Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. Immunity. 2007;27(1):111-22.

40. Doroshow DB, Bhalla S, Beasley MB, Sholl LM, Kerr KM, Gnjatic S, et al. PD-L1 as a biomarker of response to immune-checkpoint inhibitors. Nat Rev Clin Oncol. 2021;18(6):345-62.

41. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, et al. Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med. 2013;369(2):122-33.

42. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Rutkowski P, Lao CD, et al. Five-Year Survival with Combined Nivolumab and Ipilimumab in Advanced Melanoma. N Engl J Med. 2019;381(16):1535-46.

43. Postow MA, Sidlow R, Hellmann MD. Immune-related adverse events associated with immune checkpoint blockade. N Engl J Med. 2018;378(2):158-68.

44. Xu C, Chen Y-P, Du X-J, Liu J-Q, Huang C-L, Chen L, et al. Comparative safety of immune checkpoint inhibitors in cancer: systematic review and network meta-analysis. BMJ. 2018;363.

45. Ramos-Casals M, Brahmer JR, Callahan MK, Flores-Chávez A, Keegan N, Khamashta MA, et al. Immune-related adverse events of checkpoint inhibitors. Nat Rev Dis Primers. 2020;6(1):1-21.

46. Wang Y, Zhou S, Yang F, Qi X, Wang X, Guan X, et al. Treatment-Related Adverse Events of PD-1 and PD-L1 Inhibitors in Clinical Trials: A Systematic Review and Meta-analysis. JAMA Oncol. 2019;5(7):1008-19.

47. Kanjanapan Y, Day D, Butler M, Wang L, Joshua A, Hogg D, et al. Delayed immune-related adverse events in assessment for dose-limiting toxicity in early phase immunotherapy trials. Eur J Cancer. 2019;107:1-7.

48. Khoja L, Day D, Chen TW-W, Siu L, Hansen AR. Tumour-and class-specific patterns of immune-related adverse events of immune checkpoint inhibitors: a systematic review. Ann Oncol. 2017;28(10):2377-85.

49. Wolchok JD, Chiarion-Sileni V, Gonzalez R, Rutkowski P, Grob JJ, Cowey CL, et al. Overall Survival with Combined Nivolumab and Ipilimumab in Advanced Melanoma. N Engl J Med. 2017;377(14):1345-56.

50. Johnson DB, Nebhan CA, Moslehi JJ, Balko JM. Immune-checkpoint inhibitors: Long-term implications of toxicity. Nat Rev Clin Oncol. 2022;19(4):254-67.

51. Berner F, Bomze D, Diem S, Ali OH, Fässler M, Ring S, et al. Association of checkpoint inhibitor–induced toxic effects with shared cancer and tissue antigens in non–small cell lung cancer. JAMA Oncol. 2019;5(7):1043-7.

52. Johnson DB, Balko JM, Compton ML, Chalkias S, Gorham J, Xu Y, et al. Fulminant myocarditis with combination immune checkpoint blockade. N Engl J Med. 2016;375(18):1749-55.

53. Guida M, Strippoli S, Maule M, Quaglino P, Ramondetta A, Sileni VC, et al. Immune checkpoint inhibitor associated vitiligo and its impact on survival in patients with metastatic melanoma: an Italian Melanoma Intergroup study. ESMO Open. 2021;6(2):100064.

54. Iwama S, De Remigis A, Callahan MK, Slovin SF, Wolchok JD, Caturegli P. Pituitary expression of CTLA-4 mediates hypophysitis secondary to administration of CTLA-4 blocking antibody. Science Translational Med. 2014;6(230):230ra45.

55. Dubin K, Callahan MK, Ren B, Khanin R, Viale A, Ling L, et al. Intestinal microbiome analyses identify melanoma patients at risk for checkpoint-blockade-induced colitis. Nat Comm. 2016;7(1):1-8.

56. Smithy JW, Faleck DM, Postow MA. Facts and hopes in prediction, diagnosis, and treatment of immune-related adverse events. Clin Cancer Res. 2022;28(7):1250-7.

57. Eun Y, Kim IY, Sun J-M, Lee J, Cha H-S, Koh E-M, et al. Risk factors for immune-related adverse events associated with anti-PD-1 pembrolizumab. Scientific Rep. 2019;9(1):1-8.

58. Kartolo A, Sattar J, Sahai V, Baetz T, Lakoff JM. Predictors of immunotherapy-induced immune-related adverse events. Curr Oncol. 2018;25(5):e403-e10.

59. Johnson DB, Sullivan RJ, Ott PA, Carlino MS, Khushalani NI, Ye F, et al. Ipilimumab Therapy in Patients With Advanced Melanoma and Preexisting Autoimmune Disorders. JAMA Oncol. 2016;2(2):234-40.

60. Kähler KC, Eigentler TK, Gesierich A, Heinzerling L, Loquai C, Meier F, et al. Ipilimumab in metastatic melanoma patients with pre-existing autoimmune disorders. Cancer Immunol, Immunotherapy. 2018;67(5):825-34.

61. Menzies AM, Johnson D, Ramanujam S, Atkinson V, Wong A, Park J, et al. Anti-PD-1 therapy in patients with advanced melanoma and preexisting autoimmune disorders or major toxicity with ipilimumab. Ann Oncol. 2017;28(2):368-76.

62. Lidar M, Giat E, Garelick D, Horowitz Y, Amital H, Steinberg-Silman Y, et al. Rheumatic manifestations among cancer patients treated with immune checkpoint inhibitors. Autoimmunity Rev. 2018;17(3):284-9.

63. Mammen AL, Rajan A, Pak K, Lehky T, Casciola-Rosen L, Donahue RN, et al. Pre-existing antiacetylcholine receptor autoantibodies and B cell lymphopaenia are associated with the development of myositis in patients with thymoma treated with avelumab, an immune checkpoint inhibitor targeting programmed death-ligand 1. Ann Rheumatic Dis. 2019;78(1):150-2.

64. Kobayashi T, Iwama S, Yasuda Y, Okada N, Tsunekawa T, Onoue T, et al. Patients with antithyroid antibodies are prone to develop destructive thyroiditis by nivolumab: a prospective study. J Endocr Soc. 2018;2(3):241-51.

65. Sakakida T, Ishikawa T, Chihara Y, Harita S, Uchino J, Tabuchi Y, et al. Safety and efficacy of PD-1/PD-L1 blockade in patients with preexisting antinuclear antibodies. Clin Translational Oncol. 2020;22(6):919-27.

66. Tahir SA, Gao J, Miura Y, Blando J, Tidwell RS, Zhao H, et al. Autoimmune antibodies correlate with immune checkpoint therapy-induced toxicities. PNAS. 2019;116(44):22246-51.

67. Das R, Bar N, Ferreira M, Newman AM, Zhang L, Bailur JK, et al. Early B cell changes predict autoimmunity following combination immune checkpoint blockade. J Clin Investigation. 2018;128(2):715-20.

68. Young A, Quandt Z, Bluestone JA. The balancing act between cancer immunity and autoimmunity in response to immunotherapy. Cancer Immunol Res. 2018;6(12):1445-52.

69. Maher VE, Fernandes LL, Weinstock C, Tang S, Agarwal S, Brave M, et al. Analysis of the association between adverse events and outcome in patients receiving a programmed death protein 1 or programmed death ligand 1 antibody. J Clin Oncol. 2019;37(30):2730-7.

70. Attia P, Phan GQ, Maker AV, Robinson MR, Quezado MM, Yang JC, et al. Autoimmunity correlates with tumor regression in patients with metastatic melanoma treated with anti–cytotoxic T-lymphocyte antigen-4. J Clin Oncol. 2005;23(25):6043.

71. Haratani K, Hayashi H, Chiba Y, Kudo K, Yonesaka K, Kato R, et al. Association of immune-related adverse events with nivolumab efficacy in non–small-cell lung cancer. JAMA Oncol. 2018;4(3):374-8.

72. Ricciuti B, Genova C, De Giglio A, Bassanelli M, Dal Bello MG, Metro G, et al. Impact of immune-related adverse events on survival in patients with advanced non-small cell lung cancer treated with nivolumab: long-term outcomes from a multi-institutional analysis. J Cancer Res Clin Oncol. 2019;145(2):479-85.

73. Freeman-Keller M, Kim Y, Cronin H, Richards A, Gibney G, Weber JS. Nivolumab in resected and unresectable metastatic melanoma: characteristics of immune-related adverse events and association with outcomes. Clin Cancer Res. 2016;22(4):886-94.

74. Eggermont AM, Kicinski M, Blank CU, Mandala M, Long GV, Atkinson V, et al. Association between immune-related adverse events and recurrence-free survival among patients with stage III melanoma randomized to receive pembrolizumab or placebo: a secondary analysis of a randomized clinical trial. JAMA Oncol. 2020;6(4):519-27.

75. Von Pawel J, Syrigos K, Mazieres J, Cortinovis D, Dziadziuszko R, Gandara D, et al. Association between immune-related adverse events (irAEs) and atezolizumab efficacy in advanced NSCLC: analyses from the phase III study OAK. Ann Oncol. 2017;28:v469.

76. Fan Y, Xie W, Huang H, Wang Y, Li G, Geng Y, et al. Association of immune related adverse events with efficacy of immune checkpoint inhibitors and overall survival in cancers: a systemic review and meta-analysis. Frontiers Oncol. 2021;11:1081.

77. Zhong L, Wu Q, Chen F, Liu J, Xie X. Immune-related adverse events: promising predictors for efficacy of immune checkpoint inhibitors. Cancer Immunol, Immunotherapy. 2021;70(9):2559-76.

78. Chang L-S, Barroso-Sousa R, Tolaney SM, Hodi FS, Kaiser UB, Min L. Endocrine toxicity of cancer immunotherapy targeting immune checkpoints. Endocr Rev. 2019;40(1):17-65.

79. Melmed S, Polonsky KS, Larsen PR, Kronenberg HM. Williams Textbook of Endocrinology e-Book: Elsevier Health Sciences; 2015.

80. Helmink BA, Gaudreau P-O, Wargo JA. Immune checkpoint blockade across the cancer care continuum. Immunity. 2018;48(6):1077-80.

81. Esfahani K, Meti N, Miller WH, Jr., Hudson M. Adverse events associated with immune checkpoint inhibitor treatment for cancer. CMAJ. 2019;191(2):E40-E6.

82. Pearce EN, Farwell AP, Braverman LE. Thyroiditis. N Engl J Med. 2003;348(26):2646-55.

83. Eggermont AM, Chiarion-Sileni V, Grob JJ, Dummer R, Wolchok JD, Schmidt H, et al. Prolonged Survival in Stage III Melanoma with Ipilimumab Adjuvant Therapy. N Engl J Med. 2016;375(19):1845-55.

84. Ascierto PA, Del Vecchio M, Robert C, Mackiewicz A, Chiarion-Sileni V, Arance A, et al. Ipilimumab 10 mg/kg versus ipilimumab 3 mg/kg in patients with unresectable or metastatic melanoma: a randomised, double-blind, multicentre, phase 3 trial. Lancet Oncol. 2017;18(5):611-22.

85. Govindan R, Szczesna A, Ahn MJ, Schneider CP, Gonzalez Mella PF, Barlesi F, et al. Phase III Trial of Ipilimumab Combined With Paclitaxel and Carboplatin in Advanced Squamous Non-Small-Cell Lung Cancer. J Clin Oncol. 2017;35(30):3449-57.

86. Reck M, Luft A, Szczesna A, Havel L, Kim SW, Akerley W, et al. Phase III Randomized Trial of Ipilimumab Plus Etoposide and Platinum Versus Placebo Plus Etoposide and Platinum in Extensive-Stage Small-Cell Lung Cancer. J Clin Oncol. 2016;34(31):3740-8.

87. Weber J, Mandala M, Del Vecchio M, Gogas HJ, Arance AM, Cowey CL, et al. Adjuvant Nivolumab versus Ipilimumab in Resected Stage III or IV Melanoma. N Engl J Med. 2017;377(19):1824-35.

88. Robert C, Schachter J, Long GV, Arance A, Grob JJ, Mortier L, et al. Pembrolizumab versus Ipilimumab in Advanced Melanoma. N Engl J Med. 2015;372(26):2521-32.

89. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL, Lao CD, et al. Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. N Engl J Med. 2015;373(1):23-34.

90. Robert C, Long GV, Brady B, Dutriaux C, Maio M, Mortier L, et al. Nivolumab in previously untreated melanoma without BRAF mutation. N Engl J Med. 2015;372(4):320-30.

91. Weber JS, D'Angelo SP, Minor D, Hodi FS, Gutzmer R, Neyns B, et al. Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomised, controlled, open-label, phase 3 trial. Lancet Oncol. 2015;16(4):375-84.

92. Borghaei H, Paz-Ares L, Horn L, Spigel DR, Steins M, Ready NE, et al. Nivolumab versus Docetaxel in Advanced Nonsquamous Non-Small-Cell Lung Cancer. N Engl J Med. 2015;373(17):1627-39.

93. Brahmer J, Reckamp KL, Baas P, Crino L, Eberhardt WE, Poddubskaya E, et al. Nivolumab versus Docetaxel in Advanced Squamous-Cell Non-Small-Cell Lung Cancer. N Engl J Med. 2015;373(2):123-35.

94. Kang YK, Boku N, Satoh T, Ryu MH, Chao Y, Kato K, et al. Nivolumab in patients with advanced gastric or gastro-oesophageal junction cancer refractory to, or intolerant of, at least two previous chemotherapy regimens (ONO-4538-12, ATTRACTION-2): a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet. 2017;390(10111):2461-71.

95. Hellmann MD, Ciuleanu TE, Pluzanski A, Lee JS, Otterson GA, Audigier-Valette C, et al. Nivolumab plus Ipilimumab in Lung Cancer with a High Tumor Mutational Burden. N Engl J Med. 2018;378(22):2093-104.

96. Ferris RL, Blumenschein G, Jr., Fayette J, Guigay J, Colevas AD, Licitra L, et al. Nivolumab for Recurrent Squamous-Cell Carcinoma of the Head and Neck. N Engl J Med. 2016;375(19):1856-67.

97. Bellmunt J, de Wit R, Vaughn DJ, Fradet Y, Lee JL, Fong L, et al. Pembrolizumab as Second-Line Therapy for Advanced Urothelial Carcinoma. N Engl J Med. 2017;376(11):1015-26.

98. Cohen EEW, Soulieres D, Le Tourneau C, Dinis J, Licitra L, Ahn MJ, et al. Pembrolizumab versus methotrexate, docetaxel, or cetuximab for recurrent or metastatic head-and-neck squamous cell carcinoma (KEYNOTE-040): a randomised, open-label, phase 3 study. Lancet. 2019;393(10167):156-67.

99. Eggermont AMM, Blank CU, Mandala M, Long GV, Atkinson V, Dalle S, et al. Adjuvant Pembrolizumab versus Placebo in Resected Stage III Melanoma. N Engl J Med. 2018;378(19):1789-801.

100. Gandhi L, Rodriguez-Abreu D, Gadgeel S, Esteban E, Felip E, De Angelis F, et al. Pembrolizumab plus Chemotherapy in Metastatic Non-Small-Cell Lung Cancer. N Engl J Med. 2018;378(22):2078-92.

101. Garon EB, Rizvi NA, Hui R, Leighl N, Balmanoukian AS, Eder JP, et al. Pembrolizumab for the treatment of non-small-cell lung cancer. N Engl J Med. 2015;372(21):2018-28.

102. Herbst RS, Baas P, Kim D-W, Felip E, Pérez-Gracia JL, Han J-Y, et al. Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): a randomised controlled trial. Lancet. 2016;387(10027):1540-50.

103. Mok TSK, Wu Y-L, Kudaba I, Kowalski DM, Cho BC, Turna HZ, et al. Pembrolizumab versus chemotherapy for previously untreated, PD-L1-expressing, locally advanced or metastatic non-small-cell lung cancer (KEYNOTE-042): a randomised, open-label, controlled, phase 3 trial. Lancet. 2019;393(10183):1819-30.

104. Paz-Ares L, Luft A, Vicente D, Tafreshi A, Gumus M, Mazieres J, et al. Pembrolizumab plus Chemotherapy for Squamous Non-Small-Cell Lung Cancer. N Engl J Med. 2018;379(21):2040-51.

105. Reck M, Rodriguez-Abreu D, Robinson AG, Hui R, Csoszi T, Fulop A, et al. Pembrolizumab versus Chemotherapy for PD-L1-Positive Non-Small-Cell Lung Cancer. N Engl J Med. 2016;375(19):1823-33.

106. Shitara K, Ozguroglu M, Bang YJ, Di Bartolomeo M, Mandala M, Ryu MH, et al. Pembrolizumab versus paclitaxel for previously treated, advanced gastric or gastro-oesophageal junction cancer (KEYNOTE-061): a randomised, open-label, controlled, phase 3 trial. Lancet. 2018;392(10142):123-33.

107. Mateos MV, Blacklock H, Schjesvold F, Oriol A, Simpson D, George A, et al. Pembrolizumab plus pomalidomide and dexamethasone for patients with relapsed or refractory multiple myeloma (KEYNOTE-183): a randomised, open-label, phase 3 trial. Lancet Haematol. 2019;6(9):e459-e69.

108. Usmani SZ, Schjesvold F, Oriol A, Karlin L, Cavo M, Rifkin RM, et al. Pembrolizumab plus lenalidomide and dexamethasone for patients with treatment-naive multiple myeloma (KEYNOTE-185): a randomised, open-label, phase 3 trial. Lancet Haematol. 2019;6(9):e448-e58.

109. Motzer RJ, Tannir NM, McDermott DF, Aren Frontera O, Melichar B, Choueiri TK, et al. Nivolumab plus Ipilimumab versus Sunitinib in Advanced Renal-Cell Carcinoma. N Engl J Med. 2018;378(14):1277-90.

110. Horn L, Mansfield AS, Szczęsna A, Havel L, Krzakowski M, Hochmair MJ, et al. First-line atezolizumab plus chemotherapy in extensive-stage small-cell lung cancer. N Engl J Med. 2018;379(23):2220-9.

111. Schmid P, Adams S, Rugo HS, Schneeweiss A, Barrios CH, Iwata H, et al. Atezolizumab and nab-paclitaxel in advanced triple-negative breast cancer. N Engl J Med. 2018;379(22):2108-21.

112. Socinski MA, Jotte RM, Cappuzzo F, Orlandi F, Stroyakovskiy D, Nogami N, et al. Atezolizumab for first-line treatment of metastatic nonsquamous NSCLC. N Engl J Med. 2018;378(24):2288-301.

113. Rini BI, Powles T, Atkins MB, Escudier B, McDermott DF, Suarez C, et al. Atezolizumab plus bevacizumab versus sunitinib in patients with previously untreated metastatic renal cell carcinoma (IMmotion151): a multicentre, open-label, phase 3, randomised controlled trial. Lancet. 2019;393(10189):2404-15.

114. Antonia SJ, Villegas A, Daniel D, Vicente D, Murakami S, Hui R, et al. Overall survival with durvalumab after chemoradiotherapy in stage III NSCLC. N Engl J Med. 2018;379(24):2342-50.

115. Paz-Ares L, Dvorkin M, Chen Y, Reinmuth N, Hotta K, Trukhin D, et al. Durvalumab plus platinum–etoposide versus platinum–etoposide in first-line treatment of extensive-stage small-cell lung cancer (CASPIAN): a randomised, controlled, open-label, phase 3 trial. Lancet. 2019;394(10212):1929-39.

116. Angell TE, Min L, Wieczorek TJ, Hodi FS. Unique Cytologic Features of Thyroiditis Caused by Immune Checkpoint Inhibitor Therapy for Malignant Melanoma. Genes Dis. 2018;5(1):46-8.

117. Neppl C, Kaderli RM, Trepp R, Schmitt AM, Berger MD, Wehrli M, et al. Histology of Nivolumab-Induced Thyroiditis. Thyroid. 2018;28(12):1727-28.

118. Morganstein DL, Lai Z, Spain L, Diem S, Levine D, Mace C, et al. Thyroid abnormalities following the use of cytotoxic T-lymphocyte antigen-4 and programmed death receptor protein-1 inhibitors in the treatment of melanoma. Clin Endocrinol. 2017;86(4):614-20.

119. Delivanis DA, Gustafson MP, Bornschlegl S, Merten MM, Kottschade L, Withers S, et al. Pembrolizumab-induced thyroiditis: comprehensive clinical review and insights into underlying involved mechanisms. J Clin Endocrinol Metab. 2017;102(8):2770-80.

120. Min L, Vaidya A, Becker C. Thyroid autoimmunity and ophthalmopathy related to melanoma biological therapy. Eur J Endocrinol. 2011;164(2):303-7.

121. Mekki A, Dercle L, Lichtenstein P, Marabelle A, Michot JM, Lambotte O, et al. Detection of immune-related adverse events by medical imaging in patients treated with anti-programmed cell death 1. Eur J Cancer. 2018;96:91-104.

122. Mazarico I, Capel I, Giménez-Palop O, Albert L, Berges I, Luchtenberg F, et al. Low frequency of positive antithyroid antibodies is observed in patients with thyroid dysfunction related to immune check point inhibitors. J Endocrinological Invest. 2019;42:1443-50.

123. Middlebrooks EH, Westbrook BC, Conry RM. Acute inflammatory thyromegaly following checkpoint inhibition: A new imaging entity? Radiol Case Rep. 2018;13(1):89-91.

124. de Filette J, Jansen Y, Schreuer M, Everaert H, Velkeniers B, Neyns B, et al. Incidence of thyroid-related adverse events in melanoma patients treated with pembrolizumab. J Clin Endocrinol Metab. 2016;101(11):4431-9.

125. Orlov S, Salari F, Kashat L, Walfish PG. Induction of painless thyroiditis in patients receiving programmed death 1 receptor immunotherapy for metastatic malignancies. J Clin Endocrinol Metab. 2015;100(5):1738-41.

126. Yamauchi I, Sakane Y, Fukuda Y, Fujii T, Taura D, Hirata M, et al. Clinical Features of Nivolumab-Induced Thyroiditis: A Case Series Study. Thyroid. 2017;27(7):894-901.

127. Iyer PC, Cabanillas ME, Waguespack SG, Hu MI, Thosani S, Lavis VR, et al. Immune-Related Thyroiditis with Immune Checkpoint Inhibitors. Thyroid. 2018;28(10):1243-51.

128. Lee H, Hodi FS, Giobbie-Hurder A, Ott PA, Buchbinder EI, Haq R, et al. Characterization of Thyroid Disorders in Patients Receiving Immune Checkpoint Inhibition Therapy. Cancer Immunol Res. 2017;5(12):1133-40.

129. Osorio JC, Ni A, Chaft JE, Pollina R, Kasler MK, Stephens D, et al. Antibody-mediated thyroid dysfunction during T-cell checkpoint blockade in patients with non-small-cell lung cancer. Ann Oncol. 2017;28(3):583-9.

130. Villa NM, Farahmand A, Du L, Yeh MW, Smooke-Praw S, Ribas A, et al. Endocrinopathies with use of cancer immunotherapies. Clin Endocrinol. 2018;88(2):327-32.

131. O'Malley G, Lee HJ, Parekh S, Galsky MD, Smith CB, Friedlander P, et al. Rapid Evolution of Thyroid Dysfunction in Patients Treated with Nivolumab. Endocr Pract. 2017;23(10):1223-31.

132. Yamauchi I, Yasoda A, Matsumoto S, Sakamori Y, Kim YH, Nomura M, et al. Incidence, features, and prognosis of immune-related adverse events involving the thyroid gland induced by nivolumab. PLoS One. 2019;14(5):e0216954.

133. Scott ES, Long GV, Guminski A, Clifton-Bligh RJ, Menzies AM, Tsang VH. The spectrum, incidence, kinetics and management of endocrinopathies with immune checkpoint inhibitors for metastatic melanoma. Eur J Endocrinol. 2018;178(2):173-80.

134. Yu C, Chopra IJ, Ha E. A novel melanoma therapy stirs up a storm: Ipilimumab-induced thyrotoxicosis. Endocrinol, Diabetes Metab Case Rep. 2015:2015.

135. Yonezaki K, Kobayashi T, Imachi H, Yoshimoto T, Kikuchi F, Fukunaga K, et al. Combination therapy of ipilimumab and nivolumab induced thyroid storm in a patient with Hashimoto's disease and diabetes mellitus: a case report. J Med Case Rep. 2018;12(1):171.

136. Hakami OA, Ioana J, Ahmad S, Tun TK, Sreenan S, McDermott JH. A case of pembrolizumab-induced severe dka and hypothyroidism in a patient with metastatic melanoma. Endocrinol, Diabetes Metab Case Rep. 2019;2019.

137. Paepegaey AC, Lheure C, Ratour C, Lethielleux G, Clerc J, Bertherat J, et al. Polyendocrinopathy resulting from pembrolizumab in a patient with a Malignant Melanoma. J Endocr Soc. 2017;1(6):646-9.

138. Sakurai K, Niitsuma S, Sato R, Takahashi K, Arihara Z. Painless Thyroiditis and Fulminant Type 1 Diabetes Mellitus in a Patient Treated with an Immune Checkpoint Inhibitor, Nivolumab. Tohoku J Exp Med. 2018;244(1):33-40.

139. Ariyasu H, Inaba H, Ota T, Yamaoka H, Furukawa Y, Iwakura H, et al. Thyrotoxicosis and adrenocortical hormone deficiency during immune-checkpoint inhibitor treatment for malignant melanoma. In Vivo. 2018;32(2):345-51.

140. Tsang VH, McGrath RT, Clifton-Bligh RJ, Scolyer RA, Jakrot V, Guminski AD, et al. Checkpoint Inhibitor–Associated Autoimmune Diabetes Is Distinct From Type 1 Diabetes. J Clin Endocrinol Metab. 2019;104(11):5499-506.

141. de Filette J, Jansen Y, Schreuer M, Everaert H, Velkeniers B, Neyns B, et al. Incidence of Thyroid-Related Adverse Events in Melanoma Patients Treated With Pembrolizumab. J Clin Endocrinol Metab. 2016;101(11):4431-9.

142. Pollack RM, Kagan M, Lotem M, Dresner-Pollak R. Baseline Tsh Level Is Associated with Risk of Anti-Pd-1-Induced Thyroid Dysfunction. Endocr Pract. 2019;25(8):824-9.

143. Peiro I, Palmero R, Iglesias P, Diez JJ, Simo-Servat A, Marin JA, et al. Thyroid dysfunction induced by nivolumab: searching for disease patterns and outcomes. Endocr. 2019;64(3):605-13.

144. Johnson ED, Kerrigan K, Butler K, Patel SB. Nivolumab-induced hypothyoidism with consequent hypothyroid related myopathy. J Oncol Pharm Pract. 2020;26(1):224-27.

145. Khan U, Rizvi H, Sano D, Chiu J, Hadid T. Nivolumab induced myxedema crisis. J Immunother Cancer. 2017;5(1):1-3.

146. Ramos-Levi AM, Rogado J, Sanchez-Torres JM, Colomer R, Marazuela M. Nivolumab-induced thyroid dysfunction in patients with lung cancer. Endocrinol Diabetes Nutr. 2019;66(1):26-34.

147. Kaur A, Doberstein T, Amberker RR, Garje R, Field EH, Singh N. Immune-related adverse events in cancer patients treated with immune checkpoint inhibitors: A single-center experience. Medicine. 2019;98(41):e17348.

148. Brahmer JR, Lacchetti C, Schneider BJ, Atkins MB, Brassil KJ, Caterino JM, et al. Management of Immune-Related Adverse Events in Patients Treated With Immune Checkpoint

Inhibitor Therapy: American Society of Clinical Oncology Clinical Practice Guideline. J Clin Oncol. 2018;36(17):1714-68.

149. Patel NS, Oury A, Daniels GA, Bazhenova L, Patel SP. Incidence of Thyroid Function Test Abnormalities in Patients Receiving Immune-Checkpoint Inhibitors for Cancer Treatment. Oncologist. 2018;23(10):1236-41.

150. Narita T, Oiso N, Taketomo Y, Okahashi K, Yamauchi K, Sato M, et al. Serological aggravation of autoimmune thyroid disease in two cases receiving nivolumab. J Dermatol. 2016;43(2):210-4.

151. Gutzmer R, Koop A, Meier F, Hassel JC, Terheyden P, Zimmer L, et al. Programmed cell death protein-1 (PD-1) inhibitor therapy in patients with advanced melanoma and preexisting autoimmunity or ipilimumab-triggered autoimmunity. Eur J Cancer. 2017;75:24-32.

152. Danlos FX, Voisin AL, Dyevre V, Michot JM, Routier E, Taillade L, et al. Safety and efficacy of anti-programmed death 1 antibodies in patients with cancer and pre-existing autoimmune or inflammatory disease. Eur J Cancer. 2018;91:21-9.

153. Akturk HK, Alkanani A, Zhao Z, Yu L, Michels AW. PD-1 Inhibitor Immune-Related Adverse Events in Patients With Preexisting Endocrine Autoimmunity. J Clin Endocrinol Metab. 2018;103(10):3589-92.

154. Yano S, Ashida K, Nagata H, Ohe K, Wada N, Takeichi Y, et al. Nivolumab-induced thyroid dysfunction lacking antithyroid antibody is frequently evoked in Japanese patients with malignant melanoma. BMC Endocr Disord. 2018;18(1):36.

155. Maekura T, Naito M, Tahara M, Ikegami N, Kimura Y, Sonobe S, et al. Predictive Factors of Nivolumab-induced Hypothyroidism in Patients with Non-small Cell Lung Cancer. In Vivo. 2017;31(5):1035-9.

156. Kimbara S, Fujiwara Y, Iwama S, Ohashi K, Kuchiba A, Arima H, et al. Association of antithyroglobulin antibodies with the development of thyroid dysfunction induced by nivolumab. Cancer Sci. 2018;109(11):3583-90.

157. Jaafar J, Fernandez E, Alwan H, Philippe J. Programmed cell death-1 and programmed cell death ligand-1 antibodies-induced dysthyroidism. Endocr Connections. 2018;7(5):R196-R211.

158. Iadarola C, Croce L, Quaquarini E, Teragni C, Pinto S, Bernardo A, et al. Nivolumab induced thyroid dysfunction: Unusual clinical presentation and challenging diagnosis. Front Endocrinol. 2019;9:813.

159. Campredon P, Imbert P, Mouly C, Grunenwald S, Mazieres J, Caron P. Severe inflammatory ophthalmopathy in a euthyroid patient during nivolumab treatment. Eur Thyroid J. 2018;7(2):84-7.

160. Nakamura Y, Tanaka R, Asami Y, Teramoto Y, Imamura T, Sato S, et al. Correlation between vitiligo occurrence and clinical benefit in advanced melanoma patients treated with nivolumab: A multi-institutional retrospective study. J Dermatol. 2017;44(2):117-22.

161. Sanlorenzo M, Vujic I, Daud A, Algazi A, Gubens M, Luna SA, et al. Pembrolizumab Cutaneous Adverse Events and Their Association With Disease Progression. JAMA Dermatol. 2015;151(11):1206-12.

162. Kim HI, Kim M, Lee SH, Park SY, Kim YN, Kim H, et al. Development of thyroid dysfunction is associated with clinical response to PD-1 blockade treatment in patients with advanced non-small cell lung cancer. Oncoimmunol. 2018;7(1):e1375642.

163. Owen DH, Wei L, Bertino EM, Edd T, Villalona-Calero MA, He K, et al. Incidence, Risk Factors, and Effect on Survival of Immune-related Adverse Events in Patients With Non-Small-cell Lung Cancer. Clin Lung Cancer. 2018;19(6):e893-e900.

164. Kotwal A, Kottschade L, Ryder M. PD-L1 inhibitor-induced thyroiditis is associated with better overall survival in cancer patients. Thyroid. 2020;30(2):177-84.

165. Beaver JA, Pazdur R. The Wild West of Checkpoint Inhibitor Development. N Engl J Med. 2022;386(14):1297-1301.

166. Muir CA, Menzies AM, Clifton-Bligh R, Tsang VH. Thyroid toxicity following immune checkpoint inhibitor treatment in advanced cancer. Thyroid. 2020;30(10):1458-69.

167. Muir CA, Clifton-Bligh RJ, Long GV, Scolyer RA, Lo SN, Carlino MS, et al. Thyroid immune-related adverse events following immune checkpoint inhibitor treatment. J Clin Endocrinol Metab. 2021;106(9):e3704-e13.

168. von Itzstein MS, Gonugunta AS, Wang Y, Sheffield T, Lu R, Ali S, et al. Divergent prognostic effects of pre-existing and treatment-emergent thyroid dysfunction in patients treated with immune checkpoint inhibitors. Cancer Immunology, Immunotherapy. 2022;71(9):2169-81.

169. Muir CA, Wood CC, Clifton-Bligh RJ, Long GV, Scolyer RA, Carlino MS, et al. Association of anti-thyroid antibodies in checkpoint inhibitor associated thyroid immune related adverse events. J Clin Endocrinol Metab. 2022;107(5):e1843-e49.

170. Yoon JH, Hong AR, Kim HK, Kang H-C. Characteristics of Immune-Related Thyroid Adverse Events in Patients Treated with PD-1/PD-L1 Inhibitors. Endocrinol Metab. 2021;36(2):413.

171. Pollack R, Ashash A, Cahn A, Rottenberg Y, Stern H, Dresner-Pollak R. Immune Checkpoint Inhibitor-Induced Thyroid Dysfunction is Associated with Higher Body Mass Index. J Clin Endocrinol Metab. 2020;105(10):e3620-e27.

172. Kurimoto C, Inaba H, Ariyasu H, Iwakura H, Ueda Y, Uraki S, et al. Predictive and sensitive biomarkers for thyroid dysfunctions during treatment with immune-checkpoint inhibitors. Cancer Science. 2020;111(5):1468.

173. Lozano AX, Chaudhuri AA, Nene A, Bacchiocchi A, Earland N, Vesely MD, et al. T cell characteristics associated with toxicity to immune checkpoint blockade in patients with melanoma. Nature Med. 2022:1-10.

174. Khan Z, Hammer C, Carroll J, Di Nucci F, Acosta SL, Maiya V, et al. Genetic variation associated with thyroid autoimmunity shapes the systemic immune response to PD-1 checkpoint blockade. Nature Communications. 2021;12(1):1-12.

175. Luo J, Martucci VL, Quandt Z, Groha S, Murray MH, Lovly CM, et al. Immunotherapy-Mediated Thyroid Dysfunction: Genetic Risk and Impact on Outcomes with PD-1 Blockade in Non–Small Cell Lung Cancer. Clin Cancer Res. 2021;27(18):5131-40.

176. Álvarez-Sierra D, Marín-Sánchez A, Ruiz-Blázquez P, de Jesús Gil C, Iglesias-Felip C, González Ó, et al. Analysis of the PD-1/PD-L1 axis in human autoimmune thyroid disease: insights into pathogenesis and clues to immunotherapy associated thyroid autoimmunity. J Autoimmunity. 2019;103:102285.

177. Kotwal A, Gustafson M, Bornschlegl S, Kottschade L, Delivanis D, Dietz AB, et al. Immune checkpoint inhibitor-induced thyroiditis is associated with increased intrathyroidal T lymphocyte subpopulations. Thyroid. 2020;30(10):1440-50.

178. Schneider BJ, Naidoo J, Santomasso BD, Lacchetti C, Adkins S, Anadkat M, et al. Management of immune-related adverse events in patients treated with immune checkpoint inhibitor therapy: ASCO guideline update. J Clin Oncol. 2021;39(36):4073-126.

179. Mosaferi T, Tsai K, Sovich S, Wilhalme H, Kathuria-Prakash N, Smooke-Praw S, et al. Optimal Thyroid Hormone Replacement Dose in Immune Checkpoint Inhibitor-Associated Hypothyroidism is Distinct from Hashimoto's Thyroiditis. Thyroid. 2022;32(5):496-504.

180. Cheung Y-MM, Wang W, McGregor B, Hamnvik O-PR. Associations between immune-related thyroid dysfunction and efficacy of immune checkpoint inhibitors: a systematic review and meta-analysis. Cancer Immunol, Immunotherapy. 2022:1-18.

181. Kotwal A, Ryder M. Survival benefit of endocrine dysfunction following immune checkpoint inhibitors for nonthyroidal cancers. Curr Opin Endocrinol Diabetes Obesity. 2021;28(5):517-24.

182. Ferreira JL, Costa C, Marques B, Castro S, Victor M, Oliveira J, et al. Improved survival in patients with thyroid function test abnormalities secondary to immune-checkpoint inhibitors. Cancer Immunol, Immunotherapy. 2021;70(2):299-309.

183. Basak EA, van der Meer JW, Hurkmans DP, Schreurs MW, Oomen-de Hoop E, van der Veldt AA, et al. Overt thyroid dysfunction and anti-thyroid antibodies predict response to anti-PD-1 immunotherapy in cancer patients. Thyroid. 2020;30(7):966-73.

184. Inaba H, Ariyasu H, Iwakura H, Kurimoto C, Takeshima K, Morita S, et al. Distinct clinical features and prognosis between persistent and temporary thyroid dysfunctions by immune-checkpoint inhibitors. Endocr J. 2020:EJ20-0371.

185. Toi Y, Sugawara S, Sugisaka J, Ono H, Kawashima Y, Aiba T, et al. Profiling preexisting antibodies in patients treated with anti–PD-1 therapy for advanced non–small cell lung cancer. JAMA Oncol. 2019;5(3):376-83.

186. Haslam A, Gill J, Prasad V. Estimation of the percentage of US patients with cancer who are eligible for immune checkpoint inhibitor drugs. JAMA Network Open. 2020;2(5):e192535.

187. Robert C, Hwu W-J, Hamid O, Ribas A, Weber JS, Daud AI, et al. Long-term safety of pembrolizumab monotherapy and relationship with clinical outcome: A landmark analysis in patients with advanced melanoma. Eur J Cancer. 2020;144:182-91.

188. Gershenwald JE, Scolyer RA, Hess KR, Sondak VK, Long GV, Ross MI, et al. Melanoma staging: evidence-based changes in the American Joint Committee on Cancer eighth edition cancer staging manual. CA: Cancer J Clinicians. 2017;67(6):472-92.

189. Seymour L, Bogaerts J, Perrone A, Ford R, Schwartz LH, Mandrekar S, et al. iRECIST: guidelines for response criteria for use in trials testing immunotherapeutics. Lancet Oncol. 2017;18(3):e143-e52.

190. Barroso-Sousa R, Barry WT, Garrido-Castro AC, Hodi FS, Min L, Krop IE, et al. Incidence of endocrine dysfunction following the use of different immune checkpoint inhibitor regimens: a systematic review and meta-analysis. JAMA Oncol. 2018;4(2):173-82.

191. Dayan CM, Daniels GH. Chronic autoimmune thyroiditis. N Engl J Med. 1996;335(2):99-107.

192. Fatourechi V, Aniszewski JP, Fatourechi GZE, Atkinson EJ, Jacobsen SJ. Clinical features and outcome of subacute thyroiditis in an incidence cohort: Olmsted County, Minnesota, study. J Clin Endocrinol Metab. 2003;88(5):2100-5.

193. Shrestha RT, Hennessey J. Acute, subacute and Riedel's thyroiditis. Endotext [Internet]: MDText. com, Inc.; 2015.

194. Ross DS, Burch HB, Cooper DS, Greenlee MC, Laurberg P, Maia AL, et al. 2016 American Thyroid Association guidelines for diagnosis and management of hyperthyroidism and other causes of thyrotoxicosis. Thyroid. 2016;26(10):1343-421.

195. Olsson-Brown A, Lord R, Sacco J, Wagg J, Coles M, Pirmohamed M. Two distinct clinical patterns of checkpoint inhibitor-induced thyroid dysfunction. Endocr Connections. 2020;9(4):318-25.

196. O'Malley G, Lee HJ, Parekh S, Galsky MD, Smith CB, Friedlander P, et al. Rapid evolution of thyroid dysfunction in patients treated with nivolumab. Endocr Pract. 2017;23(10):1223-31.

197. Valpione S, Pasquali S, Campana LG, Piccin L, Mocellin S, Pigozzo J, et al. Sex and interleukin-6 are prognostic factors for autoimmune toxicity following treatment with anti-CTLA4 blockade. J Transl Med. 2018;16(1):1-10.

198. Tanaka R, Okiyama N, Okune M, Ishitsuka Y, Watanabe R, Furuta J, et al. Serum level of interleukin-6 is increased in nivolumab-associated psoriasiform dermatitis and tumor necrosis factor-α is a biomarker of nivolumab recativity. J Dermatological Sci. 2017;86(1):71-3.

199. Yamauchi I, Yasoda A, Hakata T, Yamashita T, Hirota K, Ueda Y, et al. Novel Thyroid-Specific Autoantibodies in Patients with Immune-Related Adverse Events Involving the Thyroid Gland. bioRxiv. 2021.

200. Muir CA, Tsang VHM, Menzies AM, Clifton-Bligh RJ. Immune Related Adverse Events of the Thyroid – A Narrative Review. Front Endocrinol. 2022;13.

201. Lechner MG, Ryder M. Insights into immune checkpoint inhibitor-induced thyroiditis. Nature Reviews Endocrinol. 2021;17(11):643-4.

202. Newby P, Roberts-Davies E, Brand O, Heward J, Franklyn J, Gough S, et al. Tag SNP screening of the PDCD1 gene for association with Graves' disease. Clin Endocrinol. 2007;67(1):125-8.

203. Mitchell AL, Cordell HJ, Soemedi R, Owen K, Skinningsrud B, Wolff AB, et al. Programmed death ligand 1 (PD-L1) gene variants contribute to autoimmune Addison's disease and Graves' disease susceptibility. J Clin Endocrinol Metab. 2009;94(12):5139-45.

204. Ueda H, Howson JM, Esposito L, Heward J, Chamberlain G, Rainbow DB, et al. Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. Nature. 2003;423(6939):506-11.

205. Kotsa K, Watson P, Weetman A. A CTLA-4 gene polymorphism is associated with both Graves' disease and autoimmune hypothyroidism. Clin Endocrinol. 1997;46(5):551-4.

206. Jabkowski J, Loidl A, Auinger B, Kehrer H, Sepp N, Pichler R. Pembrolizumab-Induced Thyroiditis Shows PD-L1Expressing Histiocytes and Infiltrating T Cells in Thyroid Tissue-A Case Report. Front Immunol. 2021;12:2437.

207. Lechner MG, Cheng MI, Patel AY, Hoang AT, Yakobian N, Astourian M, et al. Inhibition of IL-17A Protects against Thyroid Immune-Related Adverse Events while Preserving Checkpoint Inhibitor Antitumor Efficacy. J Immunol. 2022;209(4):696-709.

208. Degertekin CK, Yilmaz BA, Toruner FB, Kalkanci A, Iyidir OT, Fidan I, et al. Circulating Th17 cytokine levels are altered in Hashimoto's thyroiditis. Cytokine. 2016;80:13-7.

209. Shi Y, Wang H, Su Z, Chen J, Xue Y, Wang S, et al. Differentiation imbalance of Th1/Th17 in peripheral blood mononuclear cells might contribute to pathogenesis of Hashimoto's thyroiditis. Scandinavian J Immunol. 2010;72(3):250-5.

210. Van Gassen S, Callebaut B, Van Helden MJ, Lambrecht BN, Demeester P, Dhaene T, et al. FlowSOM: Using self-organizing maps for visualization and interpretation of cytometry data. Cytometry Part A. 2015;87(7):636-45.

211. Ashhurst TM, Marsh-Wakefield F, Putri GH, Spiteri AG, Shinko D, Read MN, et al. Integration, exploration, and analysis of high-dimensional single-cell cytometry data using Spectre. Cytometry Part A. 2022;101(3):237-53.

212. Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'hara R, et al. Package 'vegan'. Community ecology package, version. 2013;2(9):1-295.

213. Konopka T, Konopka MT. R-package: umap. Uniform Manifold Approximation and Projection. 2018.

214. Antonelli A, Ferrari SM, Corrado A, Di Domenicantonio A, Fallahi P. Autoimmune thyroid disorders. Autoimmunity Reviews. 2015;14(2):174-80.

215. Angell TE, Min L, Wieczorek TJ, Hodi FS. Unique cytologic features of thyroiditis caused by immune checkpoint inhibitor therapy for malignant melanoma. Genes Dis. 2018;5(1):46-8.

216. Lechner MG, Zhou Z, Hoang AT, Huang N, Ortega J, Scott LN, et al. Clonally-Expanded, Thyrotoxic Autoimmune Mediator CD8+ T cells Driven by IL21 Contribute to Checkpoint Inhibitor Thyroiditis. bioRxiv. 2022.

217. Nahar KJ, Marsh-Wakefield F, Rawson RV, Gide TN, Ferguson AL, Allen R, et al. Distinct pretreatment innate immune landscape and posttreatment T cell responses underlie immunotherapy-induced colitis. JCI Insight. 2022;7(21).

218. Wenzel B, Chow A, Baur R, Schleusener H, Wall J. Natural killer cell activity in patients with Graves' disease and Hashimoto's thyroiditis. Thyroid. 1998;8(11):1019-22.

219. Hall R, Stanbury J. Familial studies of autoimmune thyroiditis. Clin Experimental Immunol. 1967;2(Suppl):719.

220. Brix TH, Hegedüs L. Twin studies as a model for exploring the aetiology of autoimmune thyroid disease. Clin Endocrinol. 2012;76(4):457-64.

221. Tomer Y. Genetic susceptibility to autoimmune thyroid disease: past, present, and future. Thyroid. 2010;20(7):715-25.

222. Hollowell JG, Staehling NW, Flanders WD, Hannon WH, Gunter EW, Spencer CA, et al. Serum TSH, T4, and thyroid antibodies in the United States population (1988 to 1994): National Health and Nutrition Examination Survey (NHANES III). J Clin Endocrinol Metabol. 2002;87(2):489-99.

223. Lee HJ, Li CW, Hammerstad SS, Stefan M, Tomer Y. Immunogenetics of autoimmune thyroid diseases: a comprehensive review. J Autoimmunity. 2015;64:82-90.

224. Lee HJ, Stefan-Lifshitz M, Li CW, Tomer Y. Genetics and epigenetics of autoimmune thyroid diseases: translational implications. Best Practice Res Clin Endocrinol Metab. 2022:101661.

225. Saevarsdottir S, Olafsdottir TA, Ivarsdottir EV, Halldorsson GH, Gunnarsdottir K, Sigurdsson A, et al. FLT3 stop mutation increases FLT3 ligand level and risk of autoimmune thyroid disease. Nature. 2020:1-5.

226. Musumeci A, Lutz K, Winheim E, Krug AB. What makes a pDC: Recent advances in understanding plasmacytoid DC development and heterogeneity. Front Immunol. 2019;10:1222.

227. Law MH, Aoude LG, Duffy DL, Long GV, Johansson PA, Pritchard AL, et al. Multiplex melanoma families are enriched for polygenic risk. Human Molecular Genetics. 2020;29(17):2976-85.

228. Kazi JU, Rönnstrand L. FMS-like tyrosine kinase 3/FLT3: from basic science to clinical implications. Physiological Reviews. 2019;99(3):1433-66.

229. Daver N, Schlenk RF, Russell NH, Levis MJ. Targeting FLT3 mutations in AML: review of current knowledge and evidence. Leukemia. 2019;33(2):299-312.

230. Borowczyk M, Szczepanek-Parulska E, Dębicki S, Budny B, Janicka-Jedyńska M, Gil L, et al. High incidence of FLT3 mutations in follicular thyroid cancer: potential therapeutic target in patients with advanced disease stage. Therapeutic Advances Med Oncol. 2020;12:1758835920907534.

231. Bigley V, Haniffa M, Doulatov S, Wang X-N, Dickinson R, McGovern N, et al. The human syndrome of dendritic cell, monocyte, B and NK lymphoid deficiency. J Experimental Med. 2011;208(2):227-34.

232. Vasu C, Dogan R-NE, Holterman MJ, Prabhakar BS. Selective induction of dendritic cells using granulocyte macrophage-colony stimulating factor, but not fms-like tyrosine kinase receptor 3-ligand, activates thyroglobulin-specific CD4+/CD25+ T cells and suppresses experimental autoimmune thyroiditis. J Immunol. 2003;170(11):5511-22.

233. Martínez-López J, Kerick M, Ortiz-Fernández L, Acosta-Herrera M, Márquez A, Martín J. FLT3 functional low-frequency variant rs76428106-C is associated with susceptibility to systemic sclerosis. Rheumatol. 2022.

234. Dehlin M, Bokarewa M, Rottapel R, Foster SJ, Magnusson M, Dahlberg LE, et al. Intra-articular fms-like tyrosine kinase 3 ligand expression is a driving force in induction and progression of arthritis. PLoS One. 2008;3(11):e3633.

235. Perl AE, Martinelli G, Cortes JE, Neubauer A, Berman E, Paolini S, et al. Gilteritinib or chemotherapy for relapsed or refractory FLT3-mutated AML. N Engl J Med. 2019;381:1728-40.

236. Hills RK, Gammon G, Trone D, Burnett AK. Quizartinib significantly improves overall survival in FLT3-ITD positive AML patients relapsed after stem cell transplantation or after failure of salvage chemotherapy: a comparison with historical AML database (UK NCRI data). Blood. 2015;126(23):2557.

237. Chiovato L, Bassi P, Santini F, Mammoli C, Lapi P, Carayon P, et al. Antibodies producing complement-mediated thyroid cytotoxicity in patients with atrophic or goitrous autoimmune thyroiditis. J Clin Endocrinol Metab. 1993;77(6):1700-5.

238. Ajjan RA, Kemp EH, Waterman EA, Watson PF, Endo T, Onaya T, et al. Detection of binding and blocking autoantibodies to the human sodium-iodide symporter in patients with autoimmune thyroid disease. J Clin Endocrinol Metab. 2000;85(5):2020-7.

239. Brix TH, Hegedüs L, Weetman AP, Kemp HE. Pendrin and NIS antibodies are absent in healthy individuals and are rare in autoimmune thyroid disease: evidence from a Danish twin study. Clin Endocrinol. 2014;81(3):440-4.

240. Kemp EH, Sandhu HK, Watson PF, Weetman AP. Low frequency of pendrin autoantibodies detected using a radioligand binding assay in patients with autoimmune thyroid disease. J Clin Endocrinol Metab. 2013;98(2):e309-e13.

241. Sakata S, Nakamura S, Miura K. Autoantibodies Against Thyroid Hormones or Iodothyronine: Implications in Diagnosis, Thyroid Function, Treatment, and Pathogenesis. Ann Internal Med. 1985;103(4):579-89.

242. Ajjan R, Weetman A. Thyroid autoantibodies. Thyroid Dis. 2018:57.

243. Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, et al. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. J Cerebral Blood Flow Metab. 2020;40(9):1769-77.

244. Beach TG, Tago H, Nagai T, Kimura H, McGeer P, McGeer E. Perfusion-fixation of the human brain for immunohistochemistry: comparison with immersion-fixation. J Neuroscience Methods. 1987;19(3):183-92.

245. Choksi NY, Jahnke GD, St. Hilaire C, Shelby M. Role of thyroid hormones in human and laboratory animal reproductive health. Birth defects research part B: Developmental Reproductive Toxicol. 2003;68(6):479-91.

246. Gage GJ, Kipke DR, Shain W. Whole animal perfusion fixation for rodents. JoVE. 2012(65):e3564.

247. Sullivan RJ, Weber JS. Immune-related toxicities of checkpoint inhibitors: mechanisms and mitigation strategies. Nature Rev Drug Discovery. 2022;21(7):495-508.

248. Prineas JW, Parratt JD. Multiple sclerosis: Serum anti-CNS autoantibodies. Multiple Sclerosis J. 2018;24(5):610-22.

249. Ali OH, Bomze D, Ring SS, Berner F, Fässler M, Diem S, et al. BP180-specific IgG is associated with skin adverse events, therapy response, and overall survival in non-small cell lung cancer patients treated with checkpoint inhibitors. J Am Acad Dermatol. 2020;82(4):854-61.

250. Gowen MF, Giles KM, Simpson D, Tchack J, Zhou H, Moran U, et al. Baseline antibody profiles predict toxicity in melanoma patients treated with immune checkpoint inhibitors. J Translational Med. 2018;16:1-12.

251. Hassel JC, Zucht H-D, Mangana J, Dummer R, Pföhler C, Wistuba-Hamprecht K, et al. Autoantibodies as predictors for survival and immune-related adverse events in checkpoint inhibition therapy of metastasized melanoma. Am Soc Clin Oncol; 2020;10011.

Appendix 1

THYROID
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Thyroid Toxicity Following Immune Checkpoint Inhibitor Treatment in Advanced Cancer

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Background: Inhibitory antibodies against cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed cell death-1 (PD-1) have antitumor efficacy and are now standard of care in the management of multiple cancer subtypes. However, the use is complicated by the development of autoimmunity, which can occur in multiple organ systems. Thyroiditis is the most common immune-related adverse event.
Summary: Immune checkpoint inhibitor (ICI)-associated thyroiditis affects over 10% of treated patients. PD-1 inhibitors are associated with greater risk of thyroid dysfunction relative to CTLA-4 inhibitors, although the highest risk occurs with combined anti-CTLA-4 and anti-PD-1 treatment. Onset is typically rapid, within weeks to months and both hyperthyroidism and hypothyroidism can occur. The most frequent pattern of thyroid dysfunction is transient hyperthyroidism with evolution to hypothyroidism over four to six weeks. Most cases are asymptomatic and resolve without dedicated treatment. There is no sex or age predominance, and predictive risk factors have not been reliably identified. Thyroid autoantibodies are variably present and are not clearly related to the risk or progression of thyroid dysfunction following treatment with an ICI. Observational data suggest that development of ICI-associated thyroiditis may predict improved survival.
Conclusions: ICI-associated thyroiditis is a distinct clinical entity. Mechanisms underlying etiology remain largely unknown. Awareness among health professionals is important to limit morbidity and avoid unnecessary periods of untreated hypothyroidism.

Keywords: cytotoxic T lymphocyte antigen-4, programmed cell death-1, immune checkpoint, immune-related adverse event, thyroiditis

Introduction

TARGETED CANCER IMMUNOTHERAPY TO ENHANCE T cell activation has revolutionized the management of cancer. Immune checkpoint inhibitors (ICIs) are the best studied of currently available cancer immunotherapies. In multiple cancer types such as melanoma, non-small cell lung cancer, renal cell cancer, and others, ICIs have become standard of care with multiple large randomized controlled trials confirming benefit in terms of tumor response, progression-free survival (PFS), and overall survival (OS) (1).

Immune checkpoint antagonists primarily enhance antitumor immune responses via inhibition of the negative regulatory effects of cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed cell death-1 (PD-1) pathways, although other pathways are also under investigation (2). The CTLA-4 and PD-1 pathways are nonredundant and regulate immune activation via unique mechanisms (2). Features of different ICIs are presented in Table 1.

Cytotoxic T lymphocyte antigen-4

Immune responses by way of T cell activation, differentiation, and proliferation are dependent on co-stimulatory signals between the T cell and the antigen-presenting cell. The first signal is through recognition of antigen-derived peptides presented via major histocompatibility complex proteins to T cell receptors on CD8+ and CD4+ positive T cells. The second signal amplifies the effect of the first and occurs following recognition of B7 proteins (CD80, CD86) by CD28, also present on the T cell membrane (3).

CTLA-4 is a CD28 homolog (1,2). Both CTLA-4 and CD28 are expressed on the cell surface of CD4+ and CD8+ T lymphocytes. They have opposite effects on T cell activation, and as such, CTLA-4 competes with CD28 for binding to B7 proteins expressed by antigen-presenting cells (2). Once bound, CD28 promotes T lymphocyte activation and clonal expansion. In contrast, CTLA-4 dampens T cell signaling and thereby suppresses immune activation (2). The affinity of

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Table 1. Characteristics and Properties of Single-Agent Immune Checkpoint Inhibitors

	Ipilimumab	*Nivolumab*	*Pembrolizumab*
Trade name	Yervoy	Opdivo	Keytruda
FDA approval	2011	2014	2014
Antibody target	CTLA-4	PD-1	PD-1
Antibody type	Recombinant human IgG1 kappa monoclonal antibody	Fully humanized IgG4 kappa monoclonal antibody	Recombinant human IgG4 kappa monoclonal antibody
Molecular weight, kDa	148	146	149
Adult dosing schedules	3–10 mg/kg q3 weekly × 4 doses	240 mg q2 weekly 480 mg q4 weekly	200 mg q3 weekly
Elimination half-life, days	15	25	22

CTLA-4, cytotoxic T lymphocyte antigen-4; FDA, Food and Drug Administration; PD-1, programmed cell death-1.

CTLA-4 for B7 proteins on antigen-presenting cells is greater than that of CD28 (2). As such, surface expression of CTLA-4 is inducible and increases following T cell activation to prevent uncontrolled T cell activity (2). The primary role of CTLA-4 appears to be maintenance of central tolerance, as evidenced by CTLA-4-knockout mice that develop systemic immune activation and fatal multiorgan autoimmunity (4).

Programmed cell death-1

In comparison, PD-1 is a primary regulator of peripheral tolerance and limits the activity of effector T cells at a tissue level (2). PD-1, unlike CTLA-4, exerts its effect via interaction with its own specific ligands, programmed cell death ligand (PD-L)1 and PD-L2 (2). Once bound, PD-1 inhibits CD28 co-stimulatory signaling and dampens T cell activation (5). Similar to CTLA-4, PD-1 expression is very low in resting T lymphocytes of healthy individuals (5). Following T cell activation, PD-1 surface expression is induced to limit uncontrolled proliferation, effector function, and survival (5). In cancer, tumor cells often express PD-L1, which may directly lead to T cell inactivation and avoidance of anticancer immunity (2). As might be expected, animal PD-1-knockout models result in a less severe phenotype than CTLA-4 knockouts (6).

Immune-related adverse events

The improvements in cancer outcomes observed with ICIs come with unintended off-target effects, namely new-onset autoimmunity termed immune-related adverse events (irAEs). Immune-related complications occur in up to 80% of CTLA-4- and PD-1-treated patients (3). Antitumor efficacy is improved with combined CTLA-4 and PD-1 blockade compared with single-agent monotherapy, but the risk of irAEs increases in tandem (3). Manifestations range from mild to life-threatening, and the spectrum of irAEs differs between CTLA-4 and PD-1 inhibition (7). Immune adverse effects have been reported in multiple organ systems, with the endocrine system appearing particularly susceptible. Hypophysitis and thyroiditis are the most common endocrine manifestations, with the former more likely following anti-CTLA-4 treatment and the latter with anti-PD-1-based therapy (8). Endocrine irAEs are often irreversible, unlike irAEs that occur in other organ systems. This is particularly interesting in the setting of thyroid irAEs, as autoimmune thyroiditis normally requires years to decades of immune-mediated lymphocytic infiltration before hypothyroidism results, whereby progression to overt hypothyroidism following ICI treatment can develop within weeks to months (9).

Thyroid dysfunction is the most common irAE following ICI treatment. Description of thyroid irAEs across studies is not standardized, and the true prevalence and mechanism of this phenomenon are not well understood. Thyroiditis following ICI treatment provides a new model to study the origin and pathogenesis of autoimmune thyroid disease more generally. As such, we endeavored to assess the incidence, etiology clinical course, and other features of thyroid dysfunction developing as an adverse effect of ICI-based treatment.

Methods

Search strategy

We performed a review of published articles reporting irAEs affecting the thyroid in patients with advanced cancer who underwent ICI treatment. A search of MEDLINE, Embase, and PubMed databases identified relevant articles published before November 15, 2019. Additionally, we manually reviewed references of included studies for further literature of relevance. In PubMed, we searched keywords ''safety'' OR ''side effects'' OR ''adverse events'' AND ''ipilimumab'' OR ''nivolumab'' OR ''pembrolizumab'' OR ''CTLA-4'' OR ''PD-1'' with article type restricted to clinical trial, controlled clinical trial, multicenter study, observational study, and randomized controlled trial. We searched the same keywords in MEDLINE and Embase with results restricted to human and English language studies.

Selection criteria included studies that (a) investigated the use of ICIs in the treatment of cancer; (b) reported on irAEs related to thyroid function; and (c) were published in the English language. Phase 3 randomized trial data were assessed to determine the incidence of thyroid irAEs. To further characterize thyroid irAEs, all study types (including case reports) that described specific effects of immune checkpoint blockade on thyroid function were included. Exclusion criteria included (a) animal studies; (b) review articles, systematic reviews, and meta-analyses; (c) editorials and letters to the editor; and (d) phase I/II trials without a specific focus on thyroid-related outcomes.

Study characteristics

The search strategy identified 3086 studies. Following eligibility assessment and removal of duplicate articles, 288

full-text articles were selected for further assessment. Of the articles reviewed, 78 fulfilled our inclusion and exclusion criteria (Fig. 1). We extracted two additional articles by reviewing references of studies identified through our database search. Results included 30 phase III randomized clinical trials, which were used to calculate the incidence of thyroid irAEs and 50 observational, cohort, and case studies that were used to describe the clinical features of thyroid irAEs in greater detail.

Review

Incidence

Thyroid dysfunction has been observed following treatment with both CTLA-4 and PD-1 inhibitors in multiple cancer subtypes. The mean incidence of thyroid irAEs in phase III clinical trials is 10.8%, although rates vary widely between different ICIs (Fig. 2).

In phase III trials of ipilimumab, a CTLA-4 inhibitor, 3863 patients with melanoma or lung cancer were treated and thyroid dysfunction developed in 4.7% (range 2.0–10.4%) of exposed patients (10–18). Both hyperthyroidism and hypothyroidism were reported, possibly manifestations of the same disorder detected at different phases of evolution. The incidence of thyroid irAEs did not differ between 3 and 10 mg doses of ipilimumab (11). Similarly, rates of thyroid irAEs were not significantly increased when ipilimumab was combined with dacarbazine, gp100, or platinum-based chemotherapy (12–15).

Nivolumab, a PD-1 inhibitor, has been tested for treatment of multiple cancer types, including melanoma, lung, head and neck, gastric, and renal cancers. In phase III trials, 227 of 2566 (8.8%) patients treated with nivolumab developed thyroid irAEs (16,18–25). As with ipilimumab, both hyperthyroidism and hypothyroidism were reported, and the significant majority of cases were of mild severity. The prevalence of thyroid irAEs ranged from 3.7% to 15.1%, with seemingly higher rates in melanoma (11.4%) compared with other solid organ cancers (5.5%). Interestingly, in a study of melanoma patients treated with nivolumab after disease progression on ipilimumab, the incidence of thyroid irAEs was 15.1%, suggesting that sequential treatment may increase risk to a similar degree as concomitant CTLA-4 and PD-1 checkpoint blockade (20).

Pembrolizumab, a second anti-PD-1 checkpoint inhibitor, has also been used successfully to treat multiple cancer subtypes. Of 4445 patients treated in phase III clinical trials, 694 (15.6%) developed a thyroid irAE (17,26–35). This is double the incidence reported with nivolumab, despite working via inhibition of a common pathway and almost four times as frequent as with ipilimumab. The incidence of thyroid irAEs ranged from 10.6% to 27.4% and similar to other

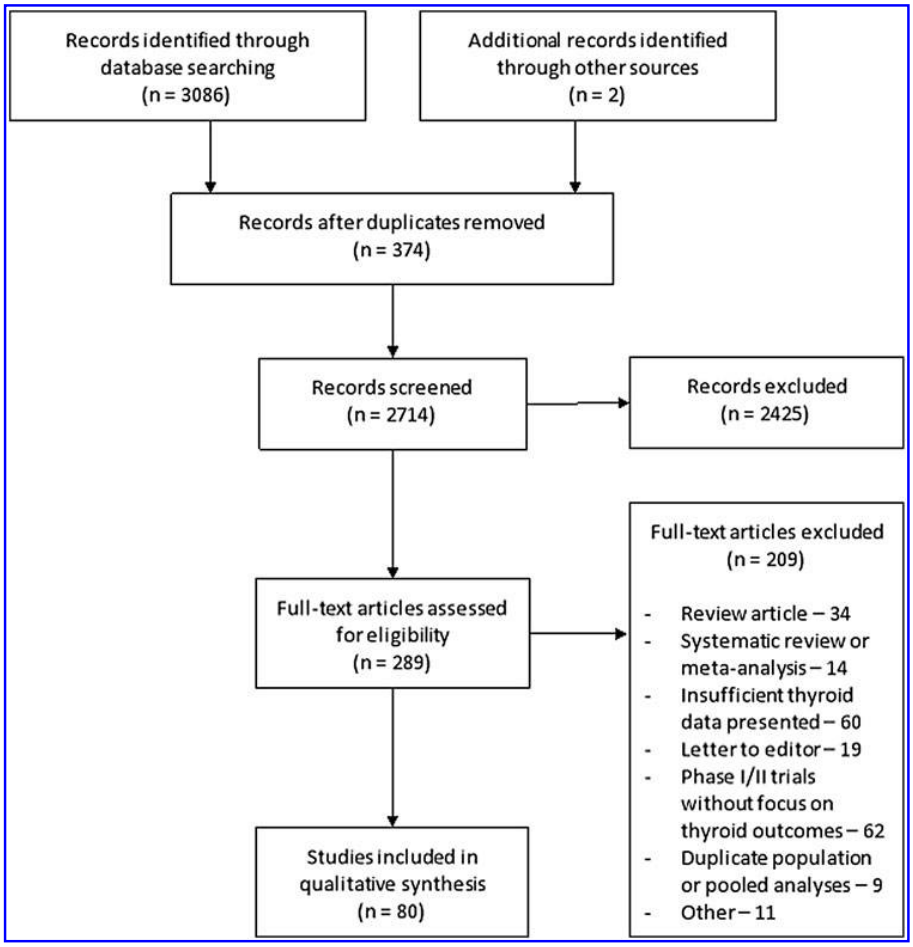

FIG. 1. Preferred reporting for systematic reviews and meta-analyses (PRISMA) diagram.

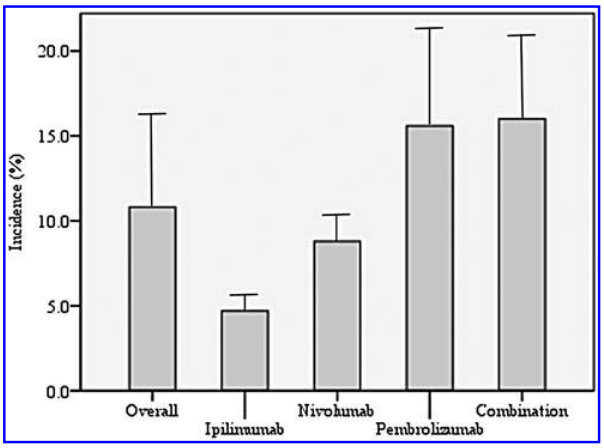

FIG. 2. Mean incidence of thyroid immune-related adverse events in phase III randomized clinical trials of immune checkpoint inhibitors for treatment of advanced cancer.

ICIs, the risk was not dose dependent and occurred at similar rates irrespective of the dose or dosing interval (17,30,31). Pembrolizumab has been used in combination with dexamethasone for the treatment of multiple myeloma in two studies (36,37). Interestingly, the incidence of pembrolizumab-induced thyroid irAEs was lower in multiple myeloma (9.1%) than in other cancer subtypes (15.6%) (36,37). At present, it is unclear whether coadministration of dexamethasone with pembrolizumab may prevent thyroid irAEs, or if another factor may be responsible for the lower rate of irAEs observed in multiple myeloma patients.

Combination treatment with ipilimumab plus nivolumab has been tested in melanoma, renal cell cancer, and lung cancer with and without high mutational burden (18,24,38). Thyroid toxicity was similar to that observed with pembrolizumab treatment, but greater than with ipilimumab or nivolumab. In 1436 patients treated with combined ipilimumab plus nivolumab, 230 (16.0%) developed a thyroid irAE. Similar to ipilimumab monotherapy, the incidence of thyroid irAEs was more common during treatment of melanoma (24.9%) than during treatment of lung cancer (11.6%) or renal cell cancer (15.5%).

PD-L1 inhibitors are another class of ICI in routine use for treatment of cancer. In phase III trials of the PD-L1 inhibitor atezolizumab, 332 of 1493 (22.2%) patients experienced a thyroid irAE (39–42). Hypothyroidism was the reported thyroid irAE in 75% of cases, with the remainder recorded as hyperthyroidism. Durvalumab, a second PD-L1 inhibitor, was also associated with a high prevalence of thyroid irAEs, affecting 100 of 740 (13.5%) treated patients in phase III randomized clinical trials (43,44). The ratio of hypothyroidism to hyperthyroidism with durvalumab was similar to that observed with atezolizumab.

Etiology

The etiology of thyroid irAEs after immune checkpoint blockade remains unknown. Two case reports have been published describing histological changes observed in thyroid irAEs. The first was a cytology specimen in a patient who developed thyroiditis during combination immunotherapy with ipilimumab and nivolumab (45). Key cytological features were lymphocytic infiltrates admixed with necrotic cells with a distinct absence of follicular cells (45). A second case underwent thyroidectomy for control of thyrotoxicosis during combined treatment with ipilimumab plus nivolumab (46). Similar to the aforementioned case, CD8+ lymphocyte-predominant infiltrates were observed in association with colloid granulomas and damaged thyroid follicles (46). Taken together, these cases suggest a destructive inflammatory thyroiditis and were histologically distinct from changes normally associated with Graves' disease.

Imaging of the thyroid in patients with ICI-associated thyroid irAEs has also suggested thyroiditis as the cause of thyroid dysfunction. Uptake scans, when performed, demonstrated diffusely reduced uptake (47–49). Thyroid ultrasound was less sensitive and specific but demonstrated heterogeneous echogenicity and hypervascularity, suggestive of thyroiditis in the majority of patients scanned (50,51). Thyroid avidity on fluorodeoxyglucose positron emission tomography (FDG-PET) was increased at the time of abnormal thyroid function in 64–100% of cases, supporting an acute hypermetabolic process (48–50). Computed tomography (CT) is uncommonly used for assessment of the thyroid but is the main modality used to monitor ICI treatment response. Acute inflammatory thyromegaly on CT has been described in two cases with a doubling–tripling of thyroid volume occurring within three days of combined ipilimumab plus nivolumab treatment and associated with localized neck swelling (52). Thyromegaly in these cases resolved spontaneously without treatment and returned to premorbid size on serial CT examination, also in keeping with a transient inflammatory process (52).

Clinical manifestations

Although the incidence of thyroid irAEs can be estimated from clinical trials, descriptions of the presentation and course of thyroid irAEs have been more difficult to elucidate. Historically, clinical trials reported hypothyroidism, hyperthyroidism, and thyroiditis as discrete entities. However, observational data suggest that each may represent a manifestation of the same process—a destructive thyroiditis originating with a thyrotoxic phase that progresses to hypothyroidism, which may or may not recover. Thyrotoxicosis usually develops early, within three to six weeks after drug exposure (Fig. 3) (47,53–61). The earliest recorded onset of thyrotoxicosis was nine days after first dose, but subclinical hyperthyroidism may be detected as early as 24 hours after treatment initiation (59). Although the vast majority of cases occur within weeks of the first dose, the onset of thyrotoxicosis greater than one year after treatment commencement has been reported (57).

As with other irAEs, combination treatment with ipilimumab plus nivolumab appeared to provoke thyrotoxicosis earlier than with anti-PD-1 monotherapy (56). The thyrotoxic phase was usually painless and self-limited, lasting on average between four and six weeks (54–56,58,62). In the majority of cases, thyrotoxicosis was asymptomatic, and specific treatment with glucocorticoids or antithyroid medication was not required. However, severe cases have been described (63,64). As such, diagnosis often occurred incidentally

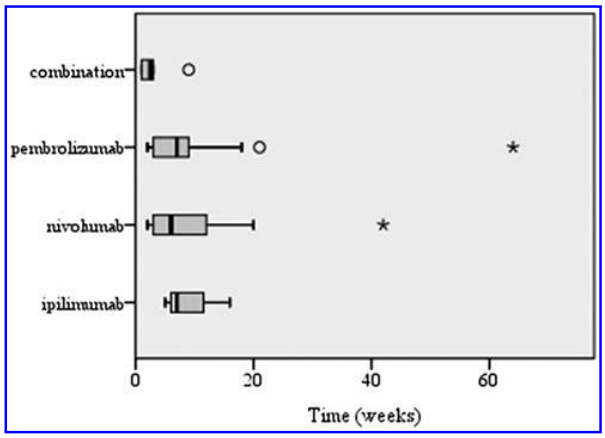

FIG. 3. Median time to development of hyperthyroidism following the first dose of immune checkpoint inhibitor. The left, middle, and right lines of the boxes correspond to the 25th, 50th, and 75th percentiles, respectively. The whiskers extend from minimum to maximum with ○ and * representing outlier and extreme outlier values, respectively.

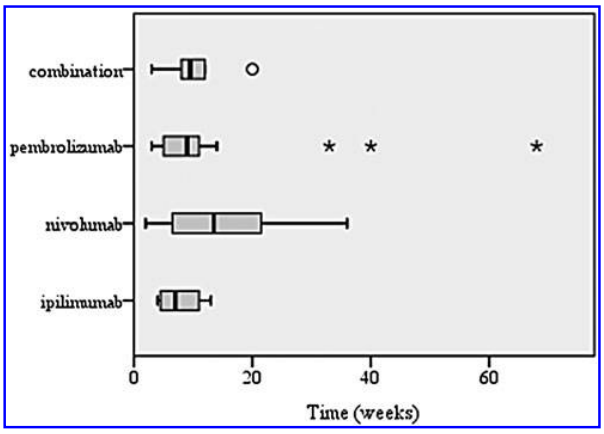

FIG. 4. Median time to development of hypothyroidism following the first dose of immune checkpoint inhibitor. The left, middle, and right lines of the boxes correspond to the 25th, 50th, and 75th percentiles, respectively. The whiskers extend from minimum to maximum with ○ and * representing outlier and extreme outlier values, respectively.

during routine monitoring of thyroid function tests. Multiple endocrine toxicities in the same patient can occur, particularly with combination therapy where up to one-third of patients who experience a thyroid irAE can develop a synchronous hypophysitis or checkpoint inhibitor-associated diabetes mellitus (57,65–69).

Following the thyrotoxic phase, a majority of cases progress to hypothyroidism, although some may recover euthyroidism without an intervening hypothyroid period (53–59,70). Early onset of thyrotoxicosis (within four weeks) may predict progression to hypothyroidism, whereas delayed onset may be less likely to be followed by an overt hypothyroid phase (61). Subclinical hyperthyroidism also portended a good prognosis, as when present, likelihood for progression to overt hypothyroidism was significantly reduced (47,59,70,71). Similar to the thyrotoxic phase, most cases of hypothyroidism were asymptomatic, although episodes of myxedema crisis have been reported (72,73).

Overt hypothyroidism without a preceding hyperthyroid phase may be the first manifestation of thyroid irAEs, and the incidence of primary hypothyroidism as the initial event varies between 10% and 60% (53,57,60,62,71). Time to initial onset of hypothyroidism is typically longer than that seen with primary hyperthyroidism (Fig. 4). It is unclear whether this represents a separate form of thyroid irAE, or if the transient thyrotoxic phase was asymptomatic and not clinically or biochemically detected. If overt hypothyroidism developed, it was irreversible in a majority of cases and long-term thyroid hormone replacement was required irrespective of whether hypothyroidism was preceded by a thyrotoxic phase or not (53,56,60,62,71,74,75). Subclinical hypothyroidism exhibited greater potential for recovery and only a minority required long-term levothyroxine (LT4) replacement (71). It is possible that milder cases may represent a nonthyroidal illness effect secondary to generalized immune activation rather than a true ICI-associated thyroiditis, although this remains to be proven. The incidence and kinetics of thyroid dysfunction after ICI treatment are summarized in Table 2.

Thyroid function monitoring

Routine monitoring of thyroid function at four to six weekly intervals is recommended for all patients on treatment with CTLA-4 and PD-1 inhibitors. Additional testing can be offered to patients who develop symptoms of potential thyroid dysfunction (fatigue, palpitations, etc.) outside the recommended intervals (76).

Overt hypothyroidism is diagnosed in the setting of an elevated thyrotropin (TSH) and low free thyroxine (fT4) concentration. Central hypothyroidism must be excluded in all patients with biochemical hypothyroidism, especially in the setting of ipilimumab or combination immunotherapy. If present, appropriate replacement of glucocorticoids in addition to thyroid hormone is essential to avoid precipitation of adrenal crises. Subclinical hypothyroidism will usually recover without treatment, whereas most cases of overt hypothyroidism will require commencement and long-term continuation of LT4. Hypothyroidism does not mandate ICI treatment interruption and can be continued during the investigation and management of thyroid dysfunction (76).

Overt hyperthyroidism is characterized by suppressed TSH and elevated fT4 levels. Most episodes of thyrotoxicosis are asymptomatic, and dedicated treatment is not required. In symptomatic cases, temporary treatment with beta-blockers can help to control symptoms. Thyrotoxic patients should be monitored more closely (two weekly intervals) to detect early transition to hypothyroidism. When thyrotoxicosis is prolonged (>6 weeks) or if high clinical suspicion, patients should be investigated for Graves' disease with measurement of thyroid-stimulating antibody levels (76). In most cases, ICI treatment can be continued throughout the period of thyrotoxicosis, although patients with grade 3 or higher toxicity should have further ICI treatment withheld until the resolution of symptoms (76).

Pre-existing hypothyroidism

Although immune checkpoint inhibition is best recognized as a cause of *de novo* thyroid dysfunction, patients with pre-

Table 2. Incidence and Kinetics of Thyroid Dysfunction After Immune Checkpoint Inhibitor Treatment in Published Observational Studies Reporting Thyroid Immune-Related Adverse Events

	Ipilimumab (N = *195*)	*Nivolumab* (N = *612*)	*Pembrolizumab* (N = *362*)	*Ipilimumab + PD-1 inhibitor* (N = *290*)
Thyroid abnormality, *n* (%)				
Overt hyperthyroidism	—	9 (1.5)	3 (0.8)	—
Overt hypothyroidism	5 (2.6)	49 (8.0)	34 (9.4)	29 (10.0)
Subclinical hyperthyroidism	20 (10.3)	7 (1.1)	—	7 (2.4)
Subclinical hypothyroidism	8 (4.1)	30 (4.9)	3 (0.8)	—
Thyrotoxic phase preceding hypothyroidism, *n* (%)	Not reported	31/49 (63.3)	25/34 (73.5)	27/29 (93.1)
Permanent hypothyroidism, *n* (%)	Not reported	40/49 (81.6)	31/34 (91.2)	29/29 (100.0)
Duration of thyrotoxic phase, weeks median (range)	Not reported	7 (2–20)	5 (4–6)	6 (2–11)

N, number of patients exposed to immune checkpoint inhibitor; *n*, number of patients affected.

existing hypothyroidism are also susceptible to thyroid irAEs. In one study of 63 patients with abnormal thyroid function before treatment with CTLA-4 or PD-1 blockade, 43 (70%) patients experienced an exacerbation of their thyroid dysfunction (77). The majority developed an exacerbation of hypothyroidism requiring an increase or commencement of LT4. However, seven patients with established hypothyroidism on LT4 became thyrotoxic, mandating temporary suspension of thyroid hormone replacement (77). Similar findings have been reported in other studies of patients with pre-existing autoimmune thyroid disease, highlighting the need for surveillance of thyroid function in all patients receiving ICIs, even in those with long established hypothyroidism (78–82).

Risk factors

The etiology of irAEs is unknown, and it remains unclear why immune toxicity develops in some patients and not in others. Thyroid irAEs are among the most common and often result in permanent hypothyroidism requiring long-term thyroid hormone replacement. Onset is difficult to predict, and reliable predictors of thyroid irAEs have yet to be identified. The role of antithyroid antibodies has received the most interest, but results have been inconsistent and do not reliably predict the development or onset of thyroid irAEs.

In a Japanese study of 200 consecutive patients treated with nivolumab, age, sex, and baseline TSH values were not predictive of overt thyroid dysfunction (61). However, in one study, thyroid avidity on FDG-PET (before nivolumab treatment) significantly increased risk for development of thyroid irAEs. Positive thyroid uptake increased risk by greater than 14 times relative to those without thyroid uptake on a baseline FDG-PET (61). A second study of 99 patients treated with anti-PD-1 or combined CTLA-4 and PD-1 inhibitors also found no relationship between age or sex and risk of thyroid irAEs (70). However, unlike the Japanese study, they did find a significant relationship between baseline TSH concentration and risk of overt thyroid dysfunction (70). The mean TSH in subjects whom developed overt thyroid dysfunction measured 2.72 mIU/L compared with 1.97 mIU/L in those who did not. Based on these data, a baseline TSH >2.19 mIU/L was 76% specific and 53% sensitive for detection of overt thyroid irAEs (70).

Antithyroid antibodies

The significance of thyroid peroxidase (TPOAb) and thyroglobulin (TgAb) antibodies in the pathogenesis of thyroid irAEs is unknown, and their significance in the risk of thyroid irAEs remains controversial. Several studies have reported a low frequency (<30%) of positive TPOAb and TgAb in association with thyroid irAEs (51,53,62,83). Others have reported increased prevalence of antithyroid antibodies in cases of thyroid irAEs relative to controls unaffected by thyroid dysfunction (58,84–86). This begs the question of whether immune checkpoint blockade is simply unmasking pre-existing subclinical Hashimoto's thyroiditis, which then progresses in an accelerated manner versus a *de novo* process directly related to treatment with anti-CTLA-4 and/or anti-PD-1 medicines. One study supports the latter and describes the onset of new TPOAb and TgAb positivity during the thyrotoxic phase of thyroid irAEs in patients who were antibody negative at baseline (58). In another, the presence of a TgAb at baseline provoked thyroid irAEs earlier than in TgAb-negative patients, and higher titers of TgAb further decreased the time to onset of thyroid dysfunction (85). Multiple other studies have not found a relationship between antithyroid antibody status and the evolution timeline of thyroiditis (56,62).

During assessment of hyperthyroidism related to anti-CTLA-4 and/or anti-PD-1 treatment, thyroid receptor antibody levels are typically not elevated (87). However, there have been several case reports of Graves' disease occurring in patients undergoing immune checkpoint blockade and two cases of euthyroid ophthalmopathy (49,87–89).

Prognostic significance

Drug toxicity following anticancer treatment with interleukin-2 and tyrosine kinase inhibitors directed against the epidermal growth factor receptor is well known to correlate with tumor response and patient survival. There has been great interest in whether the same relationship between toxicity and response exists in the setting of irAEs following ICI treatment. Several studies suggest that this may be the case and have reported benefits in PFS and OS following anti-PD-1 treatment with development of irAEs (90,91). However, such results are inherently confounded by immortal time bias and no strong evidence yet supports a strong

relationship between toxicity and cancer prognosis. The presence of multiple irAEs has also been shown to improve PFS and OS relative to patients with one or no irAEs (90). Organ-specific autoimmunity has also been studied, particularly dermatologic irAEs in melanoma patients treated with nivolumab and pembrolizumab, where there is an association with improved PFS and OS (90,92,93).

Given that thyroid irAEs are among the most common adverse effects following anti-PD-1 treatment, several studies have examined the relationship between thyroid dysfunction and clinical outcomes. Although confined to observational studies, PFS and OS were improved following the occurrence of a thyroid irAE. (58,61,91,94–96). In two studies, where thyroid irAEs were subdivided into overt and subclinical, overt thyroid dysfunction showed a trend toward improved outcomes compared with when only subclinical disease was present, suggesting that not only the presence of a thyroid irAE but also the severity may be important in the relationship between irAEs and clinical response (61,94). Thyroiditis following blockade of PD-L1 with atezolizumab or avelumab has also been reported to improve OS in cancer patients in one study (97). No studies have yet reported on the prognostic significance of thyroid irAEs following anti-CTLA-4 or combined CTLA-4 and PD-1 inhibitor treatment.

Outstanding questions

Continued research is necessary to improve our understanding of autoimmune thyroid disease following immune checkpoint inhibition. The etiology of thyroid irAEs remains unclear. Clinical manifestations are heterogeneous, and it is not possible to predict who will develop thyroid irAEs and whether they will progress to permanent hypothyroidism requiring long-term thyroid hormone replacement. Our view is that heterogeneity of thyroid irAEs probably reflects that thyroid dysfunction arises due to multiple mechanisms including *de novo* thyroiditis, activation of underlying subclinical Hashimoto's thyroiditis, and others. The role of thyroid autoantibodies is particularly intriguing, as they occur at higher frequency in thyroid irAEs than in euthyroid cancer patients. However, most studies have not found a causative association, and antibody positivity does not fully explain susceptibility. Whether, as yet, unidentified antibodies directed against the thyroid exist has not been studied. Subclinical thyroid dysfunction is probably underreported, making the true incidence of thyroid irAEs unknown. Prospective collection of thyroid function tests at frequent and regular intervals could help to clarify the true incidence of thyroid irAEs and risk of progression to permanent hypothyroidism. Finally, trials combining ICIs with other immunomodulatory therapies are ongoing. The effect of multimodal immune treatment on the risk and pattern of thyroid irAEs, and irAEs generally, is yet to be determined.

Conclusions

Inhibition of CTLA-4 and PD-1 immune checkpoints has dramatically enhanced the treatment of advanced cancer. However, treatment is associated with irAEs, of which thyroid dysfunction is the most common. Combined treatment with ipilimumab and nivolumab generally results in earlier onset and potentially more severe dysfunction. Most cases are mild and self-limited. Progression to permanent hypothyroidism can occur, and awareness among medical professionals is important to limit morbidity associated with untreated thyroid dysfunction.

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References

1. Helmink BA, Gaudreau P-O, Wargo JA 2018 Immune checkpoint blockade across the cancer care continuum. Immunity **48:**1077–1080.
2. Pardoll DM 2012 The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer **12:**252–264.
3. Esfahani K, Meti N, Miller WH, Jr., Hudson M 2019 Adverse events associated with immune checkpoint inhibitor treatment for cancer. CMAJ **191:**E40–E46.
4. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH 1995 Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity **3:**541–547.
5. Chang LS, Barroso-Sousa R, Tolaney SM, Hodi FS, Kaiser UB, Min L 2019 Endocrine toxicity of cancer immunotherapy targeting immune checkpoints. Endocr Rev **40:** 17–65.
6. Nishimura H, Nose M, Hiai H, Minato N, Honjo T 1999 Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity **11:**141–151.
7. Postow MA, Sidlow R, Hellmann MD 2018 Immune-related adverse events associated with immune checkpoint blockade. N Engl J Med **378:**158–168.
8. Khoja L, Day D, Wei-Wu Chen T, Siu LL, Hansen AR 2017 Tumour- and class-specific patterns of immune-related adverse events of immune checkpoint inhibitors: a systematic review. Ann Oncol **28:**2377–2385.
9. Pearce EN, Farwell AP, Braverman LE 2003 Thyroiditis. N Engl J Med **348:**2646–2655.
10. Eggermont AM, Chiarion-Sileni V, Grob JJ, Dummer R, Wolchok JD, Schmidt H, Hamid O, Robert C, Ascierto PA, Richards JM, Lebbe C, Ferraresi V, Smylie M, Weber JS, Maio M, Bastholt L, Mortier L, Thomas L, Tahir S, Hauschild A, Hassel JC, Hodi FS, Taitt C, de Pril V, de Schaetzen G, Suciu S, Testori A 2016 Prolonged survival in stage III melanoma with ipilimumab adjuvant therapy. N Engl J Med **375:**1845–1855.
11. Ascierto PA, Del Vecchio M, Robert C, Mackiewicz A, Chiarion-Sileni V, Arance A, Lebbe C, Bastholt L, Hamid O, Rutkowski P, McNeil C, Garbe C, Loquai C, Dreno B, Thomas L, Grob JJ, Liszkay G, Nyakas M, Gutzmer R, Pikiel J, Grange F, Hoeller C, Ferraresi V, Smylie M, Schadendorf D, Mortier L, Svane IM, Hennicken D, Qureshi A, Maio M 2017 Ipilimumab 10 mg/kg versus ipilimumab 3 mg/kg in patients with unresectable or metastatic melanoma: a randomised, double-blind, multicentre, phase 3 trial. Lancet Oncol **18:**611–622.
12. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D,

Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbe C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A, Urba WJ 2010 Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med **363:**711–723.

13. Robert C, Thomas L, Bondarenko I, O'Day S, Weber J, Garbe C, Lebbe C, Baurain J-F, Testori A, Grob J-J 2011 Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. N Engl J Med **364:**2517–2526.
14. Govindan R, Szczesna A, Ahn MJ, Schneider CP, Gonzalez Mella PF, Barlesi F, Han B, Ganea DE, Von Pawel J, Vladimirov V, Fadeeva N, Lee KH, Kurata T, Zhang L, Tamura T, Postmus PE, Jassem J, O'Byrne K, Kopit J, Li M, Tschaika M, Reck M 2017 Phase III trial of ipilimumab combined with paclitaxel and carboplatin in advanced squamous non-small-cell lung cancer. J Clin Oncol **35:** 3449–3457.
15. Reck M, Luft A, Szczesna A, Havel L, Kim SW, Akerley W, Pietanza MC, Wu YL, Zielinski C, Thomas M, Felip E, Gold K, Horn L, Aerts J, Nakagawa K, Lorigan P, Pieters A, Kong Sanchez T, Fairchild J, Spigel D 2016 Phase III Randomized trial of ipilimumab plus etoposide and platinum versus placebo plus etoposide and platinum in extensive-stage small-cell lung cancer. J Clin Oncol **34:** 3740–3748.
16. Weber J, Mandala M, Del Vecchio M, Gogas HJ, Arance AM, Cowey CL, Dalle S, Schenker M, Chiarion-Sileni V, Marquez-Rodas I, Grob JJ, Butler MO, Middleton MR, Maio M, Atkinson V, Queirolo P, Gonzalez R, Kudchadkar RR, Smylie M, Meyer N, Mortier L, Atkins MB, Long GV, Bhatia S, Lebbe C, Rutkowski P, Yokota K, Yamazaki N, Kim TM, de Pril V, Sabater J, Qureshi A, Larkin J, Ascierto PA, CheckMate C 2017 Adjuvant nivolumab versus ipilimumab in resected stage III or IV melanoma. N Engl J Med **377:**1824–1835.
17. Robert C, Schachter J, Long GV, Arance A, Grob JJ, Mortier L, Daud A, Carlino MS, McNeil C, Lotem M, Larkin J, Lorigan P, Neyns B, Blank CU, Hamid O, Mateus C, Shapira-Frommer R, Kosh M, Zhou H, Ibrahim N, Ebbinghaus S, Ribas A, investigators K-2015 Pembrolizumab versus ipilimumab in advanced melanoma. N Engl J Med **372:**2521–2532.
18. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL, Lao CD, Schadendorf D, Dummer R, Smylie M, Rutkowski P, Ferrucci PF, Hill A, Wagstaff J, Carlino MS, Haanen JB, Maio M, Marquez-Rodas I, McArthur GA, Ascierto PA, Long GV, Callahan MK, Postow MA, Grossmann K, Sznol M, Dreno B, Bastholt L, Yang A, Rollin LM, Horak C, Hodi FS, Wolchok JD 2015 Combined nivolumab and ipilimumab or monotherapy in untreated melanoma. N Engl J Med **373:**23–34.
19. Robert C, Long GV, Brady B, Dutriaux C, Maio M, Mortier L, Hassel JC, Rutkowski P, McNeil C, Kalinka-Warzocha E, Savage KJ, Hernberg MM, Lebbe C, Charles J, Mihalcioiu C, Chiarion-Sileni V, Mauch C, Cognetti F, Arance A, Schmidt H, Schadendorf D, Gogas H, Lundgren-Eriksson L, Horak C, Sharkey B, Waxman IM, Atkinson V, Ascierto PA 2015 Nivolumab in previously untreated melanoma without BRAF mutation. N Engl J Med **372:** 320–330.
20. Weber JS, D'Angelo SP, Minor D, Hodi FS, Gutzmer R, Neyns B, Hoeller C, Khushalani NI, Miller WH, Lao CD, Linette GP, Thomas L, Lorigan P, Grossmann KF, Hassel JC, Maio M, Sznol M, Ascierto PA, Mohr P, Chmielowski B, Bryce A, Svane IM, Grob J-J, Krackhardt AM, Horak C, Lambert A, Yang AS, Larkin J 2015 Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomised, controlled, open-label, phase 3 trial. Lancet Oncol **16:**375–384.
21. Borghaei H, Paz-Ares L, Horn L, Spigel DR, Steins M, Ready NE, Chow LQ, Vokes EE, Felip E, Holgado E, Barlesi F, Kohlhaufl M, Arrieta O, Burgio MA, Fayette J, Lena H, Poddubskaya E, Gerber DE, Gettinger SN, Rudin CM, Rizvi N, Crino L, Blumenschein GR, Jr., Antonia SJ, Dorange C, Harbison CT, Graf Finckenstein F, Brahmer JR 2015 Nivolumab versus docetaxel in advanced nonsquamous non-small-cell lung cancer. N Engl J Med **373:** 1627–1639.
22. Brahmer J, Reckamp KL, Baas P, Crino L, Eberhardt WE, Poddubskaya E, Antonia S, Pluzanski A, Vokes EE, Holgado E, Waterhouse D, Ready N, Gainor J, Aren Frontera O, Havel L, Steins M, Garassino MC, Aerts JG, Domine M, Paz-Ares L, Reck M, Baudelet C, Harbison CT, Lestini B, Spigel DR 2015 Nivolumab versus docetaxel in advanced squamous-cell non-small-cell lung cancer. N Engl J Med **373:**123–135.
23. Kang YK, Boku N, Satoh T, Ryu MH, Chao Y, Kato K, Chung HC, Chen JS, Muro K, Kang WK, Yeh KH, Yoshikawa T, Oh SC, Bai LY, Tamura T, Lee KW, Hamamoto Y, Kim JG, Chin K, Oh DY, Minashi K, Cho JY, Tsuda M, Chen LT 2017 Nivolumab in patients with advanced gastric or gastro-oesophageal junction cancer refractory to, or intolerant of, at least two previous chemotherapy regimens (ONO-4538-12, ATTRACTION-2): a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet **390:**2461–2471.
24. Hellmann MD, Ciuleanu TE, Pluzanski A, Lee JS, Otterson GA, Audigier-Valette C, Minenza E, Linardou H, Burgers S, Salman P, Borghaei H, Ramalingam SS, Brahmer J, Reck M, O'Byrne KJ, Geese WJ, Green G, Chang H, Szustakowski J, Bhagavatheeswaran P, Healey D, Fu Y, Nathan F, Paz-Ares L 2018 Nivolumab plus ipilimumab in lung cancer with a high tumor mutational burden. N Engl J Med **378:**2093–2104.
25. Ferris RL, Blumenschein G, Jr., Fayette J, Guigay J, Colevas AD, Licitra L, Harrington K, Kasper S, Vokes EE, Even C, Worden F, Saba NF, Iglesias Docampo LC, Haddad R, Rordorf T, Kiyota N, Tahara M, Monga M, Lynch M, Geese WJ, Kopit J, Shaw JW, Gillison ML 2016 Nivolumab for recurrent squamous-cell carcinoma of the head and neck. N Engl J Med **375:**1856–1867.
26. Bellmunt J, de Wit R, Vaughn DJ, Fradet Y, Lee JL, Fong L, Vogelzang NJ, Climent MA, Petrylak DP, Choueiri TK, Necchi A, Gerritsen W, Gurney H, Quinn DI, Culine S, Sternberg CN, Mai Y, Poehlein CH, Perini RF, Bajorin DF 2017 Pembrolizumab as second-line therapy for advanced urothelial carcinoma. N Engl J Med **376:**1015–1026.
27. Cohen EEW, Soulieres D, Le Tourneau C, Dinis J, Licitra L, Ahn MJ, Soria A, Machiels JP, Mach N, Mehra R, Burtness B, Zhang P, Cheng J, Swaby RF, Harrington KJ 2019 Pembrolizumab versus methotrexate, docetaxel, or cetuximab for recurrent or metastatic head-and-neck squamous cell carcinoma (KEYNOTE-040): a randomised, open-label, phase 3 study. Lancet **393:**156–167.
28. Eggermont AMM, Blank CU, Mandala M, Long GV, Atkinson V, Dalle S, Haydon A, Lichinitser M, Khattak A,

Carlino MS, Sandhu S, Larkin J, Puig S, Ascierto PA, Rutkowski P, Schadendorf D, Koornstra R, Hernandez-Aya L, Maio M, van den Eertwegh AJM, Grob JJ, Gutzmer R, Jamal R, Lorigan P, Ibrahim N, Marreaud S, van Akkooi ACJ, Suciu S, Robert C 2018 Adjuvant pembrolizumab versus placebo in resected Stage III melanoma. N Engl J Med **378:**1789–1801.

29. Gandhi L, Rodriguez-Abreu D, Gadgeel S, Esteban E, Felip E, De Angelis F, Domine M, Clingan P, Hochmair MJ, Powell SF, Cheng SY, Bischoff HG, Peled N, Grossi F, Jennens RR, Reck M, Hui R, Garon EB, Boyer M, Rubio-Viqueira B, Novello S, Kurata T, Gray JE, Vida J, Wei Z, Yang J, Raftopoulos H, Pietanza MC, Garassino MC, Investigators K-2018 Pembrolizumab plus chemotherapy in metastatic non-small-cell lung cancer. N Engl J Med **378:** 2078–2092.
30. Garon EB, Rizvi NA, Hui R, Leighl N, Balmanoukian AS, Eder JP, Patnaik A, Aggarwal C, Gubens M, Horn L, Carcereny E, Ahn MJ, Felip E, Lee JS, Hellmann MD, Hamid O, Goldman JW, Soria JC, Dolled-Filhart M, Rutledge RZ, Zhang J, Lunceford JK, Rangwala R, Lubiniecki GM, Roach C, Emancipator K, Gandhi L, Investigators K-2015 Pembrolizumab for the treatment of non-small-cell lung cancer. N Engl J Med **372:**2018–2028.
31. Herbst RS, Baas P, Kim D-W, Felip E, Pérez-Gracia JL, Han J-Y, Molina J, Kim J-H, Arvis CD, Ahn M-J, Majem M, Fidler MJ, de Castro G, Garrido M, Lubiniecki GM, Shentu Y, Im E, Dolled-Filhart M, Garon EB 2016 Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): a randomised controlled trial. Lancet **387:**1540–1550.
32. Mok TSK, Wu Y-L, Kudaba I, Kowalski DM, Cho BC, Turna HZ, Castro G, Srimuninnimit V, Laktionov KK, Bondarenko I, Kubota K, Lubiniecki GM, Zhang J, Kush D, Lopes G, Adamchuk G, Ahn M-J, Alexandru A, Altundag O, Alyasova A, Andrusenko O, Aoe K, Araujo A, Aren O, Arrieta Rodriguez O, Ativitavas T, Avendano O, Barata F, Barrios CH, Beato C, Bergstrom P, Betticher D, Bolotina L, Bondarenko I, Botha M, Buddu S, Caglevic C, Cardona A, Castro G, Castro H, Cay Senler F, Cerny CAS, Cesas A, Chan G-C, Chang J, Chen G, Chen X, Cheng S, Cheng Y, Cherciu N, Chiu C-H, Cho BC, Cicenas S, Ciurescu D, Cohen G, Costa MA, Danchaivijitr P, De Angelis F, de Azevedo SJ, Dediu M, Deliverski T, De Marchi PRM, de The Bustamante Valles F, Ding Z, Doganov B, Dreosti L, Duarte R, Edusma-Dy R, Emelyanov S, Erman M, Fan Y, Fein L, Feng J, Fenton D, Fernandes G, Ferreira C, Franke FA, Freitas H, Fujisaka Y, Galindo H, Galvez C, Ganea D, Gil N, Girotto G, Goker E, Goksel T, Gomez Aubin G, Gomez Wolff L, Griph H, Gumus M, Hall J, Hart G, Havel L, He J, He Y, Hernandez Hernandez C, Hespanhol V, Hirashima T, Ho CMJ, Horiike A, Hosomi Y, Hotta K, Hou M, How SH, Hsia T-C, Hu Y, Ichiki M, Imamura F, Ivashchuk O, Iwamoto Y, Jaal J, Jassem J, Jordaan C, Juergens RA, Kaen D, Kalinka-Warzocha E, Karaseva N, Karaszewska B, Kazarnowicz A, Kasahara K, Katakami N, Kato T, Kawaguchi T, Kim JH, Kishi K, Kolek V, Koleva M, Kolman P, Koubkova L, Kowalyszyn R, Kowalski D, Koynov K, Ksienski D, Kubota K, Kudaba I, Kurata T, Kuusk G, Kuzina L, Laczo I, Ladrera GEI, Laktionov K, Landers G, Lazarev S, Lerzo G, Lesniewski Kmak K, Li W, Liam CK, Lifirenko I, Lipatov O, Liu X, Liu Z, Lo SH, Lopes V, Lopez K, Lu S, Martinengo G, Mas L, Matrosova M, Micheva R, Milanova Z, Miron L, Mok T, Molina M, Murakami S, Nakahara Y, Nguyen TQ, Nishimura T, Ochsenbein A, Ohira T, Ohman R, Ong CK, Ostoros G, Ouyang X, Ovchinnikova E, Ozyilkan O, Petruzelka L, Pham XD, Picon P, Piko B, Poltoratsky A, Ponomarova O, Popelkova P, Purkalne G, Qin S, Ramlau R, Rappaport B, Rey F, Richardet E, Roubec J, Ruff P, Rusyn A, Saka H, Salas J, Sandoval M, Santos L, Sawa T, Seetalarom K, Seker M, Seki N, Seolwane F, Shepherd L, Shevnya S, Shimada AK, Shparyk Y, Sinielnikov I, Sirbu D, Smaletz O, Soares JPH, Sookprasert A, Speranza G, Srimuninnimit V, Sriuranpong V, Stara Z, Su W-C, Sugawara S, Szpak W, Takahashi K, Takigawa N, Tanaka H, Tan Chun Bing J, Tang Q, Taranov P, Tejada H, Tho LM, Torii Y, Trukhyn D, Turdean M, Turna H, Ursol G, Vanasek J, Varela M, Vallejo M, Vera L, Victorino A-P, Vlasek T, Vynnychenko I, Wang B, Wang J, Wang K, Wu Y, Yamada K, Yang C-H, Yokoyama T, Yokoyama T, Yoshioka H, Yumuk F, Zambrano A, Zarba JJ, Zarubenkov O, Zemaitis M, Zhang L, Zhang L, Zhang X, Zhao J, Zhou C, Zhou J, Zhou Q, Zippelius A 2019 Pembrolizumab versus chemotherapy for previously untreated, PD-L1-expressing, locally advanced or metastatic non-small-cell lung cancer (KEYNOTE-042): a randomised, open-label, controlled, phase 3 trial. Lancet **393:**1819–1830.
33. Paz-Ares L, Luft A, Vicente D, Tafreshi A, Gumus M, Mazieres J, Hermes B, Cay Senler F, Csoszi T, Fulop A, Rodriguez-Cid J, Wilson J, Sugawara S, Kato T, Lee KH, Cheng Y, Novello S, Halmos B, Li X, Lubiniecki GM, Piperdi B, Kowalski DM 2018 Pembrolizumab plus chemotherapy for squamous non-small-cell lung cancer. N Engl J Med **379:**2040–2051.
34. Reck M, Rodriguez-Abreu D, Robinson AG, Hui R, Csoszi T, Fulop A, Gottfried M, Peled N, Tafreshi A, Cuffe S, O'Brien M, Rao S, Hotta K, Leiby MA, Lubiniecki GM, Shentu Y, Rangwala R, Brahmer JR 2016 Pembrolizumab versus chemotherapy for PD-L1-positive non-small-cell lung cancer. N Engl J Med **375:**1823–1833.
35. Shitara K, Ozguroglu M, Bang YJ, Di Bartolomeo M, Mandala M, Ryu MH, Fornaro L, Olesinski T, Caglevic C, Chung HC, Muro K, Goekkurt E, Mansoor W, McDermott RS, Shacham-Shmueli E, Chen X, Mayo C, Kang SP, Ohtsu A, Fuchs CS; KEYNOTE-061 Investigators 2018 Pembrolizumab versus paclitaxel for previously treated, advanced gastric or gastro-oesophageal junction cancer (KEYNOTE-061): a randomised, open-label, controlled, phase 3 trial. Lancet **392:**123–133.
36. Mateos MV, Blacklock H, Schjesvold F, Oriol A, Simpson D, George A, Goldschmidt H, Larocca A, Chanan-Khan A, Sherbenou D, Avivi I, Benyamini N, Iida S, Matsumoto M, Suzuki K, Ribrag V, Usmani SZ, Jagannath S, Ocio EM, Rodriguez-Otero P, San Miguel J, Kher U, Farooqui M, Liao J, Marinello P, Lonial S 2019 Pembrolizumab plus pomalidomide and dexamethasone for patients with relapsed or refractory multiple myeloma (KEYNOTE-183): a randomised, open-label, phase 3 trial. Lancet Haematol **6:** e459–e469.
37. Usmani SZ, Schjesvold F, Oriol A, Karlin L, Cavo M, Rifkin RM, Yimer HA, LeBlanc R, Takezako N, McCroskey RD, Lim ABM, Suzuki K, Kosugi H, Grigoriadis G, Avivi I, Facon T, Jagannath S, Lonial S, Ghori RU, Farooqui MZH, Marinello P, San-Miguel J 2019 Pembrolizumab plus lenalidomide and dexamethasone for patients with treatment-naive multiple myeloma (KEYNOTE-

185): a randomised, open-label, phase 3 trial. Lancet Haematol **6:**e448–e458.
38. Motzer RJ, Tannir NM, McDermott DF, Aren Frontera O, Melichar B, Choueiri TK, Plimack ER, Barthelemy P, Porta C, George S, Powles T, Donskov F, Neiman V, Kollmannsberger CK, Salman P, Gurney H, Hawkins R, Ravaud A, Grimm MO, Bracarda S, Barrios CH, Tomita Y, Castellano D, Rini BI, Chen AC, Mekan S, McHenry MB, Wind-Rotolo M, Doan J, Sharma P, Hammers HJ, Escudier B, CheckMate I 2018 Nivolumab plus ipilimumab versus sunitinib in advanced renal-cell carcinoma. N Engl J Med **378:**1277–1290.
39. Horn L, Mansfield AS, Szczęsna A, Havel L, Krzakowski M, Hochmair MJ, Huemer F, Losonczy G, Johnson ML, Nishio M 2018 First-line atezolizumab plus chemotherapy in extensive-stage small-cell lung cancer. N Engl J Med **379:**2220–2229.
40. Schmid P, Adams S, Rugo HS, Schneeweiss A, Barrios CH, Iwata H, Diéras V, Hegg R, Im S-A, Shaw Wright G 2018 Atezolizumab and nab-paclitaxel in advanced triple-negative breast cancer. N Engl J Med **379:**2108–2121.
41. Socinski MA, Jotte RM, Cappuzzo F, Orlandi F, Stroyakovskiy D, Nogami N, Rodríguez-Abreu D, Moro-Sibilot D, Thomas CA, Barlesi F 2018 Atezolizumab for first-line treatment of metastatic nonsquamous NSCLC. N Engl J Med **378:**2288–2301.
42. Rini BI, Powles T, Atkins MB, Escudier B, McDermott DF, Suarez C, Bracarda S, Stadler WM, Donskov F, Lee JL 2019 Atezolizumab plus bevacizumab versus sunitinib in patients with previously untreated metastatic renal cell carcinoma (IMmotion151): a multicentre, open-label, phase 3, randomised controlled trial. Lancet **393:**2404–2415.
43. Antonia SJ, Villegas A, Daniel D, Vicente D, Murakami S, Hui R, Kurata T, Chiappori A, Lee KH, de Wit M 2018 Overall survival with durvalumab after chemoradiotherapy in stage III NSCLC. N Engl J Med **379:**2342–2350.
44. Paz-Ares L, Dvorkin M, Chen Y, Reinmuth N, Hotta K, Trukhin D, Statsenko G, Hochmair MJ, Özgüroğlu M, Ji JH 2019 Durvalumab plus platinum–etoposide versus platinum–etoposide in first-line treatment of extensive-stage small-cell lung cancer (CASPIAN): a randomised, controlled, open-label, phase 3 trial. Lancet **394:**1929–1939.
45. Angell TE, Min L, Wieczorek TJ, Hodi FS 2018 Unique cytologic features of thyroiditis caused by immune checkpoint inhibitor therapy for malignant melanoma. Genes Dis **5:**46–48.
46. Neppl C, Kaderli RM, Trepp R, Schmitt AM, Berger MD, Wehrli M, Seiler CA, Langer R 2018 Histology of nivolumab-induced thyroiditis. Thyroid **28:**1727–1728.
47. Morganstein DL, Lai Z, Spain L, Diem S, Levine D, Mace C, Gore M, Larkin J 2017 Thyroid abnormalities following the use of cytotoxic T-lymphocyte antigen-4 and programmed death receptor protein-1 inhibitors in the treatment of melanoma. Clin Endocrinol **86:**614–620.
48. Delivanis DA, Gustafson MP, Bornschlegl S, Merten MM, Kottschade L, Withers S, Dietz AB, Ryder M 2017 Pembrolizumab-induced thyroiditis: comprehensive clinical review and insights into underlying involved mechanisms. J Clin Endocrinol Metab **102:**2770–2780.
49. Min L, Vaidya A, Becker C 2011 Thyroid autoimmunity and ophthalmopathy related to melanoma biological therapy. Eur J Endocrinol **164:**303–307.
50. Mekki A, Dercle L, Lichtenstein P, Marabelle A, Michot JM, Lambotte O, Le Pavec J, De Martin E, Balleyguier C, Champiat S, Ammari S 2018 Detection of immune-related adverse events by medical imaging in patients treated with anti-programmed cell death 1. Eur J Cancer **96:**91–104.
51. Mazarico I, Capel I, Giménez-Palop O, Albert L, Berges I, Luchtenberg F, García Y, Fernández-Morales L, De Pedro V, Caixàs A 2019 Low frequency of positive antithyroid antibodies is observed in patients with thyroid dysfunction related to immune check point inhibitors. J Endocrinol Invest **42:**1443–1450.
52. Middlebrooks EH, Westbrook BC, Conry RM 2018 Acute inflammatory thyromegaly following checkpoint inhibition: a new imaging entity? Radiol Case Rep **13:**89–91.
53. de Filette J, Jansen Y, Schreuer M, Everaert H, Velkeniers B, Neyns B, Bravenboer B 2016 Incidence of thyroid-related adverse events in melanoma patients treated with pembrolizumab. J Clin Endocrinol Metab **101:**4431–4439.
54. Orlov S, Salari F, Kashat L, Walfish PG 2015 Induction of painless thyroiditis in patients receiving programmed death 1 receptor immunotherapy for metastatic malignancies. J Clin Endocrinol Metab **100:**1738–1741.
55. Yamauchi I, Sakane Y, Fukuda Y, Fujii T, Taura D, Hirata M, Hirota K, Ueda Y, Kanai Y, Yamashita Y, Kondo E, Sone M, Yasoda A, Inagaki N 2017 Clinical features of nivolumab-induced thyroiditis: a case series study. Thyroid **27:**894–901.
56. Iyer PC, Cabanillas ME, Waguespack SG, Hu MI, Thosani S, Lavis VR, Busaidy NL, Subudhi SK, Diab A, Dadu R 2018 Immune-related thyroiditis with immune checkpoint inhibitors. Thyroid **28:**1243–1251.
57. Lee H, Hodi FS, Giobbie-Hurder A, Ott PA, Buchbinder EI, Haq R, Tolaney S, Barroso-Sousa R, Zhang K, Donahue H, Davis M, Gargano ME, Kelley KM, Carroll RS, Kaiser UB, Min L 2017 Characterization of thyroid disorders in patients receiving immune checkpoint inhibition therapy. Cancer Immunol Res **5:**1133–1140.
58. Osorio JC, Ni A, Chaft JE, Pollina R, Kasler MK, Stephens D, Rodriguez C, Cambridge L, Rizvi H, Wolchok JD, Merghoub T, Rudin CM, Fish S, Hellmann MD 2017 Antibody-mediated thyroid dysfunction during T-cell checkpoint blockade in patients with non-small-cell lung cancer. Ann Oncol **28:**583–589.
59. Villa NM, Farahmand A, Du L, Yeh MW, Smooke-Praw S, Ribas A, Chmielowski B, Cherry G, Leung AM 2018 Endocrinopathies with use of cancer immunotherapies. Clin Endocrinol **88:**327–332.
60. O'Malley G, Lee HJ, Parekh S, Galsky MD, Smith CB, Friedlander P, Yanagisawa RT, Gallagher EJ 2017 Rapid evolution of thyroid dysfunction in patients treated with nivolumab. Endocr Pract **23:**1223–1231.
61. Yamauchi I, Yasoda A, Matsumoto S, Sakamori Y, Kim YH, Nomura M, Otsuka A, Yamasaki T, Saito R, Kitamura M, Kitawaki T, Hishizawa M, Kawaguchi-Sakita N, Fujii T, Taura D, Sone M, Inagaki N 2019 Incidence, features, and prognosis of immune-related adverse events involving the thyroid gland induced by nivolumab. PLoS One **14:** e0216954.
62. Scott ES, Long GV, Guminski A, Clifton-Bligh RJ, Menzies AM, Tsang VH 2018 The spectrum, incidence, kinetics and management of endocrinopathies with immune checkpoint inhibitors for metastatic melanoma. Eur J Endocrinol **178:**173–180.
63. Yu C, Chopra IJ, Ha E 2015 A novel melanoma therapy stirs up a storm: ipilimumab-induced thyrotoxicosis. Endocrinol Diab Metab Case Rep **2015:**140092.

64. Yonezaki K, Kobayashi T, Imachi H, Yoshimoto T, Kikuchi F, Fukunaga K, Sato S, Ibata T, Yamaji N, Lyu J, Dong T, Murao K 2018 Combination therapy of ipilimumab and nivolumab induced thyroid storm in a patient with Hashimoto's disease and diabetes mellitus: a case report. J Med Case Rep **12:**171.
65. Hakami OA, Ioana J, Ahmad S, Tun TK, Sreenan S, McDermott JH 2019 A case of pembrolizumab-induced severe dka and hypothyroidism in a patient with metastatic melanoma. Endocrinol Diab Metab Case Rep **2019:**18-0153.
66. Paepegaey AC, Lheure C, Ratour C, Lethielleux G, Clerc J, Bertherat J, Kramkimel N, Groussin L 2017 Polyendocrinopathy resulting from pembrolizumab in a patient with a malignant melanoma. J Endocrine Soc **1:**646–649.
67. Sakurai K, Niitsuma S, Sato R, Takahashi K, Arihara Z 2018 Painless thyroiditis and fulminant type 1 diabetes mellitus in a patient treated with an immune checkpoint inhibitor, nivolumab. Tohoku J Exp Med **244:**33–40.
68. Ariyasu H, Inaba H, Ota T, Yamaoka H, Furukawa Y, Iwakura H, Doi N, Yamamoto Y, Akamizu T 2018 Thyrotoxicosis and adrenocortical hormone deficiency during immune-checkpoint inhibitor treatment for malignant melanoma. in vivo **32:**345–351.
69. Tsang VH, McGrath RT, Clifton-Bligh RJ, Scolyer RA, Jakrot V, Guminski AD, Long GV, Menzies AM 2019 Checkpoint inhibitor-associated autoimmune diabetes is distinct from type 1 diabetes. J Clin Endocrinol Metab **104:** 5499–5506.
70. Pollack RM, Kagan M, Lotem M, Dresner-Pollak R 2019 Baseline Tsh level is associated with risk of anti-Pd-1-induced thyroid dysfunction. Endocr Pract **25:**824–829.
71. Peiro I, Palmero R, Iglesias P, Diez JJ, Simo-Servat A, Marin JA, Jimenez L, Domingo-Domenech E, Mancho-Fora N, Nadal E, Villabona C 2019 Thyroid dysfunction induced by nivolumab: searching for disease patterns and outcomes. Endocrine **64:**605–613.
72. Johnson ED, Kerrigan K, Butler K, Patel SB 2019 Nivolumab-induced hypothyoidism with consequent hypothyroid related myopathy. J Oncol Pharm Pract **26:**224–227.
73. Khan U, Rizvi H, Sano D, Chiu J, Hadid T 2017 Nivolumab induced myxedema crisis. J Immunother Cancer **5:**13.
74. Ramos-Levi AM, Rogado J, Sanchez-Torres JM, Colomer R, Marazuela M 2019 Nivolumab-induced thyroid dysfunction in patients with lung cancer. Endocrinol Diabetes Nutr **66:**26–34.
75. Kaur A, Doberstein T, Amberker RR, Garje R, Field EH, Singh N 2019 Immune-related adverse events in cancer patients treated with immune checkpoint inhibitors: a single-center experience. Medicine **98:**e17348.
76. Brahmer JR, Lacchetti C, Schneider BJ, Atkins MB, Brassil KJ, Caterino JM, Chau I, Ernstoff MS, Gardner JM, Ginex P, Hallmeyer S, Chakrabarty JH, Leighl NB, Mammen JS, McDermott DF, Naing A, Nastoupil LJ, Phillips T, Porter LD, Puzanov I, Reichner CA, Santomasso BD, Seigel C, Spira A, Suarez-Almazor ME, Wang Y, Weber JS, Wolchok JD, Thompson JA; National Comprehensive Cancer Network 2018 Management of immune-related adverse events in patients treated with immune checkpoint inhibitor therapy: American Society of Clinical Oncology Clinical Practice Guideline. J Clin Oncol **36:**1714–1768.
77. Patel NS, Oury A, Daniels GA, Bazhenova L, Patel SP 2018 Incidence of thyroid function test abnormalities in patients receiving immune-checkpoint inhibitors for cancer treatment. Oncologist **23:**1236–1241.
78. Narita T, Oiso N, Taketomo Y, Okahashi K, Yamauchi K, Sato M, Uchida S, Matsuda H, Kawada A 2016 Serological aggravation of autoimmune thyroid disease in two cases receiving nivolumab. J Dermatol **43:**210–214.
79. Kahler KC, Eigentler TK, Gesierich A, Heinzerling L, Loquai C, Meier F, Meiss F, Pfohler C, Schlaak M, Terheyden P, Thoms KM, Ziemer M, Zimmer L, Gutzmer R; German Dermatologic Cooperative Oncology Group 2018 Ipilimumab in metastatic melanoma patients with preexisting autoimmune disorders. Cancer Immunol Immunother **67:**825–834.
80. Gutzmer R, Koop A, Meier F, Hassel JC, Terheyden P, Zimmer L, Heinzerling L, Ugurel S, Pfohler C, Gesierich A, Livingstone E, Satzger I, Kahler KC; German Dermatooncology Group 2017 Programmed cell death protein-1 (PD-1) inhibitor therapy in patients with advanced melanoma and preexisting autoimmunity or ipilimumab-triggered autoimmunity. Eur J Cancer **75:**24–32.
81. Danlos FX, Voisin AL, Dyevre V, Michot JM, Routier E, Taillade L, Champiat S, Aspeslagh S, Haroche J, Albiges L, Massard C, Girard N, Dalle S, Besse B, Laghouati S, Soria JC, Mateus C, Robert C, Lanoy E, Marabelle A, Lambotte O 2018 Safety and efficacy of anti-programmed death 1 antibodies in patients with cancer and pre-existing autoimmune or inflammatory disease. Eur J Cancer **91:**21–29.
82. Akturk HK, Alkanani A, Zhao Z, Yu L, Michels AW 2018 PD-1 Inhibitor immune-related adverse events in patients with preexisting endocrine autoimmunity. J Clin Endocrinol Metab **103:**3589–3592.
83. Yano S, Ashida K, Nagata H, Ohe K, Wada N, Takeichi Y, Hanada Y, Ibayashi Y, Wang L, Sakamoto S, Sakamoto R, Uchi H, Shiratsuchi M, Furue M, Nomura M, Ogawa Y 2018 Nivolumab-induced thyroid dysfunction lacking antithyroid antibody is frequently evoked in Japanese patients with malignant melanoma. BMC Endocr Disord **18:**36.
84. Maekura T, Naito M, Tahara M, Ikegami N, Kimura Y, Sonobe S, Kobayashi T, Tsuji T, Minomo S, Tamiya A, Atagi S 2017 Predictive factors of nivolumab-induced hypothyroidism in patients with non-small cell lung cancer. In Vivo **31:**1035–1039.
85. Kimbara S, Fujiwara Y, Iwama S, Ohashi K, Kuchiba A, Arima H, Yamazaki N, Kitano S, Yamamoto N, Ohe Y 2018 Association of antithyroglobulin antibodies with the development of thyroid dysfunction induced by nivolumab. Cancer Sci **109:**3583–3590.
86. Kobayashi T, Iwama S, Yasuda Y, Okada N, Tsunekawa T, Onoue T, Takagi H, Hagiwara D, Ito Y, Morishita Y, Goto M, Suga H, Banno R, Yokota K, Hase T, Morise M, Hashimoto N, Ando M, Kiyoi H, Gotoh M, Ando Y, Akiyama M, Hasegawa Y, Arima H 2018 Patients with antithyroid antibodies are prone to develop destructive thyroiditis by nivolumab: a prospective study. J Endocr Soc **2:**241–251.
87. Jaafar J, Fernandez E, Alwan H, Philippe J 2018 Programmed cell death-1 and programmed cell death ligand-1 antibodies-induced dysthyroidism. Endocr Connect **7:** R196–R211.
88. Iadarola C, Croce L, Quaquarini E, Teragni C, Pinto S, Bernardo A, Fonte R, Marino M, Rotondi M, Chiovato L 2019 Nivolumab induced thyroid dysfunction: unusual clinical presentation and challenging diagnosis. Front Endocrinol **9:**813.

89. Campredon P, Imbert P, Mouly C, Grunenwald S, Mazieres J, Caron P 2018 Severe inflammatory ophthalmopathy in a euthyroid patient during nivolumab treatment. Eur Thyroid J **7:**84–87.
90. Freeman-Keller M, Kim Y, Cronin H, Richards A, Gibney G, Weber JS 2016 Nivolumab in resected and unresectable metastatic melanoma: characteristics of immune-related adverse events and association with outcomes. Clin Cancer Res **22:**886–894.
91. Haratani K, Hayashi H, Chiba Y, Kudo K, Yonesaka K, Kato R, Kaneda H, Hasegawa Y, Tanaka K, Takeda M, Nakagawa K 2018 Association of immune-related adverse events with nivolumab efficacy in non-small-cell lung cancer. JAMA Oncol **4:**374–378.
92. Nakamura Y, Tanaka R, Asami Y, Teramoto Y, Imamura T, Sato S, Maruyama H, Fujisawa Y, Matsuya T, Fujimoto M, Yamamoto A 2017 Correlation between vitiligo occurrence and clinical benefit in advanced melanoma patients treated with nivolumab: a multi-institutional retrospective study. J Dermatol **44:**117–122.
93. Sanlorenzo M, Vujic I, Daud A, Algazi A, Gubens M, Luna SA, Lin K, Quaglino P, Rappersberger K, Ortiz-Urda S 2015 Pembrolizumab cutaneous adverse events and their association with disease progression. JAMA Dermatol **151:** 1206–1212.
94. Kim HI, Kim M, Lee SH, Park SY, Kim YN, Kim H, Jeon MJ, Kim TY, Kim SW, Kim WB, Kim SW, Lee DH, Park K, Ahn MJ, Chung JH, Shong YK, Kim WG, Kim TH 2017 Development of thyroid dysfunction is associated with clinical response to PD-1 blockade treatment in patients with advanced non-small cell lung cancer. OncoImmunology **19:**e1375642.
95. Owen DH, Wei L, Bertino EM, Edd T, Villalona-Calero MA, He K, Shields PG, Carbone DP, Otterson GA 2018 Incidence, risk factors, and effect on survival of immune-related adverse events in patients with non-small-cell lung cancer. Clini Lung Cancer **19:**e893–e900.
96. Ricciuti B, Genova C, De Giglio A, Bassanelli M, Dal Bello MG, Metro G, Brambilla M, Baglivo S, Grossi F, Chiari R 2019 Impact of immune-related adverse events on survival in patients with advanced non-small cell lung cancer treated with nivolumab: long-term outcomes from a multi-institutional analysis. J Cancer Res Clin Oncol **145:** 479–485.
97. Kotwal A, Kottschade L, Ryder M 2019 PD-L1 inhibitor-induced thyroiditis is associated with better overall survival in cancer patients. Thyroid **30:**177–184.

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2. Tomoyuki Naito, Hideaki Shiraishi, Yutaka Fujiwara. 2021. Durvalumab for the treatment of PD-L1 non-small cell lung cancer. *Expert Review of Precision Medicine and Drug Development* **6**:2, 95-105. [Crossref]
3. David Tak Wai Lui, Chi Ho Lee, Vikki Tang, Carol Ho Yi Fong, Alan Chun Hong Lee, Joanne Wing Yan Chiu, Roland Ching Yu Leung, Gerry Gin Wai Kwok, Bryan Cho Wing Li, Tan To Cheung, Yu Cho Woo, Karen Siu Ling Lam, Thomas Yau. 2021. Thyroid Immune-Related Adverse Events in Patients with Cancer Treated with anti-PD1/anti-CTLA4 Immune Checkpoint Inhibitor Combination: Clinical Course and Outcomes. *Endocrine Practice* **30**. . [Crossref]
4. Jochen H. Lorch. 2020. Development of Overt Thyroid Dysfunction and Antithyroid Antibodies with Anti-PD-1 Use in Various Cancers Is Associated with Favorable Survival. *Clinical Thyroidology* **32**:5, 221-224. [Citation] [Full Text] [PDF] [PDF Plus]

Appendix 2

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REVIEW
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Immune Related Adverse Events of the Thyroid – A Narrative Review

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Immune checkpoints are small molecules present on the cell surface of T-lymphocytes. They maintain self-tolerance and regulate the amplitude and duration of T-cell responses. Antagonism of immune checkpoints with monoclonal antibodies (immune checkpoint inhibitors) is a rapidly evolving field of anti-cancer immunotherapy and has become standard of care in management of many cancer subtypes. Immune checkpoint inhibition is an effective cancer treatment but can precipitate immune related adverse events (irAEs). Thyroid dysfunction is the most common endocrine irAE and can occur in up to 40% of treated patients. Both thyrotoxicosis and hypothyroidism occur. The clinical presentation and demographic associations of thyrotoxicosis compared to hypothyroidism suggest unique entities with different etiologies. Thyroid irAEs, particularly overt thyrotoxicosis, are associated with increased immune toxicity in other organ systems, but also with longer progression-free and overall survival. Polygenic risk scores using susceptibility loci associated with autoimmune thyroiditis predict development of checkpoint inhibitor associated irAEs, suggesting potentially shared mechanisms underpinning their development. Our review will provide an up-to-date summary of knowledge in the field of thyroid irAEs. Major focus will be directed toward pathogenesis (including genetic factors shared with autoimmune thyroid disease), demographic associations, clinical presentation and course, treatment, and the relationship with cancer outcomes.

Keywords: immune related adverse events, checkpoint inhibitor, thyrotoxicosis, hypothyroidism, thyroid

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INTRODUCTION

Immune checkpoint inhibitors (ICIs) are a rapidly expanding class of medications. At present, over 2000 clinical trials have been completed or are in progress to evaluate more than 30 different ICIs (1). The main immune checkpoint targets are cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed cell death protein-1 (PD-1) or its ligand programmed cell death ligand-1 (PD-L1). The introduction of ICIs into cancer treatment algorithms has been transformative and durable responses are now possible in patients with previously limited therapeutic options. However, immune activation following ICI-treatment is associated with off target effects known as immune

related adverse events (irAEs) (2). Any organ system may be affected and thyroid dysfunction is one of the most common ICI-related toxicities (3).

IMMUNE CHECKPOINT PHYSIOLOGY

Immune checkpoints are small molecules present on the cell surface of T-lymphocytes (4). Normally, T-cell activation induces immune checkpoint expression to limit the amplitude and duration of immune responses (4). Immune checkpoints are pivotal to regulate the immune response and prevent inappropriate immune activation, such as occurs in autoimmune disease (4). However, in some situations, immune checkpoints can be counterproductive, such as malignant tumors which activate immune checkpoints to evade immune mediated tumor lysis (3). Monoclonal antibodies antagonizing CTLA-4, PD-1, and other immune checkpoints have been developed to counteract such tumor evasion strategies and stimulate anti-tumor immune responses (4). These agents convey significant therapeutic benefit but are associated with wide ranging irAEs (2–4).

IMMUNE RELATED ADVERSE EVENTS

The etiology of irAEs is yet to be fully elucidated. Potential mechanisms include activation of polyclonal T-cell populations, loss of regulatory T-cell function, and expansion/activation of self-reactive, antigen-specific T-cells (5). Irrespective of mechanism, ICI-associated irAEs are common, affecting up to 76% of treated patients (3). Any organ system can be affected, although there are distinct pharmacological class-specific differences between ICIs targeting CTLA-4 and those targeting PD-1 (3). Endocrine organs are commonly affected, with thyroid dysfunction, hypophysitis, insulin deficient diabetes, primary adrenal insufficiency, and hypoparathyroidism all reported following ICI-treatment (2, 6). In this review we will highlight the key features of thyroid irAEs and how our understanding of thyroid irAEs has informed our understanding of autoimmune thyroid disease more generally.

THYROID IMMUNE RELATED ADVERSE EVENTS

Epidemiology

Thyroid irAEs are the most common endocrine toxicity related to ICI-treatment (6). From clinical trial data, over 10% of patients treated with ICIs develop a thyroid irAE (7). Higher rates are observed following treatment with PD-1 inhibitors relative to CTLA-4 inhibitors, with the highest rates following combined anti-PD-1 and anti-CTLA-4 treatment (7). Observational studies typically report higher rates of thyroid irAEs, usually due to inclusion of subclinical thyroid dysfunction that may be overlooked in clinical trials adverse events reporting (8–11). In the two largest observational studies of thyroid irAEs to date, 42-53% experienced an ICI-associated thyroid irAE (**Table 1**) (11, 12). Thyroid dysfunction was most common following combined anti-PD-1 and anti-CTLA-4 treatment (56%) and less frequent following anti-PD-1 (38%) and anti-CTLA-4 (25%) monotherapy (11).

Clinical Manifestations

Thyroid irAEs are typically identified incidentally during routine monitoring of thyroid function as part of ICI-treatment protocols (7). Most thyroid irAEs present as a painless thyroiditis with transient thyrotoxicosis (6, 7, 11, 13). In patients with more severe (ie. biochemically overt) thyrotoxicosis, a hypothyroid phase often follows the initial thyrotoxicosis and over 40% of these patients will develop permanent hypothyroidism requiring thyroid hormone replacement (11). A smaller number of patients can present with primary hypothyroidism without a preceding thyrotoxic phase.

Onset of thyrotoxicosis usually occurs within 3 months of first ICI-exposure (6, 7). Overt and subclinical thyrotoxicosis have phenotypic differences in their presentation and disease associations. Onset of overt thyrotoxicosis typically occurs earlier and persists longer than subclinical cases (11). Overt thyrotoxicosis has also been associated with higher baseline levels of anti-thyroid antibodies which increase during ICI-inhibitor treatment (14). Treatment related changes in antibody titer have not been observed in other subtypes of thyroid irAE (14). Patients who develop overt thyrotoxicosis are more likely to develop severe irAEs and to experience two or more irAEs in extra-thyroidal organ systems (11).

Isolated hypothyroidism without a preceding thyrotoxic phase can occur. Most cases of isolated hypothyroidism are biochemically subclinical and may be related to non-ICI factors such as non-thyroidal illness syndrome. Subclinical hypothyroidism without a preceding thyrotoxic phase typically normalizes spontaneously without need for thyroid hormone replacement (11, 15). Overt hypothyroidism can have a late-onset months to years after starting ICI-treatment (7). In contrast with subclinical hypothyroidism, overt hypothyroidism is usually permanent and lifelong treatment with thyroxine is required in most cases (6, 7, 11).

Risk Factors and Disease Associations

Identification of reliable thyroid irAE risk factors has been elusive. To date, no reliable risk factors of thyroid irAEs have been identified, although clinical and biochemical associations have been documented in retrospective cohort studies (7). Female sex was associated with thyroid irAEs in the two largest studies to date (**Table 1**), although to a lesser degree than the roughly 8:1 female preponderance observed in autoimmune thyroid disease developing outside of the ICI-treatment setting (11, 12). Prevalence of baseline TPOAb and TgAb positivity is higher in patients who develop a thyroid irAE compared to those who do not (11, 16). Anti-thyroid antibody positivity is particularly associated with overt thyroid dysfunction (11, 16) and titers of TPOAb and TgAb can increase significantly during ICI-treatment in patients that

TABLE 1 | Summary of findings from the two largest studies of thyroid irAEs.

Study	Muir et al. (11)	Von Itzstein et al. (12)
Number of patients	1246 All patients had normal TSH prior to ICI-treatment and were free from pre-existing thyroid disease	1781 Many included patients had pre-existing thyroid disease prior to ICI-treatment (381 patients had abnormal TSH at baseline and 166 were receiving thyroxine; an additional 202 patients with normal TSH at baseline were also receiving thyroxine prior to ICI-treatment)
ICI-therapy; n (%)		
CTLA-4	165 (13)	56 (3)
PD-1/PD-L1	705 (57)	1445 (81)
CTLA-4 + PD-1	376 (30)	280 (16)
Cancer type; n (%)		
Melanoma	1246 (100)	238 (13)
Lung	-	512 (29)
Kidney	-	338 (19)
Other	-	599 (34)
Patients developing a thyroid irAE; n (%)	518 (42)	863 (53)
Thyroid irAE subtype; n (%)	Subclinical thyrotoxicosis n=234 (19); overt thyrotoxicosis n=154 (12); subclinical hypothyroidism without preceding thyrotoxicosis n=61 (5); overt hypothyroidism without preceding thyrotoxicosis n=39 (3)	TSH became elevated post ICI-treatment n=492 (30); TSH became low post ICI-treatment n=204 (13); TSH became both elevated and low post ICI-treatment n=167 (10)
Patients progressing to hypothyroidism following an initial thyrotoxic phase; n (%)	20/234 (9%) of patients with subclinical thyrotoxicosis; 91/154 (59%) of patients with overt thyrotoxicosis	Not reported
Patients developing permanent hypothyroidism requiring initiation of thyroid hormone replacement; n (%)	n=7 (3) patients with subclinical thyrotoxicosis; n=66 (43) patients with overt thyrotoxicosis; n=0 (0) patients with subclinical hypothyroidism; n=29 (74) patients with overt hypothyroidism	n=267 (15) new patients required thyroxine in addition to thyroxin continuation for the n=368 (21) patients already receiving thyroxine at baseline
Thyroid irAE kinetics; median (IQR)	***Time to onset:*** subclinical thyrotoxicosis – 8 wks (4-14); overt thyrotoxicosis – 5 wks (2-8); subclinical hypothyroidism – 10 wks (3-27); overt hypothyroidism – 14 wks (8-25) ***Time to restoration of euthyroidism:*** subclinical thyrotoxicosis – 4 wks (1-8); overt thyrotoxicosis – 12 wks (7-24); subclinical hypothyroidism – 3 wks (1-8); overt hypothyroidism – 10 wks (1-24)	Not reported
Patients with positive anti-TPO and anti-Tg antibodies	TPOAb was positive at baseline in 27/163 (17%) patients; 27/27 patients with TPOAb developed a thyroid irAE TgAb was positive at baseline in 42/163 (26%) patients; 41/42 patients with TgAb developed a thyroid irAE 22 of the above patients were positive for both TPOAb and TgAb	Not reported
Patient and disease characteristics associated with development of thyroid irAEs	Female sex, younger age, absence of brain metastases, combined CTLA-4 + PD-1 ICI-treatment ***Overt thyrotoxicosis*** *was uniquely associated with the development of extra-thyroidal, multi-system, and severe irAEs which were not associated with other thyroid irAE subtypes*	Female sex, Caucasian ethnicity, primary kidney malignancy*, combined CTLA-4 + PD-1 ICI-treatment
Effect of thyroid irAE on cancer survival	Presence of any thyroid irAE (all subtypes) was not associated with a benefit in OS or PFS However, the thyroid irAE subtype of ***overt thyrotoxicosis*** was associated with significant improvement in OS (HR 0.57, 95% CI 0.39-0.84) and PFS (HR 0.68, 95% CI 0.49-0.94) Improvements in OS and PFS were not present with other subtypes of thyroid irAE when tested individually	Survival was improved in patients with a thyroid irAE relative to those without (median OS 43 mths vs 26 mths); The highest OS was in patients with a normal TSH at baseline and an abnormal TSH after ICI-treatment (41 mths); The lowest OS was in patients with an abnormal TSH at baseline and a normal TSH during ICI-treatment (12 mths) In multivariate analyses, abnormal TSH prior to ICI-treatment was associated with worse survival (HR 1.62, 95% CI 1.30-2.02); initiation of thyroxine after ICI-treatment was associated with improved survival (HR 0.62, 95% CI 0.44-0.88)

irAE, immune related adverse event; TSH, thyroid stimulating hormone; ICI, immune checkpoint inhibitor; CTLA-4, cytotoxic T-lymphocyte antigen-4; PD-1, programmed cell death protein-1; PD-L1, programmed cell death ligand-1; IQR, interquartile range; TPOAb, thyroid peroxidase antibody; TgAb, thyroglobulin antibody; OS, overall survival; PFS, progression free survival.

develop overt thyrotoxicosis, which is not observed in patients with other subtypes of thyroid irAE (14). A complete list of demographic and biochemical factors that have been associated with development of thyroid irAEs are summarized in **Table 2**.

Pathophysiology

The etiology and biological drivers of irAEs remain elusive. It is possible that patients who experience irAEs may share a premorbid immunological state prior to ICI-initiation. A recent study using mass cytometry time of flight analysis identified higher levels of activated CD4+ memory T-cells and increased diversity in the T-cell receptor as pretreatment predictors of severe irAEs irrespective of the organ involved (20). These same factors also occurred with higher frequency in non-ICI treated patients with autoimmune disease relative to healthy controls, suggesting that irAEs may result from unmasking a latent or subclinical autoimmune process which predates ICI-initiation (20).

Traditionally, thyroid irAEs were thought to be a single entity spanning a spectrum of disease including isolated mild (asymptomatic or minimally symptomatic) thyrotoxicosis to biphasic thyroiditis to isolated overt and severe hypothyroidism. This indeed may be the case, although more likely thyroid irAEs arise *via* multiple mechanisms as evidenced by phenotypic differences in presentation, antithyroid antibody positivity rates, and differential association with cancer survival outcomes (11).

Genetic factors associated with lifetime risk of autoimmune thyroid disease have been implicated in the risk of developing thyroid irAEs during ICI-treatment (21, 22). Key loci have been implicated from genes regulating the immune response such as CD69 (T-cell activation), LPP (B-cell maturation), CTLA-4 (T-cell priming) and PTPN22 (T-cell and B-cell receptor signaling). When these genes and others are combined into a polygenic risk score they can identify subgroups of patients at >6-fold increased risk of thyroid irAEs and identify patients at a lower risk of cancer death (21).

The PD-1/PD-L1 axis is significantly implicated in autoimmune thyroid disease (23). Patients with autoimmune thyroiditis and Graves' disease experience a moderate increase in circulating PD-1 positive T-cells and marked increase in intrathyroidal PD-1 positive T-cells (23). Thyroid follicular cells also show high levels of PD-L1 expression, whereas multinodular goiter (non-autoimmune thyroid disease) has negligible basal expression of PD-L1 (23). The presence of high PD-L1 expression in autoimmune thyroid disease may explain why most cases are slowly progressive, as the immune stimulating effects of intrathyroidal PD-1 positive T-cells are kept in check. PD-1 positive lymphocytes and PD-L1 expression are similarly increased following ICI-treatment. However, different to autoimmune thyroid disease, blockade of the PD-1/PD-L1 axis blunts the protection against progressive immune mediated destruction *via* and therefore thyroid dysfunction is more rapidly progressive following ICI-treatment than in other autoimmune thyroid conditions (23, 24).

TABLE 2 | Demographic and biochemical factors associated with development of thyroid irAEs.

Female sex (11, 12)
Younger age (11)
Caucasian ethnicity (12)
Combined CTLA-4 + PD-1 treatment (11, 12)
Longer duration of anti-PD-1 treatment (16)
Higher body mass index (17)
Renal cell carcinoma* (12)
Baseline TSH (8, 16–18)
Baseline TPOAb positivity; treatment related increase in TPOAb titer (14, 16, 19)
Baseline TgAb positivity; treatment related increase in TPOAb titer (14, 16, 18, 19)

*CTLA-4, cytotoxic T-lymphocyte antigen-4; PD-1, programmed cell death protein-1; TSH, thyroid stimulating hormone; TPOAb, thyroid peroxidase antibody; TgAb, thyroglobulin antibody; *Likely confounded by concomitant use of tyrosine kinase inhibitors in combination with ICIs.*

Management

Screening

Thyroid function should be measured at baseline and repeated at 6-weekly intervals following commencement of ICI-treatment in asymptomatic patients. Additional measures of TSH and FT4 can be undertaken for case detection in patients that develop signs or symptoms of thyroid dysfunction outside this window (25). Most cases of clinically significant thyroid dysfunction will occur within 6-months of ICI-commencement (7). Therefore, monitoring frequency can be relaxed after the 6-month mark although continued periodic monitoring (3-6 monthly) to detect late onset cases is recommended.

Diagnosis

As most cases of thyroid irAE are asymptomatic, diagnosis is most commonly *via* thyroid function monitoring in asymptomatic patients (7). Thyrotoxicosis is diagnosed in the setting of a low TSH with a normal or elevated FT4. Thyrotoxicosis often occurs as the initial phase of a biphasic thyroiditis and is followed by progression to hypothyroidism (elevated TSH, low FT4). More rarely, isolated hypothyroidism without a preceding thyrotoxic phase can occur. Unlike thyrotoxicosis, isolated hypothyroidism may present months to years after initiation of ICI-treatment. Special attention should be paid to the finding of an inappropriately low TSH in combination with a low or low-normal FT4. This pattern of results should alert the clinician to the possibility of hypophysitis, particularly in the setting of a CTLA-4 inhibitor. When hypophysitis (central hypothyroidism) is suspected, the remaining pituitary hormone axes should be interrogated urgently to exclude cortisol and other hormone deficiencies prior to initiation of thyroid hormone replacement.

Treatment

For asymptomatic or minimally symptomatic thyrotoxicosis, treatment is usually not required. Temporary use of beta blockers such as propranolol may be considered in symptomatic patients. ICI-treatment can be continued, and most cases will resolve spontaneously within days to weeks. Thyrotoxicosis lasting longer than 6-weeks or with additional features (goiter, thyroid bruit, exophthalmos) should have TSH-receptor antibody level (TRAB) measured to exclude Graves'

disease (25). In severe or atypical presentations, imaging with a thyroid uptake scan can also be performed to differentiate between ICI-mediated thyroiditis and other etiologies of thyrotoxicosis. Thyrotoxicosis with marked symptoms or life-threatening complications is rare and necessitates suspension of ICI-treatment to allow for prompt investigation and specialist led treatment (25). Following identification of thyrotoxicosis, TSH and FT4 should be monitored every 2-3 weeks until recovery of normal thyroid function, as many patients will experience a biphasic illness which progresses to hypothyroidism. Regular monitoring allows early detection of hypothyroidism, which does not recover in the majority of cases (11). In the setting of overt hypothyroidism, treatment with thyroxine can be initiated, particularly when the TSH is >10 mIU/L, as recovery is highly unlikely in this setting (11). Alternatively, TSH and FT4 can be repeated after 4-6 weeks and if hypothyroidism persists, treatment can be initiated at that point (25). Thyroxine doses are typically higher for patients with ICI-mediated hypothyroidism than for Hashimoto's thyroiditis, suggesting that gland destruction is complete in most cases of ICI-mediated thyroiditis whereas it is slowly progressive with some residual production of endogenous thyroid hormone in patients with Hashimoto's thyroiditis (26). A simplified algorithm for the classification and management of thyroid irAEs is presented in **Figure 1**.

Special Situations

Pre-existing Hypothyroidism

Patients with established hypothyroidism may experience transient effects on thyroid function following ICI-treatment (7). Compensated hypothyroidism can be exacerbated requiring initiation or increased doses of thyroxine. More rarely, patients with hypothyroidism may paradoxically experience a transient period of thyrotoxicosis requiring temporary suspension of thyroid hormone (27).

Graves' Disease and Thyroid Eye Disease

Graves' disease (GD) has been reported following ICI-treatment (6). Whether it occurs *de novo* related to ICI-treatment, is unmasked by ICI-treatment, or is a coincidental occurrence remains unknown. Similarly, thyroid eye disease (TED) has been reported (28). Reactivation or progression of GD and TED is possible following initiation of ICI-treatment and in patients with known GD or TED, close consultation with an experienced endocrinologist and ophthalmologist is important for early identification and treatment.

Prognostic Implications

Observational studies repeatedly document an association between irAEs and improvement in cancer outcomes (29, 30). Owing to the frequency of thyroid irAEs, they are among the most well studied and strongly associated with improvement in progression free survival (PFS) and overall survival (OS) (31–33). Reported benefits are large with robust hazard ratios that are maintained following correction for immortal time bias (31, 34). However, emerging data suggest thyroid irAE subtypes are not equivalent in their association with cancer outcomes. When thyroid irAEs are classified by severity, biochemically overt thyroid dysfunction is more strongly associated with improved PFS and OS than subclinical thyroid dysfunction (11, 35, 36). Our previous work suggests this may be limited to overt thyrotoxicosis, as no survival benefit was observed in patients with overt hypothyroidism without a preceding thyrotoxic phase (11). Permanent thyroid dysfunction requiring initiation of thyroid hormone replacement has also been associated with improved cancer outcomes (12, 37). However, an ultimate requirement for thyroxine may have been a surrogate marker

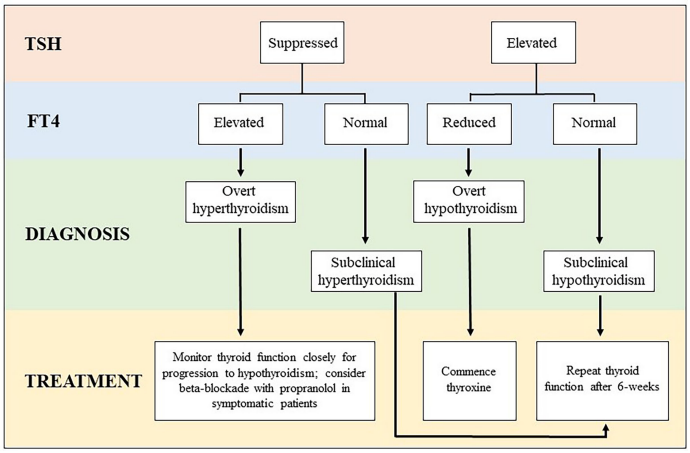

FIGURE 1 | Algorithm for classification and management of thyroid irAE subtypes. *When overt thyrotoxicosis is prolonged or severe, additional investigation with TSH receptor antibody (TRAB) and a thyroid uptake scan should be considered to exclude other etiologies of thyrotoxicosis (ie. Graves' disease, toxic adenoma, etc).

for a preceding phase of overt thyrotoxicosis rather than a beneficial prognostic factor in of itself. The significance of antithyroid antibodies on cancer outcomes following ICI-treatment is unknown and current data has conflicting results (35, 38). Given the high prevalence of anti-thyroid antibodies in patients who develop overt thyrotoxicosis, an association is likely and prospective study into potential utility as a predictive biomarker is warranted (14).

Interestingly, abnormal TSH level (elevated or low) prior to initiation of ICI-treatment has been associated with inferior cancer outcomes irrespective of thyroid function changes post-ICI initiation in one study (12). However, these results may have been confounded as significantly more patients with abnormal baseline TSH were receiving tyrosine kinase inhibitor treatment for metastatic renal cell cancer, which is known to significantly affect thyroid function (12). Abnormal TSH prior to ICI-treatment may also be a function of sicker patients displaying the non-thyroidal illness syndrome. Therefore, at present it is not known whether baseline thyroid function is truly associated with worse outcomes or if it simply reflects patients with a more aggressive cancer or a physiologic response to chronic illness in a less well patient at commencement of ICI-treatment.

FUTURE DIRECTIONS

Further research is required to better inform our understanding of thyroid irAEs and their relationship to thyroid autoimmune conditions occurring outside the cancer immunotherapy setting. Enhanced characterization of the immune phenotype and T-cell subsets driving thyroid irAEs may allow for identification of patients at risk of thyroid (and other) irAEs and inform future research to uncouple the efficacy of ICIs from the risk of developing treatment related irAEs. Understanding the effects of immune checkpoint inhibition on the thyroid gland could also inform use of these agents for treatment of primary thyroid malignancies and prevention of autoimmune thyroid disease.

CONCLUSIONS

Thyroid irAEs are a frequent complication of anti-PD-1 based ICI-treatment. Recent work has begun to unravel the molecular mechanisms underpinning development and their relationship to other autoimmune thyroid conditions. Regular monitoring of thyroid function during ICI-treatment is required, as although most cases of thyroid irAEs are asymptomatic and manageable, severe presentations with thyroid storm or myxedema can occur. Development of thyroid irAEs is associated with improved PFS and OS, especially overt thyrotoxicosis which may be a surrogate marker of patients experiencing a more robust immune response to ICI-treatment. When hypothyroidism occurs, it is usually permanent and treatment with thyroid hormone replacement will be required. Familiarity with thyroid irAEs among health professionals is important to facilitate efficient diagnosis and appropriate treatment of this increasingly common thyroid disease.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work. All authors approved it for publication.

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REFERENCES

1. Beaver JA, Pazdur R. The Wild West of Checkpoint Inhibitor Development. *N Engl J Med* (2021) 386:1297–1301. doi: 10.1056/NEJMp2116863
2. Postow MA, Sidlow R, Hellmann MD. Immune-Related Adverse Events Associated With Immune Checkpoint Blockade. *New Engl J Med* (2018) 378(2):158–68. doi: 10.1056/NEJMra1703481
3. Ramos-Casals M, Brahmer JR, Callahan MK, Flores-Chavez A, Keegan N, Khamashta MA, et al. Immune-Related Adverse Events of Checkpoint Inhibitors. *Nat Rev Dis Primers* (2020) 6(1):1–21. doi: 10.1038/s41572-020-0160-6
4. Pardoll DM. The Blockade of Immune Checkpoints in Cancer Immunotherapy. *Nat Rev Cancer* (2012) 12(4):252–64. doi: 10.1038/nrc3239
5. Korman AJ, Garrett-Thomson SC, Lonberg N. The Foundations of Immune Checkpoint Blockade and the Ipilimumab Approval Decennial. *Nat Rev Drug Discov* (2021), 1–20. doi: 10.1038/s41573-021-00345-8
6. Chang L-S, Barroso-Sousa R, Tolaney SM, Hodi FS, Kaiser UB, Min L. Endocrine Toxicity of Cancer Immunotherapy Targeting Immune Checkpoints. *Endocr Rev* (2019) 40(1):17–65. doi: 10.1210/er.2018-00006
7. Muir CA, Menzies AM, Clifton-Bligh R, Tsang VH. Thyroid Toxicity Following Immune Checkpoint Inhibitor Treatment in Advanced Cancer. *Thyroid* (2020) 30(10):1458–69. doi: 10.1089/thy.2020.0032
8. Morganstein DL, Lai Z, Spain L, Diem S, Levine D, Mace C, et al. Thyroid Abnormalities Following the Use of Cytotoxic T-Lymphocyte Antigen-4 and Programmed Death Receptor Protein-1 Inhibitors in the Treatment of Melanoma. *Clin Endocrinol* (2017) 86(4):614–20. doi: 10.1111/cen.13297
9. Scott ES, Long GV, Guminski A, Clifton-Bligh RJ, Menzies AM, Tsang VH. The Spectrum, Incidence, Kinetics and Management of Endocrinopathies With Immune Checkpoint Inhibitors for Metastatic Melanoma. *Eur J Endocrinol* (2018) 178(2):173–80. doi: 10.1530/EJE-17-0810
10. Pollack RM, Kagan M, Lotem M, Dresner-Pollak R. Baseline Tsh Level Is Associated With Risk of Anti-Pd-1-Induced Thyroid Dysfunction. *Endocr Pract* (2019) 25(8):824–9. doi: 10.4158/EP-2018-0472
11. Muir CA, Clifton-Bligh RJ, Long GV, Scolyer RA, Lo S, Carlino MS, et al. Thyroid Immune-Related Adverse Events Following Immune Checkpoint Inhibitor Treatment. *J Clin Endocrinol Metab* (2021) 106(9):e3704–e13. doi: 10.1210/clinem/dgab263
12. von Itzstein MS, Gonugunta AS, Wang Y, Sheffield T, Lu R, Ali S, et al. Divergent Prognostic Effects of Pre-Existing and Treatment-Emergent Thyroid Dysfunction in Patients Treated With Immune Checkpoint Inhibitors. *Cancer Immunol Immunother* (2022), 1–13. doi: 10.1007/s00262-022-03151-2

13. Iyer PC, Cabanillas ME, Waguespack SG, Hu MI, Thosani S, Lavis VR, et al. Immune-Related Thyroiditis With Immune Checkpoint Inhibitors. *Thyroid* (2018) 28(10):1243–51. doi: 10.1089/thy.2018.0116
14. Muir CA, Wood CCG, Clifton-Bligh RJ, Long GV, Scolyer RA, Carlino MS, et al. Association of Anti-Thyroid Antibodies in Checkpoint Inhibitor Associated Thyroid Immune Related Adverse Events. *J Clin Endocrinol Metab* (2022) 107(5):e1843–9. doi: 10.1210/clinem/dgac059
15. Peiro I, Palmero R, Iglesias P, Diez JJ, Simo-Servat A, Marin JA, et al. Thyroid Dysfunction Induced by Nivolumab: Searching for Disease Patterns and Outcomes. *Endocrine* (2019) 64(3):605–13. doi: 10.1007/s12020-019-01871-7
16. Yoon JH, Hong AR, Kim HK, Kang H-C. Characteristics of Immune-Related Thyroid Adverse Events in Patients Treated With PD-1/PD-L1 Inhibitors. *Endocrinol Metab* (2021) 36(2):413. doi: 10.3803/EnM.2020.906
17. Pollack R, Ashash A, Cahn A, Rottenberg Y, Stern H, Dresner-Pollak R. Immune Checkpoint Inhibitor-Induced Thyroid Dysfunction Is Associated With Higher Body Mass Index. *J Clin Endocrinol Metab* (2020) 105(10): e3620–7. doi: 10.1210/clinem/dgaa458
18. Kimbara S, Fujiwara Y, Iwama S, Ohashi K, Kuchiba A, Arima H, et al. Association of Antithyroglobulin Antibodies With the Development of Thyroid Dysfunction Induced by Nivolumab. *Cancer Sci* (2018) 109 (11):3583–90. doi: 10.1111/cas.13800
19. Kurimoto C, Inaba H, Ariyasu H, Iwakura H, Ueda Y, Uraki S, et al. Predictive and Sensitive Biomarkers for Thyroid Dysfunctions During Treatment With Immune-Checkpoint Inhibitors. *Cancer Sci* (2020) 111(5):1468. doi: 10.1111/cas.14363
20. Lozano AX, Chaudhuri AA, Nene A, Bacchiocchi A, Earland N, Vesely MD, et al. T Cell Characteristics Associated With Toxicity to Immune Checkpoint Blockade in Patients With Melanoma. *Nat Med* (2022) 28:1–10. doi: 10.1038/s41591-021-01623-z
21. Khan Z, Hammer C, Carroll J, Di Nucci F, Acosta SL, Maiya V, et al. Genetic Variation Associated With Thyroid Autoimmunity Shapes the Systemic Immune Response to PD-1 Checkpoint Blockade. *Nat Commun* (2021) 12 (1):1–12. doi: 10.1038/s41467-021-23661-4
22. Luo J, Martucci VL, Quandt Z, Groha S, Murray MH, Lovely CM, et al. Immunotherapy-Mediated Thyroid Dysfunction: Genetic Risk and Impact on Outcomes With PD-1 Blockade in Non–Small Cell Lung Cancer. *Clin Cancer Res* (2021) 27(18):5131–40. doi: 10.1158/1078-0432.CCR-21-0921
23. lvarez-Sierra A D, Marin-Sanchez A, Ruiz-Blazquez P, Gil CJ, Iglesias-Felip C, Gonzalez O, et al. Analysis of the PD-1/PD-L1 Axis in Human Autoimmune Thyroid Disease: Insights Into Pathogenesis and Clues to Immunotherapy Associated Thyroid Autoimmunity. *J Autoimmun* (2019) 103:102285. doi: 10.1016/j.jaut.2019.05.013
24. Kotwal A, Gustafson MP, Bornschlegl S, Kottschade L, Delivanis DA, Dietz AB, et al. Immune Checkpoint Inhibitor-Induced Thyroiditis Is Associated With Increased Intrathyroidal T Lymphocyte Subpopulations. *Thyroid* (2020) 30(10):1440–50. doi: 10.1089/thy.2020.0075
25. Schneider BJ, Naidoo J, Santomasso BD, Lacchetti C, Adkins S, Anadkat M, et al. Management of Immune-Related Adverse Events in Patients Treated With Immune Checkpoint Inhibitor Therapy: ASCO Guideline Update. *J Clin Oncol* (2021) 39(36):4073–126. doi: 10.1200/JCO.21.01440
26. Mosaferi T, Tsai K, Sovich S, Wilhalme H, Kathuria-Prakash N, Praw SS, et al. Optimal Thyroid Hormone Replacement Dose in Immune Checkpoint Inhibitor-Associated Hypothyroidism Is Distinct From Hashimoto's Thyroiditis. *Thyroid* (2022). doi: 10.1089/thy.2021.0685
27. Patel NS, Oury A, Daniels GA, Bazhenova L, Patel SP. Incidence of Thyroid Function Test Abnormalities in Patients Receiving Immune-Checkpoint Inhibitors for Cancer Treatment. *Oncologist* (2018) 23(10):1236–41. doi: 10.1634/theoncologist.2017-0375
28. Min L, Vaidya A, Becker C. Thyroid Autoimmunity and Ophthalmopathy Related to Melanoma Biological Therapy. *Eur J Endocrinol* (2011) 164(2):303–7. doi: 10.1530/EJE-10-0833
29. Haratani K, Hayashi H, Chiba Y, Kudo K, Yonesaka K, Kato R, et al. Association of Immune-Related Adverse Events With Nivolumab Efficacy in Non–Small-Cell Lung Cancer. *JAMA Oncol* (2018) 4(3):374–8. doi: 10.1001/jamaoncol.2017.2925
30. Fan Y, Xie W, Huang H, Wang Y, Li G, Geng Y, et al. Association of Immune Related Adverse Events With Efficacy of Immune Checkpoint Inhibitors and Overall Survival in Cancers: A Systemic Review and Meta-Analysis. *Front Oncol* (2021) 11:1081. doi: 10.3389/fonc.2021.633032
31. Cheung Y-MM, Wang W, McGregor B, Hamnvik O-PR. Associations Between Immune-Related Thyroid Dysfunction and Efficacy of Immune Checkpoint Inhibitors: A Systematic Review and Meta-Analysis. *Cancer Immunol Immunother* (2022) 1–18. doi: 10.1007/s00262-021-03128-7
32. Kotwal A, Ryder M. Survival Benefit of Endocrine Dysfunction Following Immune Checkpoint Inhibitors for Nonthyroidal Cancers. *Curr Opin Endocrinol Diabetes Obes* (2021) 28(5):517–24. doi: 10.1097/MED.0000000000000664
33. Ferreira JL, Costa C, Marques B, Castro S, Victor M, Oliveira J, et al. Improved Survival in Patients With Thyroid Function Test Abnormalities Secondary to Immune-Checkpoint Inhibitors. *Cancer Immunol Immunother* (2021) 70 (2):299–309. doi: 10.1007/s00262-020-02664-y
34. Kotwal A, Kottschade L, Ryder M. PD-L1 Inhibitor-Induced Thyroiditis is Associated With Better Overall Survival in Cancer Patients. *Thyroid* (2020) 30 (2):177–84. doi: 10.1089/thy.2019.0250
35. Basak EA, van derMeer JWM, Hurkmans DP, Schreurs MWJ, Oomen-de Hoop E, van derVeldt AAM, et al. Overt Thyroid Dysfunction and Anti-Thyroid Antibodies Predict Response to Anti-PD-1 Immunotherapy in Cancer Patients. *Thyroid* (2020) 30(7):966–73. doi: 10.1089/thy.2019.0726
36. Yamauchi I, Yasoda A, Matsumoto S, Sakamori Y, Kim YH, Nomura M, et al. Incidence, Features, and Prognosis of Immune-Related Adverse Events Involving the Thyroid Gland Induced by Nivolumab. *PloS One* (2019) 14 (5):e0216954. doi: 10.1371/journal.pone.0216954
37. Inaba H, Ariyasu H, Iwakura H, Kurimoto C, Takeshima K, Morita S, et al. Distinct Clinical Features and Prognosis Between Persistent and Temporary Thyroid Dysfunctions by Immune-Checkpoint Inhibitors. *Endocr J* (2020), EJ20–0371. doi: 10.1507/endocrj.EJ20-0371
38. Toi Y, Sugawara S, Sugisaka J, Ono H, Kawashima Y, Aiba T, et al. Profiling Preexisting Antibodies in Patients Treated With Anti–PD-1 Therapy for Advanced Non–Small Cell Lung Cancer. *JAMA Oncol* (2019) 5(3):376–83. doi: 10.1001/jamaoncol.2018.5860

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix 3

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Clinical Research Article

Clinical Research Article

Thyroid Immune-related Adverse Events Following Immune Checkpoint Inhibitor Treatment

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Abbreviations: CTLA-4, cytotoxic T-lymphocyte antigen-4; FT4, free thyroxine; HR, hazard ratio; ICI, immune checkpoint inhibitor; IQR, interquartile range; irAE, immune-related adverse event; OR, odds ratio; OS, overall survival; PD-1, programmed cell death protein-1; PFS, progression-free survival; TGAb, thyroglobulin antibody; TPOab, thyroid peroxidase antibody; TSH, thyroid-stimulating hormone.

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Abstract

Context: Thyroid dysfunction occurs commonly following immune checkpoint inhibition. The etiology of thyroid immune-related adverse events (irAEs) remains unclear and clinical presentation can be variable.

Objective: This study sought to define thyroid irAEs following immune checkpoint inhibitor (ICI) treatment and describe their clinical and biochemical associations.

Methods: We performed a retrospective cohort study of thyroid dysfunction in patients with melanoma undergoing cytotoxic T-lymphocyte antigen-4 (CTLA-4) and/or programmed cell death protein-1 (PD-1) based ICI treatment from November 1, 2009, to December 31, 2019. Thyroid function was measured at baseline and at regular intervals following the start of ICI treatment. Clinical and biochemical features were evaluated for associations with ICI-associated thyroid irAEs. The prevalence of thyroid autoantibodies and the effect of thyroid irAEs on survival were analyzed.

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Results: A total of 1246 patients were included with a median follow-up of 11.3 months. Five hundred and eighteen (42%) patients developed an ICI-associated thyroid irAE. Subclinical thyrotoxicosis (n = 234) was the most common thyroid irAE, followed by overt thyrotoxicosis (n = 154), subclinical hypothyroidism (n = 61), and overt hypothyroidism (n = 39). Onset of overt thyrotoxicosis occurred a median of 5 weeks (interquartile range [IQR] 2-8) after receipt of a first dose of ICI. Combination immunotherapy was strongly associated with development of overt thyrotoxicosis (odds ratio [OR] 10.8, 95% CI 4.51-25.6 vs CTLA-4 monotherapy; $P < .001$), as was female sex (OR 2.02, 95% CI 1.37-2.95; $P < .001$) and younger age (OR 0.83 per 10 years, 95% CI 0.72-0.95; $P = .007$). By comparison, median onset of overt hypothyroidism was 14 weeks (IQR 8-25). The frequency of overt hypothyroidism did not differ between different ICI types. The strongest associations for hypothyroidism were higher baseline thyroid-stimulating hormone (OR 2.33 per mIU/L, 95% CI 1.61-3.33; $P < .001$) and female sex (OR 3.31, 95% CI 1.67-6.56; $P = .01$). Overt thyrotoxicosis was associated with longer progression free survival (hazard ratio [HR] 0.68, 95% CI 0.49-0.94; $P = .02$) and overall survival (HR 0.57, 95% CI 0.39-0.84; $P = .005$). There was no association between hypothyroidism and cancer outcomes.
Conclusion: Thyroid irAEs are common and there are multiple distinct phenotypes. Different thyroid irAE subtypes have unique clinical and biochemical associations, suggesting potentially distinct etiologies for thyrotoxicosis and hypothyroidism arising in this context.

Key Words: Hyperthyroidism, hypothyroidism, thyroiditis, immune-related adverse event, immune checkpoint inhibitor

Immune checkpoint inhibitors (ICIs) are the standard of care in the management of melanoma and many other cancer types (1). Through antagonism of the cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed cell death protein-1 (PD-1) pathways, ICIs increase T-cell activation and anticancer immune responses (2). Enhancements in antitumor immunity related to ICI treatment have improved patient survival, but are associated with wide-ranging immune-related adverse events (irAEs) (3, 4). Thyroid dysfunction is the most commonly occurring endocrine irAE, affecting more than 10% of exposed patients (5). PD-1 inhibitors are associated with higher rates of thyroid dysfunction relative to CTLA-4 inhibitors, with the highest rates observed in patients undergoing combination treatment with both anti-CTLA-4 and anti-PD-1 ICIs (5).

The etiology of thyroid irAEs has generally been assumed to be secondary to destructive, immune-mediated thyroiditis. However, onset of thyroid dysfunction is highly variable and not all patients develop the classic thyroiditis-like presentation of transient thyrotoxicosis followed by a hypothyroid phase (5). Isolated thyrotoxicosis and hypothyroidism have both been reported, as well as subclinical disease that is often short-lived and transient (5). Some studies have reported thyroid irAEs to be associated with improvements in progression-free survival (PFS) and overall survival (OS) (6-9). Thyroid autoantibody positivity rates vary widely between studies with prevalence rates of 17% to 100% reported (10-14). Low-level thyroid antibody positivity has been associated with improvement in PFS and OS; however, the role of antithyroid antibodies in the pathogenesis and susceptibility to thyroid irAEs remains unknown (5, 15).

Given the heterogeneity and lack of large studies examining thyroid dysfunction following ICI treatment, the aim of the present study was to comprehensively characterize thyroid irAEs in a large cohort of patients with advanced or metastatic melanoma.

Materials and Methods

Study Design and Participants

We reviewed outcomes in a cohort of adult patients undergoing ICI treatment for advanced or metastatic melanoma between November 1, 2009, and December 31, 2019. Demographic, treatment, and cancer outcome information was extracted from a prospectively maintained central database. Measurement of serum thyroid-stimulating hormone (TSH) was performed in all subjects prior to commencement of immunotherapy and thyroid function was repeated at subsequent ICI doses every 3 to 4 weeks during the first 6 months of follow-up. In patients with documented thyroid dysfunction or symptoms of thyroid

dysfunction, testing was performed at increased frequency on an individualized basis. After the first 6 months of ICI treatment, the testing interval was extended to every 6 to 8 weeks and then to every 12 weeks after 1 year. Patients with normal thyroid function at baseline and at least 1 further measurement of thyroid function following the first dose of ICI were included. Patients with pre-existing thyroid disease (defined as abnormal thyroid function at baseline or treatment with thyroxine at baseline and patients who developed hypophysitis with central hypothyroidism were excluded.

Definitions

Thyroid irAEs were defined by detection of new thyroid dysfunction developing after treatment with an ICI and were subcategorized by the biochemical pattern of thyroid dysfunction. Overt thyrotoxicosis was defined by a TSH level below the lower reference interval with concomitant elevation of free thyroxine (FT4) above the upper reference interval. Subclinical thyrotoxicosis was defined by a TSH level below the lower reference interval in the presence of normal serum concentrations of FT4. Overt hypothyroidism was defined by a TSH level above the upper reference limit with concomitant FT4 levels below the lower reference interval or by TSH >10.0 mIU/L irrespective of FT4. Subclinical hypothyroidism was defined as normal FT4 level in the presence of TSH elevation above the upper limit of the normal reference range and <10.0 mIU/L. Euthyroid hyperthyroxinemia and hypothyroxinemia were defined as a normal TSH with FT4 concentrations above or below the normal reference interval, respectively.

Time to thyroid dysfunction was measured as the time to first documented thyroid function test abnormality following receipt of the initial dose of ICI. Duration of thyrotoxicosis or hypothyroidism was defined as the time to restoration of normal TSH following the first laboratory documented episode of abnormal thyroid function.

Assay Characteristics

Serum concentration of thyroid hormones and antibodies were made using commercially available kits from Abbott Diagnostics. TSH had a measuring range of 0.01 to 127 mIU/L with precision (coefficient of variation, CV) of 1.9% to 5.3% across a wide range of TSH values. FT4 had a measuring range of 0.0 to 77.2 pmol/L with precision (CV) of 3.6% to 7.8% across a wide range of FT4 values. Full assay characteristics are available online via Abbott Diagnostics package insert instructions. Local reference intervals for TSH and FT4 were set at 0.40 to 4.20 mIU/L and 9.0 to 19.0 pmol/L respectively. Thyroid peroxidase antibody (TPOAb) and thyroglobulin antibody (TgAb) titers were measured using the Abbott Architect platform with values of >5.61 IU/mL and >4.11 IU/mL considered positive, respectively.

Procedures and Outcomes

The primary outcome was to describe the prevalence, clinical phenotypes, and factors associated with thyroid irAEs in a large cohort of melanoma patients undergoing ICI treatment. Biobank serum specimens were retrieved to measure TPOAb and TgAb titers in a subset of patients using samples collected and stored at baseline (prior to receipt of first ICI dose). Additional analyses were performed to assess the association of thyroid irAEs with cancer survival.

Statistical Analysis

Continuous variables were summarized as median and interquartile range (IQR) or frequencies and percentages reported for categorical variables. Baseline characteristics of patients with thyroid irAEs are compared with those of patients without thyroid irAEs in Table 1 using the Mann–Whitney U-test for continuous outcome variables and Pearson's chi-squared test for categorical outcome variables. Associations between demographic factors and thyroid irAEs were assessed using multivariable logistic regression that included all factors with $P < .20$ from a univariate analysis. Results of multivariable logistic regression are presented as odds ratio (OR) and associated 95% CI. Survival differences between patients with thyroid irAEs and patients without thyroid irAEs were compared using Kaplan–Meier log-rank analysis. The effect of thyroid irAEs on OS and PFS was estimated using Cox regression adjusted with age, sex, brain metastases, and ICI type. All statistical tests were 2-tailed with the 2-sided statistically significant level set at $P = .05$. Prespecified subgroup analyses were performed in patients with overt thyrotoxicosis and overt hypothyroidism compared with patients with euthyroidism. Statistical analyses were conducted using Statistical Package for Social Sciences (SPSS) version 26 (SPSS Inc, IL, USA) and figures were created using GraphPad Prism, version 8. All patients consented for their data to be recorded in the Melanoma Institute Australia central database, and for that information to be used for research purposes. Ethics approval was provided by the Sydney Local Health District human research and ethics committee.

Table 1. Baseline characteristics

	All patients	Thyroid irAE	No thyroid irAE	*P* value
Patients; n (%)	1246 (100)	518 (42)	728 (58)	
Age (years); median (IQR)	65 (54-74)	63 (51-71)	67 (56-75)	<.001
Male; n (%)	824 (66)	306 (59)	518 (71)	<.001
Checkpoint inhibitor; n (%)				<.001
CTLA-4	165 (13)	41 (8)	124 (17)	
PD-1/PD-L1	705 (57)	266 (51)	439 (60)	
Combination CTLA-4 + PD-1	376 (30)	211 (41)	165 (23)	
AJCC stage (28); n (%)				.70
IIIa	17 (1)	6 (1)	11 (1)	
IIIb	63 (5)	29 (6)	34 (5)	
IIIc	150 (12)	55 (11)	95 (13)	
IV M1a	83 (7)	35 (7)	48 (7)	
IV M1b	167 (13)	69 (13)	98 (14)	
IV M1c	726 (58)	314 (61)	412 (57)	
ECOG status; n (%)				.14
0	744 (60)	330 (64)	414 (57)	
1	365 (29)	138 (27)	227 (31)	
2	37 (3)	14 (3)	23 (3)	
3	8 (1)	2 (<1)	6 (1)	
Brain metastases; n (%)	258 (21)	131 (25)	127 (17)	.001
Elevated LDH; n (%)	336 (27)	133 (26)	203 (28)	.29
TSH (mIU/L); median (IQR)	1.32 (0.93-1.94)	1.24 (0.81-1.90)	1.38 (0.98-1.99)	.003
FT4 (pmol/L); median (IQR)	13.1 (12.0-14.3)	13.1 (12.0-14.7)	13.0 (12.0-14.1)	.32
TPOAb positive; n (%)	27/163 (17)	27/128 (21)	0/35 (0)	<.001
TgAb positive; n (%)	42/163 (26)	41/128 (32)	1/35 (3)	<.001

Abbreviations: irAE, immune-related adverse event; AJCC, American Joint Committee on Cancer; ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase; TSH, thyroid stimulating hormone; FT4, thyroxine; TPOAb, thyroid peroxidase antibody; TgAb, thyroglobulin antibody; IQR, interquartile range.

Results

Study Population

One thousand two hundred forty-six patients met inclusion criteria. The median age was 65 years (IQR 54-74) and 824 (66%) participants were male, consistent with a typical melanoma population. Immune checkpoint inhibitor treatment consisted of the anti-CTLA-4 antibody ipilimumab (13%), anti-PD-1 antibodies nivolumab (19%) or pembrolizumab (36%), combination ipilimumab and nivolumab (23%), combination ipilimumab and pembrolizumab (7%), or the anti-PD-L1 antibodies atezolizumab/avelumab (2%). Baseline characteristics are presented in Table 1.

Incidence of Thyroid Dysfunction

The entire cohort (n = 1246) had a median follow-up of 11.3 (IQR 5-24) months. During follow-up, ICI-associated thyroid irAEs occurred in 518 (42%) patients. The highest incidence of thyroid irAEs occurred with combination CTLA-4 and PD-1 inhibitor treatment (56%), followed by anti-PD-1 (38%) and anti-CTLA-4 (25%) monotherapies (Fig. 1A).

Multiple patterns of thyroid irAEs were observed. Primary thyrotoxicosis was the most common thyroid irAE, affecting 388 (31%) patients. Of primary hyperthyroid irAEs, thyrotoxicosis was subclinical in 234 patients and overt in 154 patients (Fig. 1B). In patients with overt thyrotoxicosis, the median maximum FT4 concentration was 25.6 pmol/L (IQR 21.9-35.1). Primary hypothyroidism at onset (ie, without a preceding hyperthyroid phase) occurred in 100 (8%) patients and, again, most cases were subclinical (Fig. 1B). Euthyroid hyperthyroxinemia and hypothyroxinemia developed in 10 (<1%) and 6 (<1%) patients, respectively. Nonthyroidal illness (isolated low triiodothyronine) occurred in 14 (1%) patients (Fig. 1B). As a proportion of total thyroid irAEs, overt thyroid dysfunction occurred more commonly following combination CTLA-4 and PD-1 inhibitor treatment (47%) than after anti-PD-1 (37%) or anti-CTLA-4 (19%) based monotherapy.

Kinetics of Thyroid Dysfunction

There were kinetic differences in the temporal course of TSH and FT4 in patients with thyrotoxicosis compared with those who developed hypothyroidism from onset (Fig.

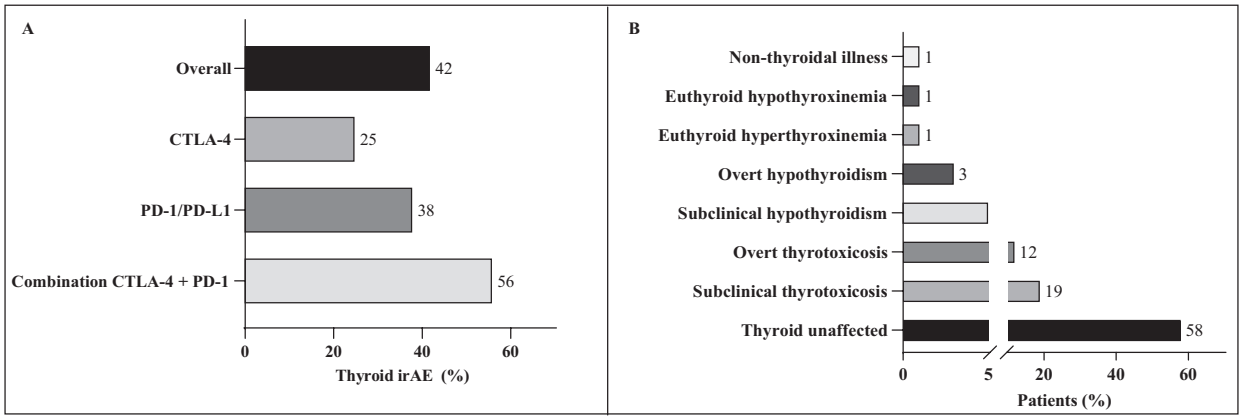

Figure 1. Incidence and biochemical subtypes of thyroid immune related adverse events following immune checkpoint inhibitor treatment. (A) Proportion of patients experiencing a thyroid immune-related adverse event by immune checkpoint inhibitor exposure. (B) Patterns and incidence rates of thyroid dysfunction developing after immune checkpoint inhibitor treatment.

2). Time to thyroid dysfunction was significantly shorter for patients with thyrotoxicosis than those with hypothyroidism (median 41 vs 90 days, $P < .001$). Of patients with thyrotoxicosis, a classical thyroiditis-like pattern of thyrotoxicosis immediately followed by a hypothyroid phase occurred in 111/388 (29%) patients. Progression to hypothyroidism was more common following overt thyrotoxicosis, with 91/154 (59%) patients progressing compared with 20/234 (9%) patients following an initial presentation with subclinical thyrotoxicosis (Table 2). Permanent hypothyroidism requiring thyroid hormone replacement was highest in patients with overt hypothyroidism at onset (74%). In patients with subclinical thyrotoxicosis, 227 (97%) returned to a euthyroid state during follow-up, whereas in patients with overt thyrotoxicosis, only 88 (57%) recovered normal thyroid function during follow-up and 66 patients (43%) progressed to permanent hypothyroidism requiring thyroid hormone replacement (Table 2).

Immune-related adverse events in extrathyroidal organ systems were common, affecting 865 (69%) patients. Rates of extrathyroidal irAEs were significantly increased in patients with overt thyrotoxicosis relative to hypothyroid and euthyroid patients (87 vs 67 vs 65%, $P < .001$). Similarly, overt thyrotoxicosis was associated with higher rates of multi-system irAEs and Common Terminology Criteria for Adverse Events (CTCAE) grade 3 or higher (more severe) irAEs compared with hypothyroid and euthyroid patients (Table 2).

Clinical and Biochemical Associations

Combination immunotherapy with anti-CTLA-4 and anti-PD-1 ICIs was associated with higher rates of thyroid irAE relative to either CTLA-4 (OR 3.76; 95% CI 2.49-5.75; $P < .001$) or PD-1 (OR 1.90; 95% CI 1.45-2.49; $P < .001$) based monotherapy. Thyroid irAEs were significantly more frequent in women (OR 1.62; 95% CI 1.27-2.08; $P < .001$) and were negatively associated with age (OR 0.88 per 10 years; 95% CI 0.81-0.96; $P = .005$) and brain metastases (OR 0.71; 95% CI 0.53-0.94; $P < .02$). The incidence of overt thyrotoxicosis was higher following combination ICI treatment compared with anti-CTLA-4 (OR 10.8; 95% CI 4.51-25.6; $P < .001$) or anti-PD-1 (OR 3.30; 95% CI 2.23-4.90; $P < .001$) monotherapy (Table 3). Overt thyrotoxicosis was more common in women (OR 2.02; 95% CI 1.37-2.95; $P < .001$) and in younger patients (OR 0.83 per 10 years; 95% CI 0.72-0.95; $P = .007$). Combination ICI treatment did not significantly influence odds of overt hypothyroidism compared to CTLA-4 (OR 7.28; 95% CI 0.89-59.4; $P = .06$) and PD-1 (OR 0.70; 95% CI 0.31-1.59; $P = .40$) based monotherapy. Baseline TSH did not predict thyroid irAEs overall. However, baseline TSH levels were significantly higher in patients with overt hypothyroidism from onset (OR 2.33 per mIU/L; 95% CI 1.61-3.33; $P < .001$). Using receiver operating characteristic curve analysis, baseline TSH values greater than 2.47 mIU/L were 90% specific and 40% sensitive for prediction of subsequent primary overt hypothyroidism.

Thyroid Autoantibodies

TPOAb and TgAb were measured in a subset of 163 patients with available biobank serum specimens collected prior to ICI treatment initiation. Overall, TPOAb, TgAb, or both were elevated in 46 patients (28%). Antithyroid antibody positivity rates were low in patients who did not develop a thyroid irAE, with only 1 patient (3%) affected by a positive TPOAb (Table 1). TPOAb and TgAb positivity rates were higher in patients who ultimately developed a thyroid irAE, affecting 21% and

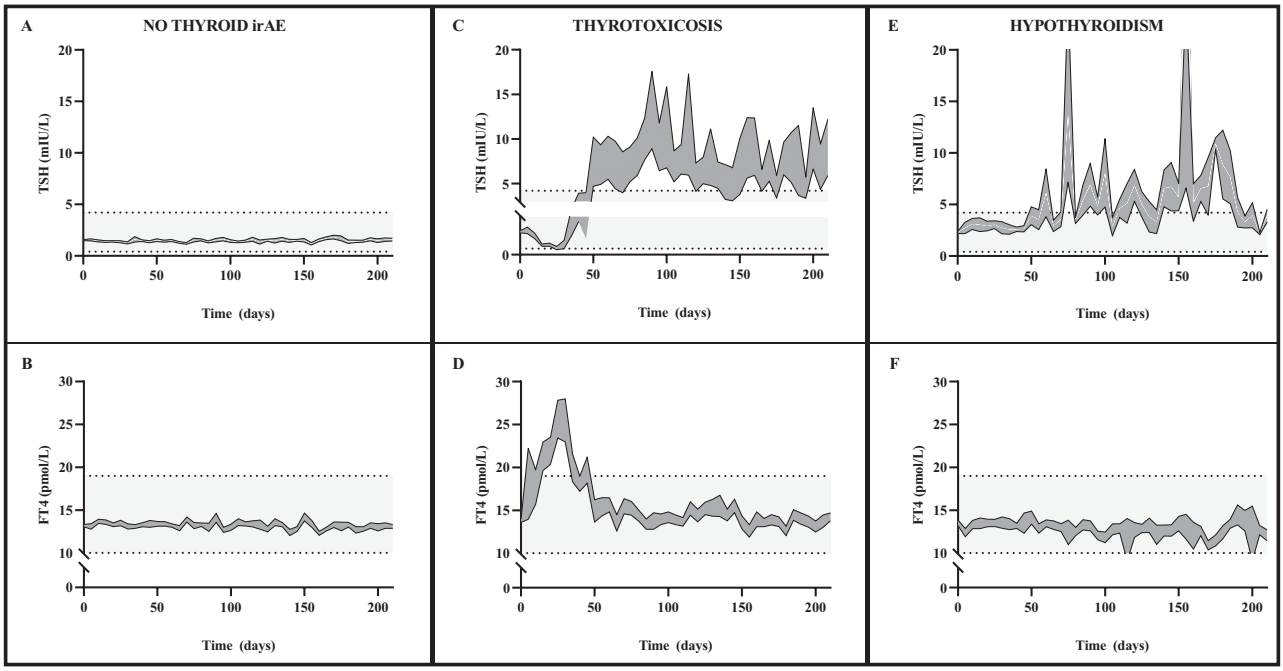

Figure 2. Kinetics of thyroid stimulating hormone and free thyroxine following immune checkpoint inhibitor treatment. (A) Change in TSH over time (mean ± SEM) in patients who remained euthyroid during follow-up. (B) Change in FT4 over time in patients who remained euthyroid during follow-up. (C) Change in TSH over time in patients who developed primary thyrotoxicosis (subclinical and overt) during follow-up. (D) Change in FT4 over time in patients who developed primary thyrotoxicosis during follow-up. (E) Change in TSH over time in patients who developed primary hypothyroidism (subclinical and overt) during follow-up. (F) Change in FT4 over time in patients who developed primary hypothyroidism during follow-up. TSH, thyroid-stimulating hormone; SEM, standard error of the mean; FT4, free thyroxine.

Table 2. Clinical phenotypes of thyroid dysfunction in patients with thyroid immune-related adverse events

	Thyrotoxicosis		Hypothyroidism		*P* value
	Subclinical	Overt	Subclinical	Overt	
Patients; n	234	154	61	39	
Age (years); median (IQR)	63 (49-71)	60 (49-69)	68 (55-74)	62 (51-73)	<.001
Male; n (%)	147 (63)	83 (54)	42 (69)	16 (41)	<.001
Checkpoint inhibitor; n (%)					<.001
CTLA-4	23 (10)	6 (4)	6 (10)	1 (3)	
PD-1/PD-L1	110 (47)	62 (40)	48 (79)	29 (74)	
Combined CTLA-4 + PD-1	101 (43)	86 (56)	7 (11)	9 (23)	
Baseline TSH (mIU/L); median (IQR)	0.95 (0.63-1.41)	1.29 (0.89-1.88)	2.32 (1.43-3.03)	1.94 (1.20-3.07)	<.001
Baseline FT4 (pmol/L); median (IQR)	12.8 (11.9-13.7)	13.6 (12.2-15.1)	12.5 (11.7-14.1)	14.1 (12.5-15.6)	.23
TPOAb positive; n (%)	3/58 (5)	18/53 (34)	2/4 (50)	4/13 (31)	
TgAb positive; n (%)	8/58 (14)	26/53 (49)	1/4 (25)	6/13 (46)	
Time to onset of thyroid dysfunction (weeks); median (IQR)	8 (4-14)	5 (2-8)	10 (3-26)	14 (8-25)	<.001
Time to normalization of TSH after onset of thyroid dysfunction (weeks); median (IQR)	4 (1-8)	12 (7-24)	3 (1-8)	10 (1-24)	.37
Progression to hypothyroidism; n (%)	20 (9)	91 (59)	n/a	n/a	n/a
Permanent hypothyroidism requiring thyroxine; n (%)	7 (3)	66 (43)	n/a	29 (74)	.13
Extra-thyroidal irAEs; n (%)	173 (74)	134 (87)	38 (62)	29 (74)	.01
Multi-system extrathyroidal irAEs; n (%)	13 (6)	24 (16)	0 (0)	2 (5)	.01
Severe (CTCAE grade 3-4) extrathyroidal irAEs; n (%)	56 (24)	37 (24)	9 (15)	5 (15)	.03

Abbreviations: TSH, thyroid-stimulating hormone; FT4, thyroxine; TPOAb, thyroid peroxidase antibody; TgAb, thyroglobulin antibody; IQR, interquartile range; irAE, immune related adverse event; CTCAE, Common Terminology Criteria for Adverse Events.

Table 3. Demographic associations with ICI-associated thyroid immune related adverse events (including subgroup analyses of overt thyroid irAEs) compared with patients that remained euthyroid during follow-up

Factor	Any thyroid irAE OR (95% CI)	*P* value	Overt thyrotoxicosis OR (95% CI)	*P* value	Overt hypothyroidism OR (95% CI)	*P* value
Age (per 10-years)	0.88 (0.81-0.96)	.005	0.83 (0.72-0.95)	.007	0.87 (0.68-1.10)	.23
Sex (female vs male)	1.62 (1.27-2.08)	<.001	2.02 (1.37-2.95)	<.001	3.31 (1.67-6.56)	.001
Immune checkpoint inhibitor						
CTLA-4+PD-1 combination vs CTLA-4	3.76 (2.49-5.75)	<.001	10.8 (4.51-25.6)	<.001	7.28 (0.89-59.4)	.06
CTLA-4+PD-1 combination vs PD-1	1.90 (1.45-2.49)	<.001	3.30 (2.23-4.90)	<.001	0.70 (0.31-1.59)	.40
Baseline TSH (per 1.0 mIU/L)	1.05 (0.91-1.22)	.48	1.08 (0.84-1.38)	.55	2.33 (1.61-3.33)	<.001
Brain metastases (absent vs present)	0.71 (0.53-0.94)	.02	0.78 (0.50-1.22)	.27	0.70 (0.31-1.59)	.39

Abbreviations: irAE, immune-related adverse event; OR, odds ratio; ICI, immune checkpoint inhibitor; CTLA-4, cytotoxic T-lymphocyte antigen-4; PD-1, programmed cell death-1.

32% of patients, respectively. In patients with thyroid irAEs and positive antibodies, 22/45 (49%) were positive for both TPOAb and TgAb, whereas no euthyroid patients were positive for both antibodies. The prevalence of antithyroid antibody positivity, including dual antibody positivity, was higher in patients with overt thyroid irAEs (thyrotoxicosis or hypothyroidism) compared with those with subclinical thyroid irAEs (Table 2).

Prognostic Significance

Median follow-up was not different between patients with thyroid irAEs (11.5 months; IQR 5.0-23.9) and patients without thyroid irAEs (11.1 months; IQR 4.8-25.3). No difference in treatment response (iRECIST criteria (16)) was observed in patients with ICI-associated thyroid irAEs relative to patients that remained euthyroid (58% vs 56%; $P = .55$). Similarly, mortality did not differ between patients with and patients without thyroid irAEs (33% vs 37%; $P = .14$). After adjustment for age, sex, brain metastases, and ICI type, OS and PFS did not differ between patients with thyroid irAEs and patients that remained euthyroid. In thyroid irAE patients, hazard ratios (HR) for OS and PFS were 0.95 (95% CI 0.77-1.16; $P = .59$) and 0.97 (95% CI 0.81-1.16; $P = .74$) respectively.

Survival analyses were performed separately for patients with overt thyrotoxicosis or overt hypothyroidism. Patients who developed overt thyrotoxicosis had longer OS (log-rank test; $P = .001$) and PFS (log-rank test; $P = .001$) than patients who did not experience a thyroid irAE. These results were confirmed by multivariable Cox regression analysis (adjusted for age, sex, brain metastases, and ICI type), with a HR of 0.57 (95% CI 0.39-0.84; $P = .005$) for OS and a HR of 0.68 (95% CI 0.49-0.94; $P = .02$) for PFS (Fig. 3). No improvement in PFS or OS was observed in association with overt hypothyroidism.

Discussion

We report clinical and biochemical characteristics of ICI-associated thyroid irAEs in the largest cohort of patients studied to date. The incidence of thyroid dysfunction in our study was substantially higher than reported in other cohorts, with sufficient patient numbers to examine this condition (12, 15, 17). We confirmed that combined ICI treatment resulted in more frequent and severe thyroid irAEs (5, 18). Notably, with the power of our large dataset we were able to clearly delineate subsets of thyroid dysfunction and describe new clinical and biochemical associations. Our findings suggest that ICI-associated overt thyrotoxicosis (with or without subsequent hypothyroidism) and hypothyroidism (without preceding thyrotoxicosis) may be etiologically distinct, rather than different presentations of the same disease process.

Whereas previous studies have failed to identify clinical factors related to thyroid irAEs, our study identified multiple new demographic characteristics that were associated with incident thyroid irAEs. In our cohort, female sex was a strong predictor of thyroid irAEs. An association between sex and thyroid irAE risk had not previously been reported, possibly due to small sample sizes and lack of statistical power (13, 19). A predilection of thyroid irAEs toward women is similar to the female preponderance of autoimmune thyroid disease observed in the general population (20). Age also acted as an independent risk factor for thyroid irAEs, with younger patients experiencing higher rates of thyrotoxicosis, but not hypothyroidism. These findings are consistent with other forms of thyroiditis, such as

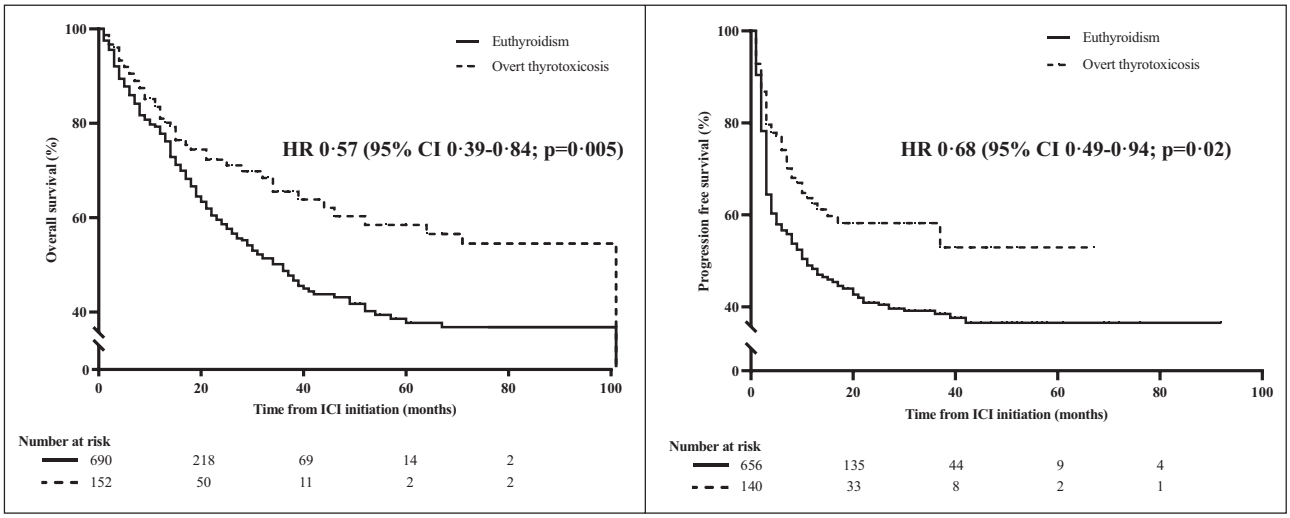

Figure 3. Overall survival and progression-free survival in patients with thyroid immune related adverse events. (A) Overall survival in patients with overt primary thyrotoxicosis relative the patients who remained euthyroid. (B) Progression-free survival in patients with overt primary thyrotoxicosis relative the patients who remained euthyroid.

subacute thyroiditis, which typically develop between the third and fifth decades (21, 22).

Kinetic changes of TSH and FT4 that occurred following ICI treatment demonstrated several distinct patterns. In patients with overt thyrotoxicosis, 93% presented with abnormal thyroid function within 3 months of ICI treatment initiation compared to only 38% of patients presenting with overt hypothyroidism as the primary manifestation of ICI-associated thyroid irAE. Whereas the time to onset of thyrotoxicosis was generally uniform, there was marked heterogeneity in the time to onset of hypothyroidism, ranging from 1 week to 70 weeks after the start of ICI treatment. The mechanisms underpinning late-onset hypothyroidism are poorly characterized. However, antithyroid antibody positivity rates in hypothyroid patients approached 50%, suggesting that late onset hypothyroidism may be secondary to ICI-mediated acceleration of lymphocytic infiltration of the thyroid in the context of a subclinical Hashimoto's thyroiditis that predated ICI treatment.

The role of antithyroid antibodies in the pathogenesis of ICI-associated thyroid irAEs remains controversial and poorly characterized (5). We measured baseline TPOAb and TgAb status using stored serum samples in 163 ICI-treated patients using a single assay and within a single run. Our results clearly demonstrated an association between TPOAb and/or TgAb positivity at baseline and the incidence of ICI-associated thyroid irAEs. Interestingly, these findings share similarity to other causes of thyroiditis (ie, postpartum), where antithyroid antibodies are present in up to 50% of affected patients but may not be pathognomonic (23). TPOAb and TgAb positivity was particularly prominent in patients who developed overt thyroid dysfunction, suggesting that pre-existing thyroid autoimmunity may be a potent risk factor for future development of ICI-associated thyroid toxicity. This hypothesis is strengthened by our additional findings that TPOAb and TgAb titers and dual antibody positivity rates were higher in patients with overt thyroid irAEs than patients with subclinical or no thyroid irAE.

As expected, thyrotoxicosis was significantly associated with the type of ICI treatment received. Combination therapy resulted in significantly higher rates of both overt and subclinical thyrotoxicosis relative to CTLA-4 and PD-1 inhibitors used alone. However, no such association was observed with respect to overt hypothyroidism, where the incidence of thyroid irAEs was similar across all ICI types. Baseline TSH concentrations were significantly higher in patients who developed overt hypothyroidism as their initial thyroid irAE. The association of hypothyroidism and baseline TSH had been observed previously and again may suggest progression of a pre-existing, subclinical Hashimoto's thyroiditis accelerated by ICI treatment, rather than a de novo ICI-mediated thyroiditis (19, 24, 25). Our data are consistent with this hypothesis, although our finding of differential thyroid irAE sensitivity to ICI type and high prevalence of antithyroid antibodies needs to be validated in other large ICI patient cohorts.

Overall, thyroid irAEs were associated with increased rates of extrathyroidal irAEs (hepatitis, colitis, etc.) including multisystem and CTCAE grade 3 or higher (more severe) irAEs relative to euthyroid patients. Increased rates of nonthyroidal irAEs were driven mainly by patients with overt thyrotoxicosis.

Primary hypothyroidism alone was not associated with extrathyroidal, multisystem, or severe irAEs, which in this group of patients occurred at similar rates to that observed in patients who remained euthyroid. Taken together, our data suggest that overt thyrotoxicosis could be a surrogate marker of more robust immune response to ICI treatment. Overt thyrotoxicosis was associated with high rates of extrathyroidal immune toxicity, but also improved cancer survival outcomes. Similar autoinflammatory sequalae and survival improvements were not observed in association with other thyroid irAE subtypes. Previous studies have inconsistently reported benefit in PFS and OS associated with thyroid irAEs (7, 9, 26, 27). Improvements in PFS and OS have been reported with overt relative to subclinical thyroid irAEs, but no studies had examined the differential effect of thyrotoxicosis and hypothyroidism with respect to cancer survival (6, 15). Differences in clinical and biochemical presentation of ICI-associated thyroid irAEs in our study support the hypothesis that ICI-associated thyrotoxicosis and hypothyroidism represent distinct clinical entities with differing underlying pathogenesis—thyrotoxicosis as an acute destructive thyroiditis and hypothyroidism as a progression of pre-existing autoimmune lymphocytic thyroiditis.

Our study had limitations inherent to the retrospective study design. Although patients were followed prospectively, and thyroid function tests were usually performed on a 3- to 4-weekly schedule, not all patients had tests performed at uniform time points, and it is possible that some cases of thyroid dysfunction may have been missed. We did not routinely test for TSH receptor antibodies in hyperthyroid patients and therefore Graves' disease cannot be excluded. However, all patients in our study developed transient thyrotoxicosis which was self-limited and resolved without treatment. We believe this makes Graves' disease highly unlikely. Stored serum at baseline was not available for all patients. As such, antithyroid antibodies were measured in a subset of patients only. However, we believe these results can be generalized to the larger cohort, as patients were sampled from all subgroups of thyroid dysfunction and demographically were not significantly different from untested patients of their respective thyroid irAE subtypes.

In summary, this study comprehensively details the clinical and biochemical features of ICI-associated thyroid irAEs. As the use of ICIs continue to increase, irAEs (thyroid and nonthyroid) will become an increasingly important component of endocrine and oncology practice. Our study reports new associations of thyroid irAEs with age, sex, TPOAb/TgAb, extrathyroidal irAEs, and other factors, which in totality suggest there are multiple mechanisms underpinning development of thyroid dysfunction following ICI treatment.

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Author Contributions: C.A.M. designed the study, acquired the data, performed data analyses, and drafted/approved all versions of the manuscript. R.C.B., V.H.M.T., and A.M.M. designed the study, aided with interpretation of data, and edited/approved the manuscript. G.V.L., R.A.S., and M.S.C. aided with interpretation of data and edited/approved the manuscript. S.N.L. performed data analyses, aided with data interpretation, and edited/approved the manuscript.

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Disclosures: A.M.M. has served on advisory boards for BMS, MSD, Novartis, Roche, Pierre-Fabre, and QBiotics. G.V.L. is consultant advisor for Aduro Biotech Inc, Amgen Inc, Array Biopharma Inc., Boehringer Ingelheim International GmbH, Bristol-Myers Squibb, Hexel AG, Highlight Therapeutics S.L., Merck Sharpe & Dohme, Novartis Pharma AG, OncoSec, Pierre Fabre, QBiotics Group Limited, Regeneron Pharmaceuticals Inc., SkylineDX B.V., Specialised Therapeutics Australia Pty Ltd. R.A.S. has received fees for professional services from Qbiotics, Novartis, Merck Sharp & Dohme, NeraCare, AMGEN Inc., Bristol-Myers Squibb, Myriad Genetics, and GlaxoSmithKline; M.S.C. is a consultant advisor for Bristol Myers Squibb, MSD, Amgen, Novartis, Pierre Fabre, Roche, Sanofi, Merck, Ideaya, Regeneron, Nektar, Eisai; Oncosec and Qbiotics and honoraria from Bristol Myers Squibb, MSD, Novartis. C.A.M., R.C.B., S.N.L., and V.H.M.T. have nothing to declare.

Data Availability: Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

References

1. Helmink BA, Gaudreau PO, Wargo JA. Immune checkpoint blockade across the cancer care continuum. *Immunity.* 2018;**48**(6):1077-1080.
2. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer.* 2012;**12**(4):252-264.
3. Postow MA, Sidlow R, Hellmann MD. Immune-related adverse events associated with immune checkpoint blockade. *N Engl J Med.* 2018;**378**(2):158-168.
4. Robert C, Hwu WJ, Hamid O, et al. Long-term safety of pembrolizumab monotherapy and relationship with clinical

outcome: a landmark analysis in patients with advanced melanoma. *Eur J Cancer*. 2021;**144**:182-191.
5. Muir CA, Menzies AM, Clifton-Bligh R, Tsang VHM. Thyroid toxicity following immune checkpoint inhibitor treatment in advanced cancer. *Thyroid*. 2020;**30**(10):1458-1469.
6. Yamauchi I, Yasoda A, Matsumoto S, et al. Incidence, features, and prognosis of immune-related adverse events involving the thyroid gland induced by nivolumab. *PLoS One*. 2019;**14**(5):e0216954.
7. Haratani K, Hayashi H, Chiba Y, et al. Association of immune-related adverse events with nivolumab efficacy in non-small-cell lung cancer. *JAMA Oncol*. 2018;**4**(3):374-378.
8. Osorio JC, Ni A, Chaft JE, et al. Antibody-mediated thyroid dysfunction during T-cell checkpoint blockade in patients with non-small-cell lung cancer. *Ann Oncol*. 2017;**28**(3):583-589.
9. Kotwal A, Kottschade L, Ryder M. PD-L1 inhibitor-induced thyroiditis is associated with better overall survival in cancer patients. *Thyroid*. 2020;**30**(2):177-184.
10. Delivanis DA, Gustafson MP, Bornschlegl S, et al. Pembrolizumab-induced thyroiditis: comprehensive clinical review and insights into underlying involved mechanisms. *J Clin Endocrinol Metab*. 2017;**102**(8):2770-2780.
11. de Filette J, Jansen Y, Schreuer M, et al. Incidence of thyroid-related adverse events in melanoma patients treated with pembrolizumab. *J Clin Endocrinol Metab*. 2016;**101**(11):4431-4439.
12. Iyer PC, Cabanillas ME, Waguespack SG, et al. Immune-related thyroiditis with immune checkpoint inhibitors. *Thyroid*. 2018;**28**(10):1243-1251.
13. Kimbara S, Fujiwara Y, Iwama S, et al. Association of antithyroglobulin antibodies with the development of thyroid dysfunction induced by nivolumab. *Cancer Sci*. 2018;**109**(11):3583-3590.
14. Yamauchi I, Sakane Y, Fukuda Y, et al. Clinical features of nivolumab-induced thyroiditis: a case series study. *Thyroid*. 2017;**27**(7):894-901.
15. Basak EA, van der Meer JWM, Hurkmans DP, et al. Overt thyroid dysfunction and anti-thyroid antibodies predict response to anti-PD-1 immunotherapy in cancer patients. *Thyroid*. 2020;**30**(7):966-973.
16. Seymour L, Bogaerts J, Perrone A, et al.; RECIST working group. iRECIST: guidelines for response criteria for use in trials testing immunotherapeutics. *Lancet Oncol*. 2017;**18**(3):e143-e152.
17. Scott ES, Long GV, Guminski A, Clifton-Bligh RJ, Menzies AM, Tsang VH. The spectrum, incidence, kinetics and management of endocrinopathies with immune checkpoint inhibitors for metastatic melanoma. *Eur J Endocrinol*. 2018;**178**(2):173-180.
18. Barroso-Sousa R, Barry WT, Garrido-Castro AC, et al. Incidence of endocrine dysfunction following the use of different immune checkpoint inhibitor regimens: a systematic review and meta-analysis. *JAMA Oncol*. 2018;**4**(2):173-182.
19. Pollack RM, Kagan M, Lotem M, Dresner-Pollak R. Baseline TSH level is associated with risk of anti-PD-1-induced thyroid dysfunction. *Endocr Pract*. 2019;**25**(8):824-829.
20. Dayan CM, Daniels GH. Chronic autoimmune thyroiditis. *N Engl J Med*. 1996;**335**(2):99-107.
21. Fatourechi V, Aniszewski JP, Fatourechi GZ, Atkinson EJ, Jacobsen SJ. Clinical features and outcome of subacute thyroiditis in an incidence cohort: Olmsted County, Minnesota, study. *J Clin Endocrinol Metab*. 2003;**88**(5):2100-2105.
22. Shrestha RT, Hennessey J. *Acute, Subacute and Riedel's Thyroiditis*. *In Endotext [Internet]*. MDText.com, Inc.; 2015.
23. Ross DS, Burch HB, Cooper DS, et al. 2016 American thyroid association guidelines for diagnosis and management of hyperthyroidism and other causes of thyrotoxicosis. *Thyroid*. 2016;**26**(10):1343-1421.
24. Morganstein D, Lai Z, Spain L, Diem S, Levine D, Mace C, et al. Thyroid abnormalities following the use of cytotoxic T-lymphocyte antigen-4 and programmed death receptor protein-1 inhibitors in the treatment of melanoma. *Clin Endocrinol*. 2017;**86**:614-620.
25. Pollack R, Ashash A, Cahn A, Rottenberg Y, Stern H, Dresner-Pollak R. Immune checkpoint inhibitor-induced thyroid dysfunction is associated with higher body mass index. *J Clin Endocrinol Metab*. 2020;**105**(10):1-8.
26. Kim HI, Kim M, Lee SH, et al. Development of thyroid dysfunction is associated with clinical response to PD-1 blockade treatment in patients with advanced non-small cell lung cancer. *Oncoimmunology*. 2017;**7**(1):e1375642.
27. Freeman-Keller M, Kim Y, Cronin H, Richards A, Gibney G, Weber JS. Nivolumab in resected and unresectable metastatic melanoma: characteristics of immune-related adverse events and association with outcomes. *Clin Cancer Res*. 2016;**22**(4):886-894.
28. Gershenwald JE, Scolyer RA, Hess KR, Sondak VK, Long GV, Ross MI, et al. Melanoma staging: evidence-based changes in the American Joint Committee on Cancer eighth edition cancer staging manual. *CA: Cancer J Clin*. 2017;**67**:472-492.

Appendix 4

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Clinical Research Article

Association of Antithyroid Antibodies in Checkpoint Inhibitor–Associated Thyroid Immune–Related Adverse Events

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Abstract

Context: The significance of thyroid peroxidase (TPOAb) and thyroglobulin antibody (TgAb) in the pathogenesis of thyroid immune-related adverse events (irAEs) is unknown.

Objective: To characterize the association of anti-thyroid antibodies with the development of thyroid immune related adverse events.

Methods: A retrospective cohort study was conducted of patients with melanoma receiving immune checkpoint inhibitor (ICI) treatment. TPOAb, TgAb, and interleukin-6 (IL-6) were measured retrospectively using tumor-banked samples at baseline and at time of diagnosis of a thyroid irAE. In euthyroid patients (without thyroid irAEs) measures were repeated 30 to 60 days after ICI commencement, which was similar to the median time to onset of thyroid irAEs in other patients.

Results: A total of 122 patients were included—31 remained euthyroid, 47 developed subclinical thyrotoxicosis, 37 developed overt thyrotoxicosis, and 7 developed overt hypothyroidism without preceding thyrotoxicosis. Baseline elevation of TPOAb or TgAb was present in 19 (16%) and 28 (23%) patients, respectively. Positive TPOAb or TgAb at baseline was 97% and 100% specific for eventual development of a thyroid irAE, respectively. During ICI treatment, overt thyrotoxicosis, but not other subtypes of thyroid irAE, was associated with statistically significant increases in the titer of TgAb and TPOAb. Baseline IL-6 levels were not associated with thyroid irAE onset but statistically significantly increased during treatment in patients who developed overt hypothyroidism.

Conclusions: TPOAb and TgAb positivity at baseline was more prevalent in patients who developed thyroid irAEs. Statistically significant increases or new antibody positivity was observed in association with overt thyrotoxicosis. TPOAb and TgAb positivity or increases during ICI treatment may be a useful biomarker to identify patients at increased risk of thyroid irAEs, particularly overt thyrotoxicosis.

Key Words: hyperthyroidism, thyroiditis, thyroid peroxidase antibody, interleukin-6, immune checkpoint inhibitor, immune-related adverse event

Abbreviations: AITD, autoimmune thyroid disease; FT4, free thyroxine; ICI, immune checkpoint inhibitor; IL-6, interleukin-6; IQR, interquartile range; irAE, immune-related adverse event; OS, overall survival; PD-1, programmed cell death protein-1; PFS, progression-free survival; TgAb, thyroglobulin antibody; TPOAb, thyroid peroxidase antibody; TRAb, thyrotropin-receptor antibody; TSH, thyrotropin.

Immune checkpoint inhibitors (ICIs) are associated with wide ranging immune-related adverse events (irAEs) (1). The thyroid is particularly susceptible, and thyroid dysfunction is one of the most common irAEs (2, 3). The etiology of thyroid irAEs is not fully understood and multiple mechanisms may underly disease onset (4, 5). The influence of thyroid peroxidase antibody (TPOAb) and thyroglobulin antibody (TgAb) positivity on risk for thyroid irAEs remains unknown.

We recently described thyroid irAEs in a large cohort of ICI-treated patients with melanoma (4). We showed that TPOAb and TgAb were mainly found in association with biochemically overt thyroid irAEs, particularly overt thyrotoxicosis where we also found an association with longer progression-free survival (PFS) and overall survival (OS) (4). Based on these findings, the present study aimed to further characterize the association of TPOAb/TgAb and changes

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in TPOAb/TgAb during treatment with thyroid irAEs, with a particular interest in patients with overt thyrotoxicosis. Thyrotropin-receptor antibody (TRAb) and interleukin-6 (IL-6), a proinflammatory cytokine associated with irAEs in other nonendocrine organ systems, were also studied.

Materials and Methods

Study Design and Participants

We included adult patients undergoing ICI treatment for melanoma with biobank serum samples to study the prevalence and kinetics of TPOAb, TgAb, and IL-6. Demographic, treatment, and cancer outcome information was extracted from a prospectively maintained central database. As part of routine care, measurement of serum thyrotropin (TSH) was performed in all patients before commencement of immunotherapy and thyroid function was repeated at subsequent ICI doses every 3 to 4 weeks. Biobank samples collected and stored at baseline (before receipt of first ICI dose) and at onset of thyroid irAE (or 30-60 days after treatment initiation in patients who remained euthyroid) were retrieved and used to measure TPOAb, TgAb, TRAb, and IL-6 levels.

Definitions

Thyroid irAEs were defined by new thyroid dysfunction developing after treatment with an ICI and were subcategorized by the biochemical pattern of thyroid dysfunction. Overt thyrotoxicosis was defined by a TSH level below the lower reference interval with concomitant elevation of free thyroxine (FT4) above the upper reference interval. Subclinical thyrotoxicosis was defined by a TSH level below the lower reference interval in the presence of normal serum concentrations of FT4. Overt hypothyroidism was defined by a TSH level above the upper reference limit with concomitant FT4 levels below the lower reference interval and no preceding thyrotoxic phase.

Assay Characteristics

Serum concentration of thyroid hormones were measured using the Abbott Architect I-2000 immunoassay (TSH catalog No. 7K62, RRID: AB_2883972, https://scicrunch.org/resources/Any/search?q=AB_2883972&l=AB_2883972; FT4 catalog No. 7K65, RRID: AB_2801665, https://scicrunch.org/resources/Any/search?q=AB_2801665&l=AB_2801665). Local reference intervals for TSH and FT4 were set at 0.40 to 4.20 mIU/L and 9.0 to 19.0 pmol/L, respectively. TPOAb and TgAb titers were measured using Abbott Architect with values greater than 5.61 IU/mL and greater than 4.11 IU/mL considered positive, respectively (TPOAb catalog No. 2K47, RRID: AB_2904180, https://scicrunch.org/resources/Any/search?q=AB_2904180&l=AB_2904180; TgAb catalog No. 2K46, RRID: AB_2904179, https://scicrunch.org/resources/Any/search?q=AB_2904179&l=AB_2904179). TRAb levels were measured using Roche Diagnostics cobas e601 analyzer (catalog No. 04388780190, RRID: AB_2801453, https://scicrunch.org/resources/Any/search?q=AB_2801453&l=AB_2801453), with a measuring range of 0.8 to 40.0 IU/L (coefficient of variation 7.6%, 1.9%, and 0.9% for TRAb levels of 1.7, 5.9, and 24.6 IU/L, respectively). TRAb titers greater than 1.8 IU/L were considered positive. IL-6 was measured using Siemens IMMULITE 2000-XPI enzyme-labeled, chemiluminescent immunometric assay (catalog No. L2K6P2, RRID: AB_2904178, https://scicrunch.org/resources/Any/search?q=AB_2904178&l=AB_2904178) with the reference range set at 0.0 to 3.4 pg/mL.

Procedures and Outcomes

The primary outcome was change in TPOAb and TgAb from baseline during ICI-treatment and whether increases in TPOAb/TgAb were associated with the development of a thyroid irAE. Change in TPOAb or TgAb from baseline were considered present if there was new antibody positivity or a greater than 50% increase in antibody titer for patients already positive at baseline. Other outcomes of interest included IL-6 levels at baseline and change in IL-6 level during ICI-treatment. In addition, TRAb levels were measured in patients at time of development of thyroid irAE to exclude Graves disease.

Statistical Analysis

Baseline characteristics are presented as median and interquartile range (IQR) or frequency and percentages. Changes in TPOAb, TgAb, and IL-6 from baseline were tested using the Wilcoxon signed rank test. All statistical tests were 2-tailed with 2-sided statistical significance level was set at P equal to .05. Statistical analyses were conducted using Statistical Package for Social Sciences (SPSS) version 26 (SPSS Inc) and figures were created using GraphPad Prism, version 8. All patients consented for their data to be recorded in the Melanoma Institute Australia central database, and for that information to be used for research purposes. Ethical approval was provided by the Sydney Local Health District human research and ethics committee.

Results

Patient Characteristics

Paired serum samples at baseline and on treatment were obtained for 122 patients. Samples came from patients with subclinical thyrotoxicosis (n = 47), overt thyrotoxicosis (n = 37), overt hypothyroidism (n = 7), and persistent euthyroidism (n = 31). The median follow-up of the study cohort was 13.8 months (IQR, 5.6-25.2 months). At baseline, 19 patients (16%) had TPOAb elevated, and 28 patients (23%) had TgAb elevated above the upper reference interval. Sixteen patients (13%) were positive both for TPOAb and TgAb. Baseline characteristics of all patients are presented in Table 1 and stratified by thyroid irAE subtype in Table 2.

Thyroglobulin Antibody

TgAb positivity at baseline was 100% specific (31% sensitive) for development of a thyroid irAE. Five patients (11%) with subclinical thyrotoxicosis had a positive TgAb at baseline. By comparison, 20 patients (54%) with overt thyrotoxicosis and 3 patients (43%) with overt hypothyroidism had a positive TgAb at baseline (Fig. 1A). No patients that remained euthyroid had a positive TgAb at baseline. In patients with a positive baseline TgAb, median TgAb titer was statistically significantly higher in patients with overt thyroid irAEs compared to patients with subclinical irAEs (see Table 2, $P < .001$). In patients with overt thyrotoxicosis, progression to hypothyroidism following the initial thyrotoxic phase was statistically significantly higher in TgAb-positive patients compared to TgAb-negative patients (85.0% vs 41.2%;

$P = .01$). No patients remained thyrotoxic, and the remainder spontaneously returned to euthyroidism.

During ICI treatment, 34 patients experienced a greater than 50% increase in TgAb titer or became positive from a negative baseline level. In 24 of 34 (71%) patients, increases or new positivity in TgAb were observed in association with overt thyrotoxicosis. TgAb positivity at baseline had a positive predictive value of 71.4% and a positive likelihood ratio of 6.0 for future development of overt thyrotoxicosis. Conversely, the absence of TgAb had a negative predictive value of 81.9% and a negative likelihood ratio of 0.5 for future development of overt thyrotoxicosis. In patients with overt thyrotoxicosis, TgAb titer at the time of thyroid dysfunction was statistically significantly increased compared to baseline (37.6 vs 6.55 IU/mL; $P < .001$). Treatment-related increases in TgAb were not observed in patients who remained euthyroid or in patients with subclinical thyrotoxicosis and overt hypothyroidism (Fig. 2A).

Table 1. Baseline characteristics

	All patients (N = 122)
Sex, n (%)	
Male	73 (60)
Female	49 (40)
Age, y; median (IQR)	65 (53-75)
Checkpoint inhibitor, n (%)	
CTLA-4	3 (2)
PD-1/PD-L1	75 (62)
CTLA-4 + PD-1	44 (36)
TSH, mIU/L; median (IQR)	1.27 (0.91-1.82)
FT4, pmol/L; median (IQR)	13.1 (12.1-14.4)
TPOAb positive; n (%)	19 (16)
TPOAb titer, IU/mL; median (IQR)	0.33 (0.10-0.75)
TgAb positive; n (%)	28 (23)
TgAb titer, IU/mL; median (IQR)	0.77 (0.00-3.32)
Dual antibody positive; n (%)	16 (13)
Interleukin-6 elevated; n (%)	65 (53)
Interleukin-6, pg/mL; median (IQR)	3.7 (1.3-6.0)

Abbreviations: CTLA-4, cytotoxic lymphocyte antigen-4; FT4, free thyroxine; IQR, interquartile range; irAE, immune-related adverse event; PD-1, programmed cell death protein-1; TgAb, thyroglobulin antibody; TPOAb, thyroid peroxidase antibody; TSH, thyrotropin.

Thyroid Peroxidase Antibody

TPOAb positivity at baseline was 97% specific (20% sensitive) for identification of patients destined to develop a thyroid irAE. Two patients (4%) with subclinical thyrotoxicosis had a positive TPOAb at baseline. By comparison, 13 patients (35%) with overt thyrotoxicosis and 3 patients (43%) with overt hypothyroidism had a positive TPOAb at baseline (Fig. 1B). Only 1 patient (3%) who remained euthyroid had a positive TPOAb at baseline. In patients with a positive baseline TPOAb, median TPOAb titer was statistically significantly higher in patients with overt thyroid irAEs compared to patients with subclinical irAEs (see Table 2, $P < .001$). In patients with overt thyrotoxicosis, progression to hypothyroidism following the initial thyrotoxic phase was not statistically significantly different in TPOAb-positive patients compared to TPOAb-negative patients (76.9% vs 58.3%; $P = .26$). No patients remained thyrotoxic, and the remainder spontaneously returned to euthyroidism.

During ICI-treatment, 16 patients experienced a greater than 50% increase in TPOAb titer or became positive from a negative baseline level. In 13 of 16 (81%) patients, increases

Table 2. Baseline characteristics by thyroid irAE subtype

	Euthyroid	Subclinical thyrotoxicosis	Overt thyrotoxicosis	Overt hypothyroidism
No.	31	47	37	7
Sex, n (%)				
Male	23 (74)	28 (60)	20 (54)	2 (29)
Female	8 (26)	19 (40)	17 (46)	5 (71)
Age, y; median (IQR)	68 (58-77)	69 (57-75)	55 (44-64)	74 (56-81)
Checkpoint inhibitor; n (%)				
CTLA-4	2 (6)	1 (2)	0 (0)	0 (0)
PD-1/PD-L1	22 (71)	27 (57)	20 (54)	6 (86)
CTLA-4 + PD-1	7 (23)	19 (41)	17 (46)	1 (14)
TSH, mIU/L; median (IQR)	1.56 (1.16-1.90)	0.96 (0.58-1.40)	1.32 (0.92-1.77)	3.29 (1.94-3.33)
FT4, pmol/L; median (IQR)	12.5 (11.1-14.0)	13.1 (12.5-14.2)	13.7 (12.1-15.3)	13.6 (12.5-14.3)
TPOAb positive, n (%)	1 (3)	2 (4)	13 (35)	3 (43)
TPOAb titer, IU/mL; median (IQR)	0.17 (0.09-0.39)	0.24 (0.07-0.43)	0.57 (0.34-78.9)	0.38 (0.18-278.6)
TgAb positive, n (%)	0 (0)	5 (11)	20 (54)	3 (43)
TgAb titer, IU/mL; median (IQR)	0.80 (0.00-1.45)	0.41 (0.00-0.92)	6.55 (0.91-43.6)	0.40 (0.00-426.7)
Dual antibody positive, n (%)	0 (0)	2 (4)	11 (30)	3 (43)
Interleukin-6 elevated, n (%)	17 (55)	21 (45)	23 (62)	4 (57)
Interleukin-6, pg/mL; median (IQR)	3.6 (1.0-6.3)	3.1 (1.0-5.7)	4.1 (2.1-6.2)	4.7 (3.0-7.0)

Abbreviations: CTLA-4, cytotoxic lymphocyte antigen-4; FT4, free thyroxine; IQR, interquartile range; irAE, immune-related adverse event; PD-1, programmed cell death protein-1; TgAb, thyroglobulin antibody; TPOAb, thyroid peroxidase antibody; TSH, thyrotropin.

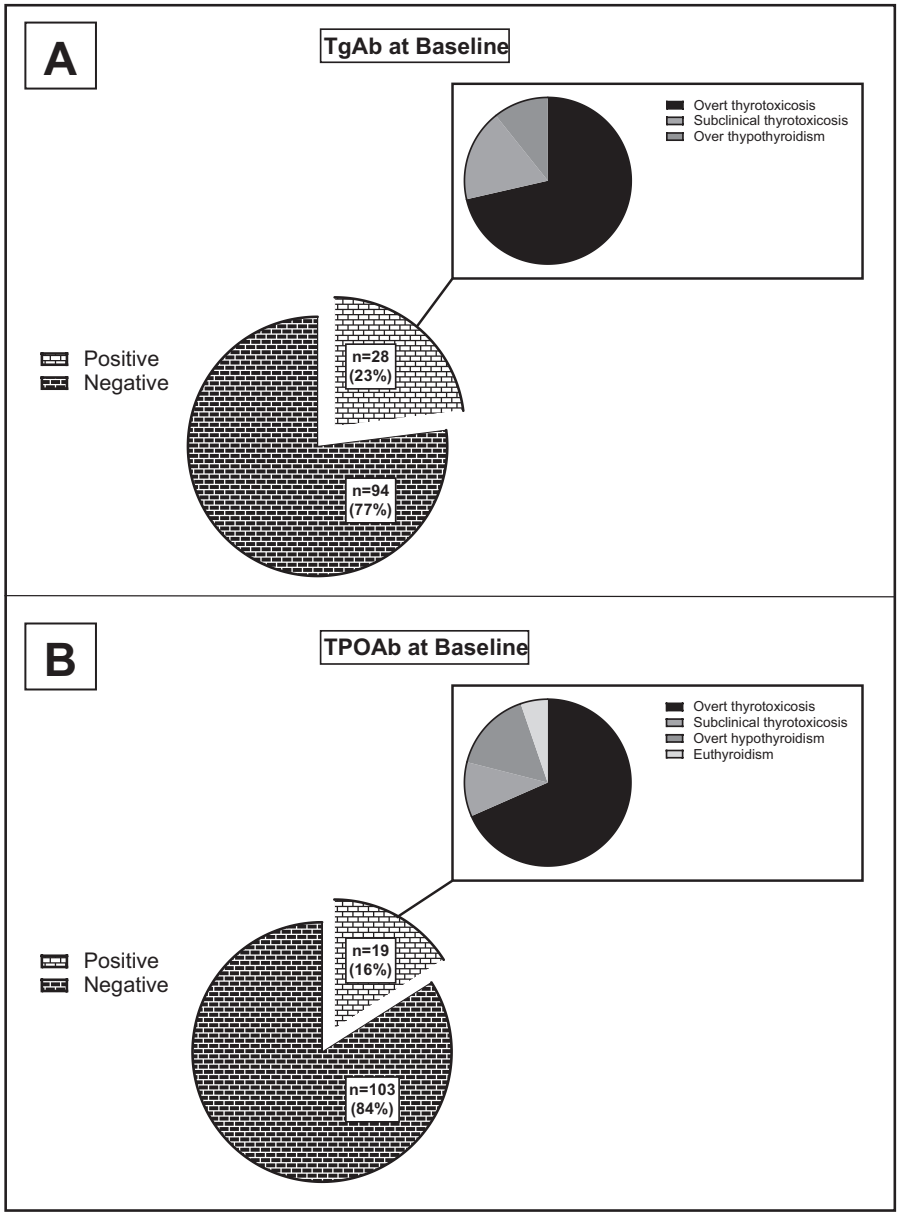

Figure 1. Prevalence of thyroid peroxidase (TPOAb) and thyroglobulin antibody (TgAb) at baseline. A, Proportion of patients with positive TgAb at baseline by thyroid immune-related adverse event (irAE) subtype. B, Proportion of patients with positive TPOAb at baseline by thyroid irAE subtype.

or new positivity in TPOAb were observed in association with overt thyrotoxicosis. TPOAb positivity at baseline had a positive predictive value of 68.4% and a positive likelihood ratio of 5.0 for future development of overt thyrotoxicosis. Conversely, the absence of TPOAb had a negative predictive value of 76.6% and a negative likelihood ratio of 0.7 for overt thyrotoxicosis. In patients with overt thyrotoxicosis, TPOAb titer at the time of thyroid dysfunction was statistically significantly increased compared to baseline ($P < .001$). Statistically significant treatment-related increases in TPOAb were not observed in patients who remained euthyroid or in patients with subclinical thyrotoxicosis and overt hypothyroidism (Fig. 2B).

Thyrotropin-Receptor Antibody

TRAb was measured at the time of thyroid irAE development in all patients. TRAb positivity was not documented in any patient.

Interleukin-6

Elevation of IL-6 at baseline was present in 65 (53%) patients. Median baseline IL-6 measured 3.70 pg/mL (IQR, 1.3-6.0 pg/mL). Rates of IL-6 elevation and absolute IL-6 levels were not different in patients with positive TPOAb or TgAb and those without. Similarly, IL-6 levels were not different across different subtypes of thyroid irAE (see Table 2). During ICI treatment there was a statistically significant increase in IL-6 levels from baseline in patients with overt hypothyroidism ($P = .03$). Treatment-related increases in IL-6 were not observed in patients who remained euthyroid or in patients with subclinical or overt thyrotoxicosis (Fig. 3).

Discussion

This study examined the prevalence and change in TPOAb and TgAb during ICI treatment in patients with melanoma.

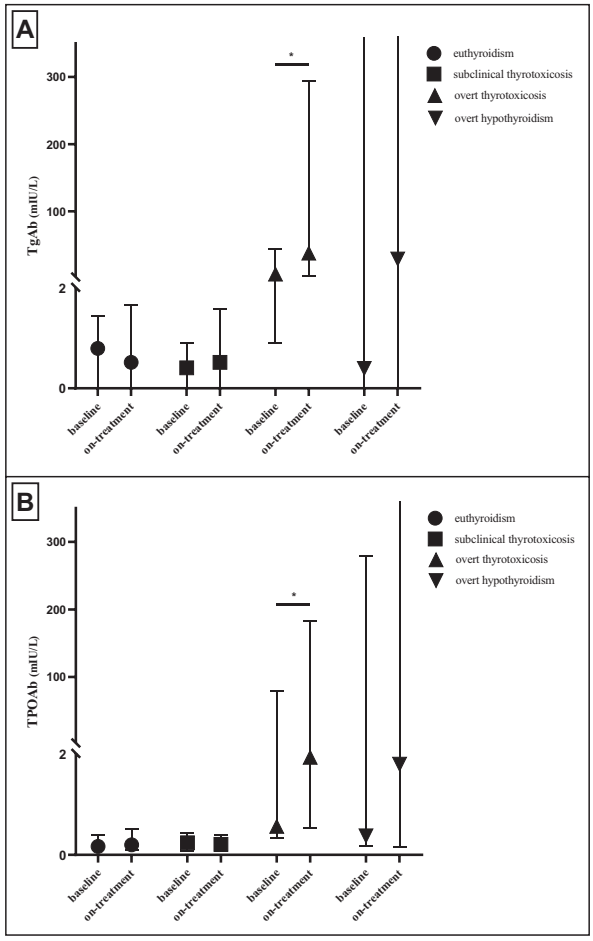

Figure 2. Change in thyroid peroxidase and thyroglobulin antibody (TgAb) titer following exposure to immune checkpoint inhibitor (ICI) treatment. A, Median and interquartile range of TgAb at baseline and during ICI-treatment by thyroid immune-related adverse event (irAE) subtype) (*$P < .001$). B, Median and interquartile range of thyroid peroxidase antibody (TPOAb) at baseline and during ICI-treatment by thyroid irAE subtype (*$P < .001$).

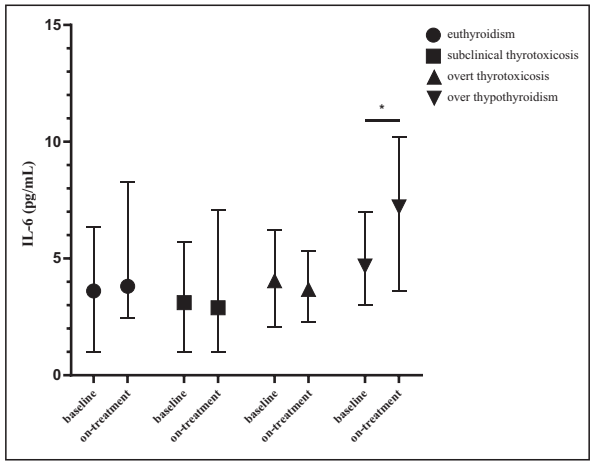

Figure 3. Change in interleukin-6 (IL-6) level following exposure to immune checkpoint inhibitor (ICI) treatment. Median and interquartile range of IL-6 at baseline and during ICI-treatment by thyroid immune-related adverse event subtype (*$P = .03$).

We found the prevalence of TPOAb and TgAb positivity to be higher in patients who developed overt thyroid dysfunction (thyrotoxicosis or hypothyroidism) compared to patients who remained euthyroid or those with subclinical thyrotoxicosis. However, only overt thyrotoxicosis was associated with large increases or new positivity in TPOAb and TgAb during ICI treatment. In patients with overt thyrotoxicosis, TgAb positivity was associated with progression to permanent hypothyroidism following an initial thyrotoxic phase. TPOAb positivity was also higher in patients who developed hypothyroidism following an initial thyrotoxic phase, although the association did not attain statistical significance in our cohort.

Thyroid irAE onset is variable and can be missed because symptoms may be minimal or conflated with those related to the underlying malignancy (2). Pretreatment selection of at-risk patients would be clinically useful, as overt thyrotoxicosis commonly progresses to permanent hypothyroidism and treatment with thyroid hormone replacement may be delayed (4). Predictive biomarkers to identify patients at risk of thyroid irAEs could promote timely diagnosis and reduce risk of thyroid irAE-related morbidity.

TPOAb or TgAb are positive in more than 90% of patients with autoimmune thyroiditis, but their relevance to thyroid irAE pathogenesis is poorly characterized (6). The clinical course of thyroid irAEs often mimics painless sporadic thyroiditis, a subtype of autoimmune thyroid disease (AITD). Thyroid irAEs are associated with higher TPOAb and TgAb positivity rates relative to the general population, but do not approach the 90% prevalence observed in AITD and therefore irAE pathogenesis cannot be solely antibody related (7-10). In our study, all but one patient with positive TPOAb or TgAb went on to develop thyroid dysfunction, although all patients with thyroid irAEs did not have positive antithyroid antibodies. It may be that a subgroup of thyroid irAE patients with positive TPOAbs or TgAbs are predisposed to AITD, which is unmasked following inhibition of immune checkpoints important for the maintenance of self-tolerance in the thyroid (11). This is supported by other studies showing early onset of thyroid irAE in antibody-positive patients (10, 12).

IrAEs correlate with improvements in PFS and OS in immunotherapy-treated cancer patients (13). Thyroid irAEs are often less severe than irAEs in other organ systems but have also been shown to be associated with improved cancer survival, particularly when thyroid dysfunction is overt (4, 14-17). Limited data have been published on the importance of antithyroid antibodies with respect to PFS and OS. A retrospective analysis of 137 patients with advanced non–small cell lung cancer treated with programmed cell death protein-1 (PD-1) inhibitors did not find improved OS or PFS in patients with positive antithyroid antibodies (18). However, TPOAb and TgAb were associated with improved PFS and OS in patients with levels above compared to below the median value in a second cohort of 168 anti–PD-1–treated patients (16). Our previous work in a large cohort of more than 1200 patients demonstrated that overt thyrotoxicosis irAEs were associated with increased immune toxicity in other organ systems as well as improved cancer survival and is hypothesized to be a surrogate marker of patients who demonstrate more robust immune activation following ICI treatment (4). In our present study, we found higher incidence and titers of TPOAbs and TgAbs in patients with overt thyrotoxicosis but were not powered to examine the effect of antibody positivity on cancer survival.

Dermatologic irAEs and colitis have been associated with low baseline IL-6 levels or increases in IL-6 during ICI treatment (19, 20). We did not find an association between baseline IL-6 and thyroid irAEs in our study. This is consistent with one other study examining the role of IL-6 in thyroid irAEs (12). However, we did observe a statistically significant increase in IL-6 at time of diagnosis in patients who developed overt hypothyroidism that was not observed with other thyroid irAE subtypes, potentially supporting our hypothesis that thyrotoxicosis and hypothyroidism arise via distinct mechanisms in the setting of ICI-associated thyroid dysfunction.

Strengths of our study include the use of uniformly stored samples, which were processed together in a single run to limit analytical variation. Limitations of our study are that it may have been underpowered to measure associations of TPOAb and TgAb with overt hypothyroidism. Although numerically TPOAb and TgAb values did increase in patients with overt hypothyroidism during ICI treatment, samples were available for only 7 patients. Although highly specific, TPOAb and TgAb lacked sensitivity, which reduces their utility as a clinically useful biomarker. Antithyroid antibodies other than TPOAbs and TgAbs may be involved in the pathogenesis of thyroid irAEs (21). We did not test nonclassical antithyroid antibody levels and cannot exclude that they may have contributed to the development of thyroid irAEs in antibody-negative patients.

In conclusion, ICI-treatment indications are rapidly expanding and thyroid toxicity is a common side effect of anti–cytotoxic lymphocyte antigen-4 (anti–CTLA-4) and anti-PD-1–based treatment (1). Predictive biomarkers will be important to identify patients at risk of irAEs, and our data suggest that baseline TPOAb and TgAb are highly specific and may be useful to identify patients at risk of overt thyrotoxicosis. Prospective studies are warranted to further evaluate the utility of TPOAbs and TgAbs as predictive biomarkers.

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Author Contributions

C.A.M. designed the study, acquired the data, performed data analyses, and drafted/approved all versions of the manuscript. R.C.B., V.H.M.T., and A.M.M. designed the study, aided with the interpretation of data, and edited/approved the manuscript. G.V.L., R.A.S., and M.S.C. aided with the interpretation of data and edited/approved the manuscript. G.V.L., A.M.M., and R.A.S. contributed the ICI-treated patients and biospecimens.

Disclosures

A.M.M. has served on advisory boards for BMS, MSD, Novartis, Roche, Pierre-Fabre, and QBiotics. G.V.L. is a consultant advisor for Aduro Biotech Inc, Amgen Inc, Array Biopharma Inc, Boehringer Ingelheim International GmbH, Bristol-Myers Squibb, Hexel AG, Highlight Therapeutics S.L., Merck Sharpe & Dohme, Novartis Pharma AG, OncoSec, Pierre Fabre, QBiotics Group Limited, Regeneron Pharmaceuticals Inc, SkylineDX B.V., and Specialised Therapeutics Australia Pty Ltd. R.A.S. has received fees for professional services from Evaxion, Provectus Biopharmaceuticals Australia, Qbiotics, Novartis, Merck Sharp & Dohme, NeraCare, AMGEN Inc, Bristol-Myers Squibb, Myriad Genetics, and GlaxoSmithKline; M.S.C. is a consultant advisor for Bristol-Myers Squibb, MSD, Amgen, Novartis, Pierre Fabre, Roche, Sanofi, Merck, Ideaya, Regeneron, Nektar, Eisai, Oncosec, and Qbiotics, and has received honoraria from Bristol Myers Squibb, MSD, and Novartis. C.A.M., R.C.B., C.C.G.W., and V.H.M.T. have nothing to disclose.

Data Availability

The data sets analyzed during the present study are not publicly available. Requests for data sharing can be made to the corresponding author, including a research proposal that must be approved by the principal investigators of participating centers.

References

1. Postow MA, Sidlow R, Hellmann MD. Immune-related adverse events associated with immune checkpoint blockade. *N Engl J Med*. 2018;378(2):158-168.
2. Muir CA, Menzies AM, Clifton-Bligh R, Tsang VH. Thyroid toxicity following immune checkpoint inhibitor treatment in advanced cancer. *Thyroid*. 2020;30(10):1458-1469.
3. Chang L-S, Barroso-Sousa R, Tolaney SM, Hodi FS, Kaiser UB, Min L. Endocrine toxicity of cancer immunotherapy targeting immune checkpoints. *Endocr Rev*. 2019;40(1):17-65.
4. Muir CA, Clifton-Bligh RJ, Long GV, *et al*. Thyroid immune-related adverse events following immune checkpoint inhibitor treatment. *J Clin Endocrinol Metab*. 2021;106(9):e3704-e3713.
5. Olsson-Brown A, Lord R, Sacco J, Wagg J, Coles M, Pirmohamed M. Two distinct clinical patterns of checkpoint inhibitor-induced thyroid dysfunction. *Endocr Connect*. 2020;9(4):318-325.
6. Pearce EN, Farwell AP, Braverman LE. Thyroiditis. *N Engl J Med*. 2003;348(26):2646-2655.
7. Iyer PC, Cabanillas ME, Waguespack SG, *et al*. Immune-related thyroiditis with immune checkpoint inhibitors. *Thyroid*. 2018;28(10):1243-1251.
8. O'Malley G, Lee HJ, Parekh S, *et al*. Rapid evolution of thyroid dysfunction in patients treated with nivolumab. *Endocr Pract*. 2017;23(10):1223-1231.
9. Yamauchi I, Sakane Y, Fukuda Y, *et al*. Clinical features of nivolumab-induced thyroiditis: a case series study. *Thyroid*. 2017;27(7):894-901.
10. Kimbara S, Fujiwara Y, Iwama S, *et al*. Association of antithyroglobulin antibodies with the development of thyroid dysfunction induced by nivolumab. *Cancer Sci*. 2018;109(11):3583-3590.

11. Álvarez-Sierra D, Marín-Sánchez A, Ruiz-Blázquez P, *et al*. Analysis of the PD-1/PD-L1 axis in human autoimmune thyroid disease: insights into pathogenesis and clues to immunotherapy associated thyroid autoimmunity. *J Autoimmun*. 2019;103:102285.
12. Kurimoto C, Inaba H, Ariyasu H, *et al*. Predictive and sensitive biomarkers for thyroid dysfunctions during treatment with immune-checkpoint inhibitors. *Cancer Sci*. 2020;111(5):1468-1477.
13. Ramos-Casals M, Brahmer JR, Callahan MK, *et al*. Immune-related adverse events of checkpoint inhibitors. *Nat Rev Dis Primers*. 2020;6(1):38.
14. Kim HI, Kim M, Lee SH, *et al*. Development of thyroid dysfunction is associated with clinical response to PD-1 blockade treatment in patients with advanced non–small cell lung cancer. *Oncoimmunology*. 2017;7(1):e1375642.
15. Yamauchi I, Yasoda A, Matsumoto S, *et al*. Incidence, features, and prognosis of immune-related adverse events involving the thyroid gland induced by nivolumab. *PLoS One*. 2019;14(5):e0216954.
16. Basak EA, van der Meer JW, Hurkmans DP, *et al*. Overt thyroid dysfunction and anti-thyroid antibodies predict response to anti-PD-1 immunotherapy in cancer patients. *Thyroid*. 2020;30(7):966-973.
17. Kotwal A, Kottschade L, Ryder M. PD-L1 inhibitor-induced thyroiditis is associated with better overall survival in cancer patients. *Thyroid*. 2020;30(2):177-184.
18. Toi Y, Sugawara S, Sugisaka J, *et al*. Profiling preexisting antibodies in patients treated with anti–PD-1 therapy for advanced non–small cell lung cancer. *JAMA Oncol*. 2019;5(3):376-383.
19. Valpione S, Pasquali S, Campana LG, *et al*. Sex and interleukin-6 are prognostic factors for autoimmune toxicity following treatment with anti-CTLA4 blockade. *J Transl Med*. 2018;16(1):94.
20. Tanaka R, Okiyama N, Okune M, *et al*. Serum level of interleukin-6 is increased in nivolumab-associated psoriasiform dermatitis and tumor necrosis factor-α is a biomarker of nivolumab recativity. *J Dermatol Sci*. 2017;86(1):71-73.
21. Yamauchi I, Yasoda A, Hakata T, *et al*. Novel thyroid-specific autoantibodies in patients with immune-related adverse events involving the thyroid gland. *bioRxiv* 2021.

Appendix 5

Medication-induced thyroid dysfunction

A guide to management

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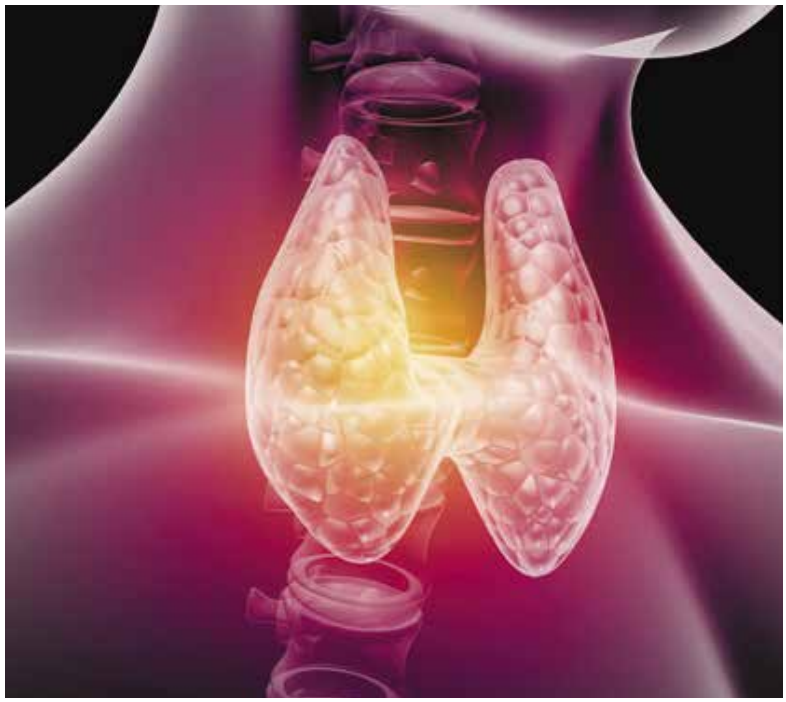
Medications can affect thyroid hormone production and function or interpretation of thyroid laboratory results. Awareness of these medication effects by GPs allows for early detection of thyroid abnormalities, effective monitoring and prompt intervention when indicated.

Key points

- **Medications can affect thyroid hormone production and function, or the interpretation of thyroid laboratory results.**
- **Patients presenting with thyroid dysfunction should be asked about the use of medications, including over-the-counter preparations.**
- **Thyroid stimulating hormone levels should be measured in all patients before starting a medication that may cause thyroid dysfunction.**
- **Prompt investigation and appropriate treatment of medication-induced thyroid dysfunction is crucial to limit thyroid-related morbidity.**



Thyroid hormone synthesis and secretion is regulated by a classic endocrine negative feedback loop (Figure). However, other factors can contribute to the development of thyroid dysfunction. For example, iodine is a crucial component of thyroid hormone synthesis and iodine deficiency can result in progressive hypothyroidism. Exposure to large amounts of iodine can also disrupt thyroid hormone homeostasis and inhibit thyroid hormone production (Wolff-Chaikoff effect) or stimulate thyroid hormone production independent of thyroid stimulating hormone ([TSH] Jod Basedow effect).[1]

Numerous medications can cause thyroid dysfunction or interfere with the interpretation of thyroid function tests (TFTs). Each step involved in thyroid hormone synthesis, secretion, transport and metabolism is susceptible to drug interactions.[2] Some medications, such as amiodarone and checkpoint inhibitors, can affect multiple steps in this process, whereas biotin-containing compounds can interfere with thyroid function assays resulting in spurious laboratory results.

All patients should be screened for pre-existing thyroid disease by measuring the TSH level before starting on medications that may cause thyroid dysfunction. If the TSH level is abnormal, thyroid hormone

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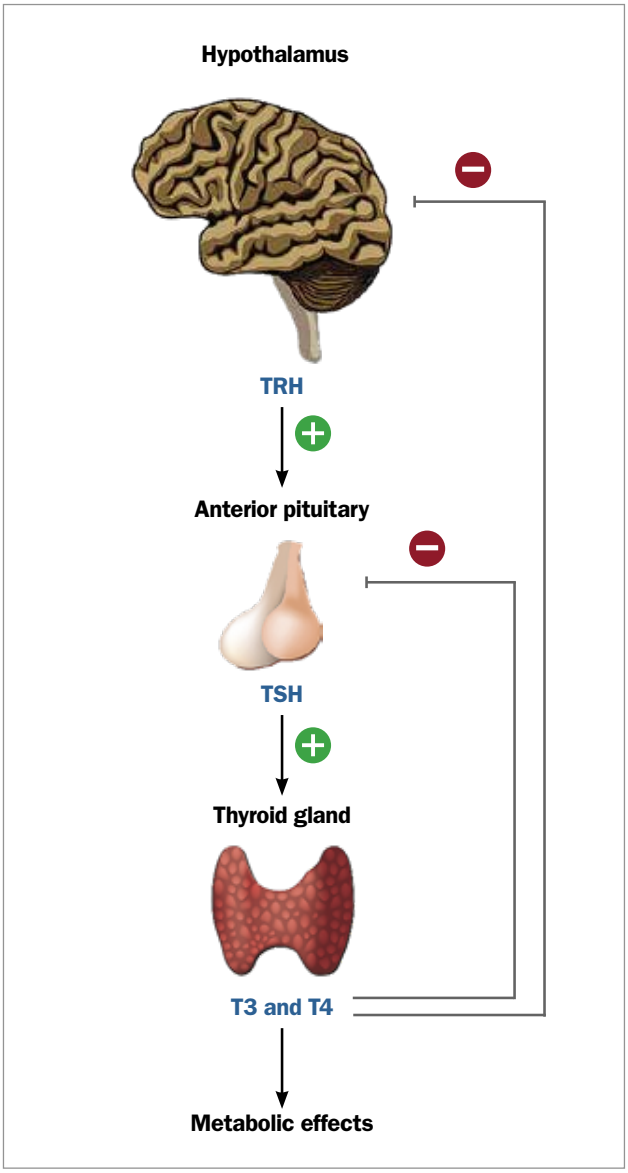


Figure. Hypothalamic–pituitary–thyroid feedback loop. Thyroid hormone production is controlled by TSH secreted by the pituitary gland in response to TRH secreted from the hypothalamus. TRH and TSH are inhibited by thyroid hormone via a negative feedback loop, which maintains thyroid hormone levels within a relatively fixed range.

Abbreviations: T3 = free tri-iodothyronine; T4 = free thyroxine; TRH = thyrotrophin-releasing hormone; TSH = thyroid-stimulating hormone.

levels (free thyroxine [FT4] and free tri-iodothyronine T3 [FT3]) and antithyroid antibodies should be measured. In asymptomatic patients, six-monthly TFT surveillance is sufficient to screen for the development of thyroid dysfunction.

This review focuses on four important medications – amiodarone, lithium, checkpoint inhibitors and biotin-containing products – that may be encountered routinely by GPs in clinical practice.

Medications that can cause thyroid dysfunction

Amiodarone

Amiodarone causes both hypothyroidism and hyperthyroidism, which affects 14 to 18% of treated patients.[3,4] Amiodarone is comprised of 37.2% iodine, which is released during its metabolism. A single 200 mg tablet releases 45 times the recommended daily intake of iodine.[4]

Amiodarone-induced hypothyroidism typically develops within six to 12 months of starting treatment.[3] Although it can occur in patients with no underlying thyroid abnormality, the risk is increased in those with abnormal TSH levels before starting amiodarone or positive antithyroid peroxidase antibodies.[5] Treatment of amiodarone-induced hypothyroidism is with levothyroxine replacement. After starting levothyroxine, four- to six-weekly thyroid function monitoring is sufficient, with the goal of normalising the TSH level over three to six months.

Amiodarone can induce two distinct types of hyperthyroidism, termed type 1 and type 2 amiodarone-induced thyrotoxicosis. Differentiation between the two subtypes may be difficult, making diagnosis and treatment challenging. A simplified algorithm for screening, diagnosing and managing amiodarone-induced thyroid dysfunction is shown in the Flowchart.

Type 1 amiodarone-induced thyrotoxicosis

Type 1 amiodarone-induced thyrotoxicosis is caused by increased synthesis of thyroid hormone. Typically, this develops in patients with pre-existing or latent subclinical thyroid disease and tends to occur early after amiodarone is started.[6] Increased production of thyroid hormone is attributed to the increased availability of iodine from amiodarone, which can provoke hyperthyroidism particularly in the setting of an abnormal gland (e.g. multinodular goitre).

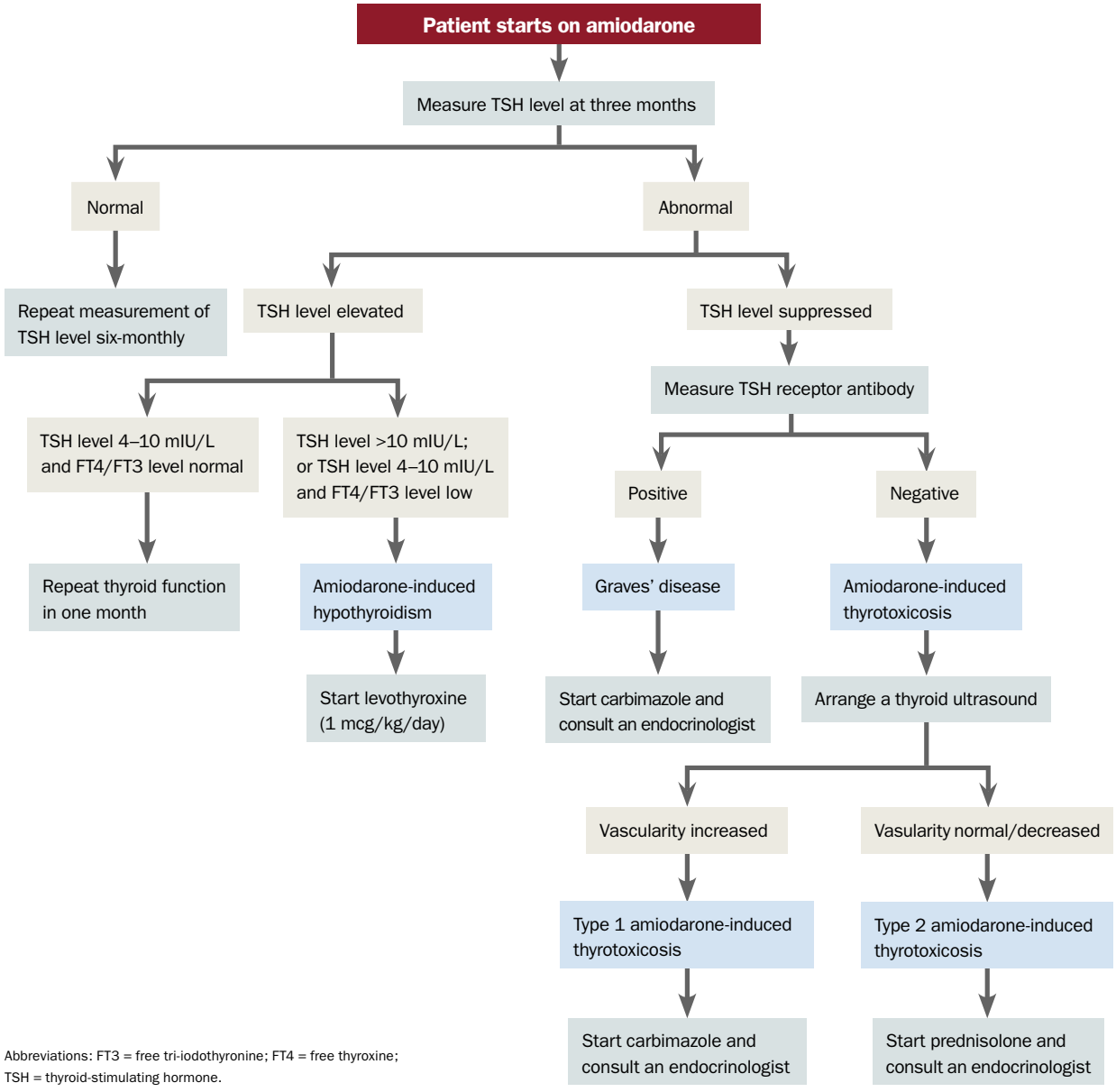
Radioiodine uptake scans are typically unhelpful in the setting of amiodarone because of interference from the high iodine load. However, ultrasound, which usually has no role in the diagnosis of hyperthyroidism, can be beneficial as increased thyroid vascularity is suggestive of the diagnosis.[7] Doppler imaging for assessment of thyroid vascularity should be stipulated on the imaging request form, as vascularity is not routinely assessed and may not be reported if not specifically requested.

A trial of antithyroid medication (carbimazole) is first-line treatment for type 1 amiodarone-induced thyrotoxicosis. The starting dose depends on the severity of thyrotoxicosis and in severe cases, doses of up to 15 mg three times daily may be required with prompt referral of the patient to an endocrinologist.

Type 2 amiodarone-induced thyrotoxicosis

Type 2 amiodarone-induced thyrotoxicosis results from toxic effects of amiodarone on the thyroid gland precipitating a destructive thyroiditis. Patients typically have no underlying thyroid disease.[8] Clinically, it is characterised by an initial hyperthyroid phase lasting weeks to months, followed by a hypothyroid phase or recovery of

Screening, diagnosing and managing amiodarone-induced thyroid dysfunction



Abbreviations: FT3 = free tri-iodothyronine; FT4 = free thyroxine; TSH = thyroid-stimulating hormone.

normal thyroid function. In contrast to type 1 amiodarone-induced thyrotoxicosis, oral prednisolone, with doses up to 40 mg daily is first-line treatment.[5]

Management of amiodarone-induced thyrotoxicosis if first-line treatment is suboptimal

A significant proportion of patients with amiodarone-induced thyrotoxicosis have a suboptimal response to initial medical treatment, reported to be up to 48% in one study.[9] In these patients, both type 1 and type 2 amiodarone effects may be present and cotreatment with antithyroid medication and prednisone should be trialled. Additionally, in very sick patients or if an ultrasound cannot be obtained, dual treatment with both carbimazole and corticosteroids should be strongly considered. Endocrinology input is recommended. In most cases, there is no immediate benefit to stopping amiodarone because of its long half-life of about 100 days.[10] In severe cases or if there is failure to respond to medical treatment, urgent thyroidectomy should be considered early.

Lithium

Lithium causes hypothyroidism in up to 20% of patients through inhibition of thyroid hormone release from the thyroid gland.[11] It is important to monitor a patient's mental state closely, as thyroid dysfunction may exacerbate or destabilise psychiatric symptoms. Female sex, age over 40 years and positive thyroid peroxidase antibodies are associated with increased risk of lithium-related thyroid dysfunction.[12] Levothyroxine replacement (1.0 to 1.5 mcg/kg/day) should be initiated in symptomatic patients with biochemical hypothyroidism (elevated TSH level, low FT4 level) and strongly considered in subclinical hypothyroidism, particularly if the TSH level is over 10 mIU/L, irrespective of FT4 and FT3 levels.

Immune checkpoint inhibitors

Immune checkpoint inhibitors have become standard of care in multiple cancer subtypes and are now in widespread clinical use. The most frequently used checkpoint inhibitors include: ipilimumab (CTLA-4 inhibitor); nivolumab and pembrolizumab (PD-1 inhibitors); and atezolizumab, avelumab and durvalumab (PD-L1 inhibitors). Treatment with checkpoint inhibitors can be associated with development of immune-related adverse events in multiple organ systems, although the thyroid is particularly susceptible.[13]

Up to 40% of patients treated with checkpoint inhibitors develop thyroid dysfunction, particularly when PD-1 and CTLA-4 inhibitors are used in combination.[14] Both hyperthyroidism and hypothyroidism can occur. In most patients, no treatment is required, and checkpoint inhibitors can be continued during the period of thyroid dysfunction. The most frequent presentation is transient thyrotoxicosis, developing one to two months after treatment initiation.[14] Many of these patients (about 50%) will progress to hypothyroidism after four to six weeks, which is often permanent. Levothyroxine replacement should be started in patients with a TSH level above 10 mIU/L, as the likelihood of recovery in this setting is rare.[14] After the first six months of treatment, annual testing of thyroid function is sufficient in asymptomatic patients to identify uncommon cases of late-onset hypothyroidism without a preceding thyrotoxic phase.[13]

Alemtuzumab

Alemtuzumab, a monoclonal antibody used to treat relapsing-remitting multiple sclerosis, rapidly depletes lymphocytes through inhibition of its target, CD52, followed by gradual immune reconstitution. During reconstitution, autoimmune thyroid disease can develop in 30 to 40% of patients.[15] Median onset of thyroid dysfunction is about 18 months after treatment, with most presenting within the first three years.[16]

Graves' disease and associated TSH-receptor antibody positive hyperthyroidism is the most common manifestation, representing two-thirds of alemtuzumab-associated thyroid dysfunction.[16] However, up to 15% of Graves' cases can have coexisting blocking antibodies, and Graves' disease in this context may present with a fluctuating course of rapid transition between periods of hyperthyroidism and hypothyroidism.[16] Endocrinology input is recommended for patients with hyperthyroidism, as coexistence of blocking and stimulating antibodies may require a 'block and replace' treatment strategy using both thyroxine and antithyroid medication.[16]

A smaller proportion of patients will develop Hashimoto's thyroiditis with hypothyroidism and positive thyroid peroxidase antibodies.[16] Following treatment with alemtuzumab, thyroid function (TSH, FT4, FT3 levels) should be monitored every three months for the first four years.[17] After this period, testing is required only for patients with signs or symptoms suggestive of thyroid dysfunction.

Biotin

Biotin is a water-soluble vitamin widely available in nutritional hair and nail supplements. It can also be found in some treatments for inherited mitochondrial disorders and multiple sclerosis. The amount of biotin varies considerably in over-the-counter preparations and can be as high as 10 mg per tablet.[18] In a recent study, 29% of adults reported using biotin-containing supplements, with most being in the form of over-the-counter multivitamin preparations.[19]

Biotin interferes with biotinylated laboratory reagents used in TSH, FT4, FT3 and TSH receptor antibody assays.[20] The direction and degree of interference depend on the type of assay used, but low TSH levels with elevated FT4 and FT3 levels mimicking hyperthyroidism occurs most commonly.[2] TSH-receptor antibody levels may also be elevated due to biotin use, artefactually suggesting Graves' disease. Interference is typically seen with high doses but can occur with doses as low as 10 mg per day.[21] GPs should be suspicious of biotin use in patients with abnormal thyroid levels who do not have clinical manifestations of thyroid disease. Retesting 48 hours after stopping biotin-containing supplements or contacting the pathology laboratory to request testing for biotin interference is recommended.[22]

Conclusion

The thyroid plays a pivotal role in the regulation of whole-body metabolism and energy expenditure. Medications can impact on thyroid function through multiple mechanisms resulting in a spectrum of thyroid dysfunction. Awareness by GPs of the effects of these commonly used medications will enhance monitoring, diagnosis and treatment of drug-related thyroid disorders. In addition, understanding how drug interactions, such as biotin, can produce spurious laboratory thyroid function results will reduce unnecessary imaging and treatment of patients without organic thyroid disorders. **ET**

References

A list of references is included in the online version of this article (www.endocrinologytoday.com.au).

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Medication-induced thyroid dysfunction

A guide to management

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References

1. Wolff J, Chaikoff I. Plasma inorganic iodide as a homeostatic regulator of thyroid function. J Biol Chem 1948; 174: 555.
2. Burch HB. Drug effects on the thyroid. N Engl J Med 2019; 381: 749-761.
3. Harjai KJ, Licata AA. Effects of amiodarone on thyroid function. Ann Intern Med 1997; 126: 63-73.
4. Martino E, Bartalena L, Bogazzi F, Braverman L. The effects of amiodarone on the thyroid. Endocr Rev 2001; 22: 240-254.
5. Basaria S, Cooper DS. Amiodarone and the thyroid. Am J Med 2005; 118: 706-714.
6. Tomisti L, Rossi G, Bartalena L, Martino E, Bogazzi F. The onset time of amiodarone-induced thyrotoxicosis (AIT) depends on AIT type. Eur J Endocrinol 2014; 171: 363-868.
7. Daniels G. Amiodarone-induced thyrotoxicosis. J Clin Endocrinol Metab 2001; 86: 3-8.
8. Ross D, Burch H, Cooper D, et al. 2016 American Thyroid Association guidelines for diagnosis and management of hyperthyroidism and other causes of thyrotoxicosis. Thyroid 2016; 26: 1343-1421.
9. Isaacs M, Costin M, Bova R, et al. Management of amiodarone-induced thyrotoxicosis at a cardiac transplantation centre. Front Endocrinol 2018; 9: 482.
10. Latini R, Tognoni G, Kates RE. Clinical pharmacokinetics of amiodarone. Clin Pharmacokinet 1984; 9: 136-156.
11. Lamb L, Samaras K. Endocrinopathies of lithium: common and treatable. Endocrinol Today 2020; 9(3): 29-32.
12. Kraszewska A, Chlopocka-Wozniak M, Abramowicz M, Sowinski J, Rybakowski JK. A cross-sectional study of thyroid function in 66 patients with bipolar disorder receiving lithium for 10-44 years. Bipolar Disord 2015; 17: 375-80.
13. Muir C, Menzies A, Clifton-Bligh R, Tsang V. Thyroid toxicity following immune checkpoint inhibitor treatment in advanced cancer. Thyroid 2020; 30: 1458-1469.
14. Muir C, Clifton-Bligh R, Long G, et al. Thyroid immune-related adverse events following immune checkpoint inhibitor treatment. J Clin Endocrinol Metab 2021: 106: e3704-e3713.
15. Scappaticcio L, Castellana M, Virili C, et al. Alemtuzumab-induced thyroid events in multiple sclerosis: a systematic review and meta-analysis. J Endocrinological Investigation 2020; 43: 219-229.
16. Pariani N, Willis M, Muller I, et al. Alemtuzumab-induced thyroid dysfunction exhibits distinctive clinical and immunological features. J Clin Endocrinol Metab 2018; 103: 3010-3018.
17. Muller I, Moran C, Lecumberri B, et al. 2019 European Thyroid Association guidelines on the management of thyroid dysfunction following immune reconstitution therapy. Eur Thyroid J 2019; 8: 173-185.
18. Kummer S, Hermsen D, Distelmaier F. Biotine treatment mimicking Graves' disease. N Engl J Med 2016; 375: 704-706.
19. Kantor ED, Rehm CD, Du M, White E, Giovannucci EL. Trends in dietary supplement use among US adults from 1999-2012. JAMA 2016; 316: 1464-1474.
20. Favresse J, Burlacu M, Maiter D, Gruson D. Interferences with thyroid function immunoassays: clinical implications and detection algorithm. Endocr Rev 2018; 39: 830-850.
21. Li D, Radulescu A, Shrestha RT, et al. Association of biotin ingestion with performance of hormone and nonhormone assays in healthy adults. JAMA 2017; 318: 1150-1160.
22. Wijeratne NG, Doery JC, Lu ZX. Positive and negative interference in immunoassays following biotin ingestion: a pharmacokinetic study. Pathology 2012; 44: 674-675.